Studies on the Importance of the Microbiome of Cooling Towers on *Legionella* spp. Ecology

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ABSTRACT

Engineered water systems are important infrastructures for bringing water to urbanized areas. Gastrointestinal pathogens were usually the cause of disease associated with these systems during the 19th century. For instance, typhoid, dysentery, and cholera were common diseases contracted through contaminated water in the United States and Europe during this period. These types of diseases have been significantly reduced with the improvements of detection and sanitation measures created over the past century. However, waterborne diseases are still an issue to this day and the classical gastrointestinal pathogens have been replaced by pathogens that naturally inhabit water systems, such as *Pseudomonas* spp. and *Legionella* spp. Amongst these pathogens, *Legionella pneumophila* is one of the most important cause of waterborne disease in developed countries. It is the causative agent of Legionnaires' disease, a severe pneumonia. *L. pneumophila* is a natural bacterial inhabitant of water systems where it parasitizes and grows in protozoan host species, such as amoeba and ciliates. This adaptation to grow intracellularly has also allowed it to grow within human macrophages. In this way, Legionnaires' disease is contracted by the inhalation of aerosols contaminated with *L. pneumophila*, which leads to infections of the lung macrophages and subsequent pneumonia.

Cooling towers, used for air conditioning and ventilation systems, are a major source of large outbreaks of Legionnaires' disease, however the reasons for this are not well understood. Indeed, colonization of cooling towers by *L. pneumophila* is variable, as some cooling towers are perpetually colonized, and others are almost never colonized. As *L. pneumophila* is an intracellular parasite, it is believed that the microbiome of cooling towers may play a significant role in the colonization, survival, and proliferation of *L. pneumophila* in these systems. Consequently, our

main objective was to characterize the bacterial and eukaryotic communities of cooling towers and understand their role in *L. pneumophila* ecology. Another objective was to isolate and characterize bacteria from cooling towers that could stimulate or inhibit *L. pneumophila* and to study their role in the ecology of *L. pneumophila* in cooling towers. In order to do this, we characterized the bacterial community of 18 different cooling towers in southern Québec, Canada, using a *16S rRNA* amplicon sequencing approach. The findings revealed that the bacterial community of the towers was moulded by several physicochemical factors such as water source and application of chlorine. The continuous application of chlorine was associated with the establishment of a *Pseudomonas* population. The *Pseudomonas* counts were negatively correlated with most other taxa identified in the cooling towers, including *Legionella*. As a result, we concluded that continuous application of chlorine could be an effective way to reduce levels of *L. pneumophila* in cooling towers. As a second step, we characterized the eukaryotic community using an *18S rRNA* sequencing approach and examined its interplay with the bacterial community and the *Legionella* community. The results revealed that cooling towers contain a diverse community of eukaryotes. Several eukaryotic and bacterial taxa formed a complex network based on co-occurrence. The network revealed that several taxa could potentially affect *L. pneumophila* ecology. This was demonstrated through the study of the interaction of a *Brevundimonas* sp. isolate and the ciliate community. These two microbial groups could promote the growth of *L. pneumophila* through direct and indirect mechanisms, such as nutritional supplementation or promoting host population growth. Finally, our last experiment isolated, identified, and characterized through whole genome sequencing several bacterial isolates that could inhibit *L. pneumophila* on plate. The analysis of the genomes of these isolates revealed a number of potential antimicrobial gene clusters that could explain the inhibition. Overall, the results suggest that the permissiveness of *L. pneumophila* colonization,

survival, proliferation in cooling towers is dependent on the presence of positive and negative interacting species composing the microbiomes of these environments. Manipulating these microbiomes so that they are not permissive to *L. pneumophila* could be a potential method to control Legionnaires' disease outbreaks.

RÉSUMÉ

Les systèmes d'eaux urbains sont des infrastructures importantes pour l'apport d'eau aux régions peuplées. Les maladies gastro-intestinales, tels que la fièvre typhoïde, le choléra, ou la dysenterie, due aux pathogènes d'origine fécal étaient originalement associées avec ces systèmes pendant le 19ième siècle. Depuis, ces maladies ont été largement réduites par l'amélioration des méthodes de détection et d'assainissement durant le dernier siècle. Cependant, les maladies reliées à l'eau sont toujours d'actualité. En effet, les pathogènes gastro-intestinaux de jadis ont été remplacés par des pathogènes opportunistes habitant naturellement dans les systèmes d'eau urbains, tels que *Pseudomonas* spp. et *Legionella* spp. Parmi ces pathogènes, *Legionella pneumophila* est l'une des plus importantes causes de maladie associé à l'eau dans les pays développés. Cette bactérie est l'agente causatrice de la maladie du légionnaire, une pneumonie sévère. *L. pneumophila* est une bactérie aquatique trouvée dans les systèmes d'eaux où elle parasite et croit dans des protozoaires, tels que les amibes ou les ciliés. La croissance intracellulaire est une adaptation qui permet à *L. pneumophila* de croitre aussi dans les macrophages humains. Ainsi, l'inhalation d'aérosols contaminés avec *L. pneumophila* est la méthode de transmission de la maladie. L'entrée de *L. pneumophila* dans les poumons permet à la bactérie d'infecter les macrophages et de causer la pneumonie.

Les tours aeroréfrigérantes, utilisées pour les systèmes de climatisation et de ventilation, sont une source majeure d'éclosion de la maladie du légionnaire. Cependant les raisons pour ceci ne sont pas bien comprises. En effet, la colonisation des tours par *L. pneumophila* est variable, avec certaines tours étant perpétuellement colonisées et d'autre l'étant presque jamais. Étant donné que *L. pneumophila* est un parasite intracellulaire, le microbiome des tours est soupçonné comme

étant un facteur majeur pour la colonisation, la survie, et la prolifération de *L. pneumophila*. Ainsi, nous avions pour objectif principal de caractériser la communauté bactérienne et eucaryote des tours aeroréfrigérantes et de comprendre leur rôle dans l'écologie de *L. pneumophila*. Nous avions aussi comme objectif d'isoler et de caractériser des bactéries qui pourraient stimuler ou inhiber la croissance de *L. pneumophila* et de comprendre le rôle de ces isolats dans l'écologie de *L. pneumophila*. Dans un premier temps, nous avons séquencé la communauté bactérienne de 18 tours au Québec, Canada, en utilisant une approche par séquençage d'amplicons du gène de l'*ARNr 16S*. Les résultats ont démontré que la communauté bactérienne est structurée par plusieurs facteurs physicochimiques, tels que la source d'eau et l'application du chlore. L'application en continu du chlore était associée avec l'établissement d'une population de *Pseudomonas* dans les tours. Cette population était corrélée négativement avec les autres taxons identifiés dans les tours, incluant avec *Legionella*. En conséquence, il est raisonnable de penser que l'application en continu au chlore pourrait être un moyen de réduire les niveaux de *L. pneumophila*. Dans un deuxième temps, nous avons aussi caractérisé la population eucaryote en utilisant une approche par séquençage d'amplicons de l'*ARNr 18S* et examiné son interaction avec la communauté bactérienne et la population de Légionnelles. Les résultats ont démontré que les tours d'eau contiennent une grande diversité d'organismes eucaryotes. Plusieurs taxons eucaryotes et bactériens forment un réseau basé sur des corrélations de cooccurrences. Le réseau a démontré que plusieurs taxons pouvaient affecter l'écologie de *L. pneumophila*. Ceci a été démontré par l'interaction d'un isolat bactérien de *Brevundimonas* et de la communauté de ciliés. Ces deux groupes microbiens pouvaient stimuler la croissance de *L. pneumophila* par des interactions directes et indirectes, tels que par la supplémentation de nutriment ou par l'augmentation du nombre de cellules hôtes. Dans un dernier temps, nous avons isolé, identifié, et caractérisé par

séquençage du génome plusieurs isolats bactériens capables d'inhiber *L. pneumophila* sur gélose. L'analyse des génomes des isolats a démontré un grand nombre de groupes de gènes antimicrobiens potentiels parsemant les génomes qui pouvaient expliquer l'inhibition. De façon générale, les résultats suggèrent que la permissivité de colonisation, de survie, et de prolifération de *L. pneumophila* dans les tours d'eau est dépendante de la présence d'espèces interagissant positivement et négativement dans les microbiomes des tours. La manipulation de ces microbiomes, pour qu'elles soient non permissive pour *L. pneumophila*, pourrait être une méthode potentielle pour contrôler les éclosions de maladie du légionnaire.

CONTRIBUTION TO KNOWLEDGE

The work presented here provides a better understanding of the ecology of *Legionella pneumophila*, the causative agent of Legionnaires disease (LD), and the mechanisms leading to an outbreak from a cooling tower. In recent years the microbiome of cooling towers has been suspected to play a major role in *L. pneumophila* ecology. The major findings of the research presented in this thesis are as follows:

1. We have characterized the bacterial and eukaryotic communities of cooling towers. The findings suggest that several physicochemical parameters can shape these communities. As a result, the manipulation of these factors could potentially be used as prebiotic or probiotic approach to create a non-permissive microbiome that inhibits *L. pneumophila* from colonizing the cooling tower ecosystem. Thus, potentially reducing the number of outbreaks of LD associated with cooling towers.
2. We are the first group to demonstrate that *L. pneumophila* ecology depends on a complex network of bacterial and eukaryotic organisms. Several of the organisms of this network could be used as novel potential biomarkers for *L. pneumophila* surveillance programs, such as *Brevundimonas*, *Pseudomonas*, or *Sediminibacterium*.
3. We have also discovered several organisms that can interact with *L. pneumophila*. Most of these interactions are novel. Seven bacterial isolates were found to inhibit *L. pneumophila* on plate and one was found to stimulate the growth of *L. pneumophila*. These findings revealed that the permissiveness of the resident microbiome to *L. pneumophila* is probably dependent on the levels of positive or negative interacting species within it. The genomes

of these bacteria were sequenced in order to investigate the genes that could be linked with the stimulation or the inhibition of *L. pneumophila*.

4. Finally, we have demonstrated that the interaction between a *Brevundimonas* isolate and the ciliate community were important for *L. pneumophila* ecology. The data suggested that trophic chains between bacterial prey and protozoan predators is an important element in the proliferation of *L. pneumophila* in cooling towers. Furthermore, the results suggested that ciliates may play a more important role than previously acknowledged in cooling towers ecosystems.

CONTRIBUTION OF AUTHORS

Contribution of authors for chapter 3:

I am the lead author of the article. I contributed to the experimental design and I was involved with the preparation of the genomic DNA library for next generation sequencing. I processed the samples, analyzed the data, and wrote the draft of the article. Émilie Bédard was responsible for organising the sampling campaign, managing some of the physicochemical tests, contributing to the design of the experiment, and reviewed the draft manuscript. Lyle G. Whyte provided some of the primers for the sequencing run and reviewed the draft manuscript. Jennifer Ronholm provided some of the primers for sequencing, and helped with creating the DNA library for sequencing, running the samples on the sequencing platform, and reviewing the draft manuscript. Michèle Prévost and Sébastien P. Faucher contributed to the experimental design, writing and editing of the manuscript.

Contribution of authors for chapter 4:

Emilie Bédard, Michèle Prévost, Sébastien P. Faucher, and I contributed to the design of the study. I performed the *18S rRNA* gene amplicon sequencing, whole genome sequencing and the ciliate co-culture experiments. I also processed and analysed the sequencing data. MengQi Hue isolated the *Brevundimonas* isolate. Deeksha Shetty and Fiona Chan Pak Choon performed the *Brevundimonas* stimulation assay. I analysed the data and wrote the first draft of the manuscript. Sebastien P. Faucher, Emilie Bédard, Michèle Prévost, and I edited the manuscript.

Contribution of authors for chapter 5:

I contributed to the experimental design, performed the inhibition assay, the whole genome sequencing of the different strains, the processing and analysis of the data, and wrote the manuscript. Sébastien P. Faucher contributed to the experimental design, writing and editing of the manuscript.

LIST OF ABBREVIATION

ANI: Average Nucleotide Identity

ANOSIM: Analysis of Similarity

AYE: ACES-Yeast Extract

BCYE: Buffered Charcoal Yeast Extract

BGCs: Biosynthetic Gene Clusters

bp: Base Pairs

CDC: Centre for Disease Control (USA)

CFU/L: Colony Forming Units per Litre

CYE: Charcoal Yeast Extract

DNA: Deoxyribonucleotide Acid

DOC: Dissolved Organic Carbon

ECDC: European Centre for Disease Control and Prevention

EWSs: Engineered Water systems

GU/L: Genomic Units per Litres

HCl: Hydrochloric Acid

HPC: Heterotrophic Plate Count

LCV: Legionella Containing Vacuole

LD: Legionnaires' Disease

LDA: Linear Discriminant Analysis

LEfSE: Linear Discriminant Analysis Effect Size

M: Modularity

MENA: Microbial Ecological Network Analysis

MIF: Mature Infectious Form

MiGA: Microbial Genome Atlas

mgCL_2/L: Milligram of Chlorine per Litre

NaOH: Sodium Hydroxide

NMDS: Non-metric Multi-dimensional Scaling

NRPS: Non-Ribosomal Peptide Synthetase

OD: Optical Density

OPPP: Opportunistic Premise Plumbing Pathogen

OTU: Operational Taxonomic Unit

P: p-value

P_i: Among-module connectivity

PEPC: Phosphoenolpyruvate Carboxylase

PKS: Polyketide synthase

pM: Picomolar

PRPP: Phosphoribosyl Diphosphate

qPCR: Quantitative Polymerase Chain Reaction

R: R-value for analysis of similarity (ANOSIM)

rRNA: Ribosomal Ribonucleic Acids

r_s: Spearman's rank correlation coefficient

SBT: Sequence Base Type

spp.: Species

TCA cycle: Tricarboxylic Acid cycle

TIVSS: Type 4 Secretion System

TSS: Total Suspend Solids

T_x/T_0: Time x / Time 0

VSS: Volatile Suspended Solids

Zi: Within-module Connectivity

CHAPTER 1. INTRODUCTION

Legionnaires' disease (LD) is a severe pneumonia caused by several bacterial species of the genus *Legionella*. The species *Legionella pneumophila*, which is a natural inhabitant of engineered water systems, is by far the most important cause of the disease. Several other species of the genus *Legionella* can also cause LD, such as *L. longbeachae* or *L. anisa*. LD is contracted when aerosols contaminated with the bacteria are inhaled. Once inside the lungs, the bacteria can infect the lung macrophages and cause the pneumonia. Water systems, such as potable water distribution systems, cooling towers, shower heads, or fountains, are important sources of *L. pneumophila*, as these environments can produce aerosols and harbour the bacteria. Cooling towers are an especially interesting case, as they are one of the more important sources of large outbreaks of LD.

For an outbreak to occur, *L. pneumophila* must first colonize and survive the water ecosystem it finds itself in. Following this, the surviving bacteria must grow their population to sufficient levels in order to increase their probability of being dispersed in the surrounding environment. In the case of cooling towers, this is fraught with difficulty, as *L. pneumophila* must deal with very low levels of nutrients, harsh physicochemical properties (such as the use of disinfectants), and the presence of inhibitive organisms. To bypass these hurdles, *L. pneumophila* uses its ability to grow, as a parasite, inside of different protozoan host species. These species are usually amoebic or ciliated species but can span various other groups of Protozoa.

In general, it is believed that *L. pneumophila* can only grow in nature if the host population is present. This is supported by the fact that host species have been identified experimentally and the bacterium's highly fastidious growth nature on laboratory growth media (up to now only one

growth medium is known to support cultures of *L. pneumophila*). In addition, host species are important factors for survival and colonization of systems, especially in cooling towers. Indeed, several host species can produce cysts, which are spore like forms that are highly tolerant to physical and chemical stresses, such as disinfectants. These cysts are known to harbour *L. pneumophila*, allowing the bacterium to be protected from various stresses that would normally kill it. This can be useful to bypass certain disinfection procedures and be spread through various engineered water systems, such as from the hot water distribution system to a cooling tower. As a consequence, it is believed that host species are obligatory requirement for outbreaks of LD. However, several studies have detected high levels of *L. pneumophila* in different engineered water systems without the presence of hosts in these same systems. It has also been shown that *L. pneumophila* can grow without the presence of hosts in experimental settings. For instance, several mutualistic or commensal relationships have been identified between *L. pneumophila* and different bacterial species. These mutualistic interactions have shown to promote survival and sometimes even growth (though minimal) of *L. pneumophila* in conditions that would otherwise not permit it. Though these findings have only been shown in experimental models, they suggest that *L. pneumophila* growth is not solely dependent on the presence of host species, and that several other biotic components are also important for colonization, survival, and growth of the bacterium.

To further expand on the idea that *L. pneumophila* outbreaks are not only dependent on the presence of host species, we have chosen to conduct an exploratory investigation into the microbiome of cooling towers and its effect on *L. pneumophila* ecology. This is to say we wanted to understand how and if certain specific organisms, other than known hosts, are important for colonization, survival, and proliferation of *L. pneumophila* in the cooling tower ecosystem. So far very little research has examined whether or not specific microbiomes, that is to say a specific set

of organisms, are required for outbreaks of LD to occur, and this even less in the case in cooling towers (one of the main sources of large outbreaks). Indeed, several communities have not been studied that could potentially affect *L. pneumophila*. For instance, since protozoa are microbial grazers, they must feed on different microbial communities (usually bacterial). Predation can depend on several factors, such as physiological state of the prey, size, and species of microorganisms. As a result, the growth and establishment of the protozoan host community may require specific prey communities. This suggests that, indirectly, the prey community could affect *L. pneumophila*, as a right set of prey organisms would be required for the establishment and increase of protozoan host community. However, so far research on this subject is lacking. Another example that demonstrates the importance of other microbial communities, than the host community, is the presence of several negatively interacting species with *L. pneumophila*. These negatively interacting species can either directly inhibit *L. pneumophila*, through competition or predation, or can have an indirect effect by inhibiting organisms that positively interact with the bacterium, such as host species. As a result, microbiomes harbouring these organisms may not be permissive to *L. pneumophila* growth or survival. Identifying and understanding how *L. pneumophila* interacts with these different communities may be crucial to better understanding the exact mechanisms that lead to an outbreak in cooling towers.

It is important to note that though the biotic components are important for *L. pneumophila* ecology, the abiotic factors are also crucial. These factors comprise the chemical and physical parameters of the cooling towers but can also encompass other unique factors of these systems, such as design of the tower or managerial practices. The effect of some of these factors on *L. pneumophila* has been examined in other water systems but has seldomly been looked into in cooling towers. This is especially true for features that are unique to cooling towers, such as design

and managerial practices. Unique factors of cooling towers may shape certain important communities differently than in other water systems. As a result, these should be better investigated in conjunction with the biotic factors to have a more comprehensive view of *L. pneumophila* colonization, survival, and proliferation in cooling towers.

From the reasoning put forth, it would seem *L. pneumophila* outbreaks are complex phenomena dependent on numerous abiotic and biotic elements. Though host species are an important factor, it would seem that several other microbial communities may also play an import role in *L. pneumophila* ecology, and that certain microbiomes of cooling towers may be more permissive for growth than others. The permissiveness may depend on the presence of specific species that either allow or promote the growth of *L. pneumophila*. Additionally, the presence of these positive interacting species may be due to the presence of certain abiotic factors in the cooling tower. Consequently, we hypothesise that the colonization, growth, and proliferation of *L. pneumophila* in cooling towers is depend on the presence of specific microbiome, with specific groups of organisms. This hypothesis led to the following objectives:

1. Characterize the bacterial and eukaryotic community of 18 cooling towers and their relationship with *L. pneumophila* ecology.
2. Identify the factors that shape the bacterial and eukaryotic communities and understand how these factors can affect *L. pneumophila* ecology.
3. Identify biomarkers that could potentially lead to predicting the presence or absence of *L. pneumophila* in cooling towers
4. Isolate, identify, and characterize species that can affect *L. pneumophila* either negatively or positively.

CHAPTER 2. LITERATURE REVIEW

I- INTRODUCTION

Throughout history, water management has been an important step for the foundation of great civilizations. As humans settled from nomadic hunter-gatherer bands to sedentary agrarian civilizations, water distribution and its management was of significant importance to these new societies. The goals were to bring clean fresh water to urban areas for public or private use, while at the same time, discarding the wastewater created. In order to distribute water in their cities, several ancient civilizations developed ingenious systems. The ancient Romans created cisterns to accumulate and store water from underground sources. Through a system of pipes, the water was then distributed to public areas, such as fountains and baths, and sometimes to private households (Cech, 2009). More impressively, by the fourth century B.C, aqueducts were being built in roman cities, and by 300 B.C., Rome had 14 aqueducts bringing in 150 million liters of water every day to the city (Cech, 2009). Other civilizations also had complex water distribution systems. The Xia dynasty (2070- 1600 B.C) of China built dams, dikes and other systems along the Huang He River in order to control flooding and irrigate crops (Cech, 2009).

The development and upkeep of a well-maintained water system was an important indicator of the success of a society. Indeed, proper water management was an important factor for efficient irrigation and improved crop yields. This helped feed a higher population and decrease events of famine and revolt, as well as increase human creativity and innovation. Ancient Egypt is a good example of such benefit. During its long history, Ancient Egypt was divided into periods of stable kingdoms alternating with relatively unstable periods, known as “Intermediate periods”. Stable periods were partly due to an increased agricultural output due to better control over the Nile River

and improvements in irrigation control (Allen, 1997; Mays, 2010). This helped to create one of the first agriculturally based economies in the Antiquity. Interestingly, it is believed that the first Intermediate period was partly due to climatic changes that reduced the flooding levels of the Nile valley consequently reducing crop yield (Dalfes et al., 2013). Furthermore, water management is also important for supplying water to the population, either for drinking or other uses. Several ancient civilizations created water systems to ensure the hydration of their population. For example, *Qanats* are sloping underground channels used to bring drinking water from aquifers and wells to cities in the Middle East. These systems have been in place since the 1st millennium B.C, and interestingly, some are still in use today in Iran (Cech, 2009). In the middle of the third millennium BC, the ancient city of Mohenjo-Daro, in the Indus valley, had a complex network of wells, which supplied the city with water for consumption and bathing, and allowed for the city to flourish (Jansen, 1989). Around 700 wells were discovered in the city, when archaeologist first discovered the ruin, and are believed to be an engineering marvel at the time, allowing water distribution to almost all of the households in the city (Jansen, 1989). Consequently, water supply and distribution systems were important factors for growth of civilizations. If properly managed, EWSs could allow a particular society to flourish, however, the contrary could have a disastrous impact on that society. Nevertheless, delivering water is one thing, but delivering clean and potable water is another thing.

Water is of vital importance for life. Proper water management is necessary to solve the problem of delivering potable and clean water to urban areas. History is fraught with examples of epidemics originating from miss-management of water systems. Up to the 19th century, gastrointestinal pathogens, such as *Escherichia coli*, *Salmonella enterica*, *Vibrio cholera*, and certain viruses were a major source of waterborne disease (Wang et al., 2013). However, with new

advances in technologies and increased scientific investigation, these outbreaks would decrease significantly over the next century. Indeed, the physician John Snow first discovered the link between contaminated water and cholera, during an outbreak in London in 1854 (McGuire, 2006). At the same time, the *Germ Theory* of disease, promoted by Pasteur and Koch, was replacing the conventional *Miasma Theory* of disease (Loomis and Wing, 1990). All of these new insights led to the implementation of disinfection procedures to water systems, starting with the implementation of a centralised water system to control waterborne diseases (McGuire, 2006). By 1897, these centralised systems would use sand filtration and chlorination to decontaminate drinking water in Europe and North America (Ashbolt, 2004). These advances in sanitation would be one of the greatest public health policies for mankind and would significantly decrease disease and death. For example, the implementation of strict disinfection procedure of drinking water system in the USA in the early 20th century decreased the cases of typhoid fever by almost 3 folds from 1900 to 1920, and by a 1000 fold from 1900 to 2006 (CDC, 1999).

Nowadays, waterborne diseases associated with gastrointestinal pathogens have very low incidence in developed countries. However, there seems to be a shift towards new waterborne disease associated with opportunistic pathogens or "Opportunistic Premise Plumbing Pathogens" (OPPPs) that naturally inhabit water systems (Falkinham III et al., 2015; Wang et al., 2013). Examples of such pathogens are *Legionella* spp., *Mycobaterium* spp., and *Pseudomonas* spp. Out of all these pathogens, *Legionella pneumophila* is of great importance as it has become, since 2013, the most common cause of disease associated with drinking water in the USA (Brunkard, 2011; Falkinham III et al., 2015). *L. pneumophila* is a Gram-negative bacterium that naturally inhabits aquatic environments. It is the causative agent of Legionnaires' disease (LD), a potentially deadly pneumonia, and Pontiac Fever, a flu like disease (Edelstein, 2008). This disease was first

recognized when in 1976 a mysterious illness killed several veterans that had attended the American Legion Convention during the USA Bicentennial, in Philadelphia at the Bellevue-Stratford Hotel. Out of more than 2000 attendants, 182 people were made ill, out of which 29 died after returning home from the convention (Winn, 1988). The outbreak led to a national inquiry by the Center for Disease Control (CDC), directed by Joseph E. McDade and David W. Fraser and their team. Their investigation led to the isolation of the bacterium from patient using guinea pigs (Fraser et al., 1977; Winn, 1988). The source of contamination was not identified, though airborne routes were suspected (Winn, 1988). The correlation between aerosols and LD was only made later, in 1979, when it was discovered that *L. pneumophila* could colonize hospital shower heads (Edelstein, 2008). Since then, more and more engineered water systems (EWS) have been identified as potential sources for the disease. A few examples of these systems that can disseminate *L. pneumophila* are water distribution systems, cooling towers, water fountains, misters, and whirlpool spas (Bédard et al., 2016; Haupt et al., 2012; Jernigan et al., 1996; Mahoney et al., 1992; Palmore et al., 2009; van Heijnsbergen et al., 2015).

Due to the ubiquitous nature of EWSs in urban areas, LD cases seem to be increasing in the developed world. The European Centre for Disease Prevention and Control (ECDC) and the CDC in the USA both reported increasing incidents of LD cases in recent years, with the CDC reporting a 249% increase in legionellosis case from 2000 to 2011, and the ECDC reporting 1.5 fold increase in cases from 2013 to 2017 (Dooling et al., 2015; ECDC, 2019). Travel-associated cases have also been reported by the ECDC, which represent 21% of all European cases of LD (ECDC, 2019). This fact suggests that LD is a worldwide phenomenon; however, the actual numbers of cases worldwide are probably underreported. Several factors could increase rates of LD in the future, such as increased urbanization, ageing population, climate change, antibiotic

resistance, increased monitoring, and increases in population (ECDC, 2019). Consequently, as LD is becoming a pressing issue, understanding the ecology of *L. pneumophila* in EWSs is an important step into identifying solutions to decrease risks of LD.

II- LEGIONELLA BIOLOGY AND ECOLOGY

1. LEGIONELLOSIS

Legionellosis encompasses two types of diseases, Legionnaires' disease (LD) and Pontiac fever. These diseases are caused by several species of the bacterial genus *Legionella*, such as *L. pneumophila*. LD is a form of atypical pneumonia. The symptoms can include fever, chills, cough (dry, with sputum, or bloody), difficulty breathing, aches, tiredness, loss of appetite, chest pain, diarrhea, and vomiting (Edelstein, 2008). Half of patients may get neurological issues, such as confusion and impaired cognition (Edelstein, 2008). LD is contracted by inhalation of droplets contaminated with *L. pneumophila*. Once inhaled, the bacteria enter the lungs and infect alveolar macrophages. *Legionella* species will grow inside the macrophages and cause cellular death. This in turn causes an immune response, which creates inflammation, destruction of the tissues and results in the pneumonia. Legionnaires' disease is not believed to be spread from person to person. Consequently, the human host is usually believed to be a dead end for *Legionella* species, as the disease either causes the death of the host or of the bacteria. However, a single case of suspected human to human transmission was recently reported (Borges et al., 2016; Correia et al., 2016). As a result, the human host as a reservoir is still being debated. On the other hand, Pontiac fever is an upper respiratory tract infection with symptoms similar to influenza. Pontiac fever is not a type of pneumonia, and the symptoms typically fade away after a few days (Edelstein, 2008). As of yet,

there is no standard definition or laboratory test for Pontiac fever, making diagnosis and causation of this disease difficult to pinpoint and understand.

Cases of legionellosis are contracted through the inhalation of water aerosols contaminated with the bacteria. EWSs are the major disseminator of the bacteria, as these systems can produce aerosols and harbour *Legionella* species. This includes but is not restricted to showerheads, water distribution systems, humidifiers, cooling towers, misters, fountains, and potable water distribution systems (van Heijnsbergen et al., 2015). In certain cases potting soil and compost have been linked to dissemination of *L. longbeachae*, mainly in Australia and New Zealand (Whiley and Bentham, 2011).

LD cases have been increasing both in Europe and in North America and, as of now, *Legionella* is the most frequently reported etiology among water borne disease in the USA (Beer et al., 2015). Most cases of Legionnaires' disease are sporadic, with potable distribution systems being the main source of dissemination. However, large outbreaks also occur. Though no explicit threshold defines a large outbreak, reported outbreaks can range anywhere from two reported cases to a couple hundred cases, with most outbreaks reporting between 10 to 200 cases (Table 1). The largest outbreak ever reported was in Murcia, Spain, in July of 2001, where more than 800 cases were suspected and 449 confirmed cases were reported (García-Fulgueiras et al., 2003). Cooling towers are usually reported as the source of large outbreaks, though, other sources have also been attributed to large outbreaks, such as whirlpool spas. It is also estimated that in certain cases cooling towers are responsible for 28 % of sporadic cases of LD (Fitzhenry et al., 2017).

Table 1: Examples of Legionnaires' disease outbreak.

Year	City	Cases	Deaths	Mortality Rate	Source	Reference
1976	Philadelphia (USA)	182	29	16%	Air Conditioning system	(Winn, 1988)
1985	Stafford (UK)	68	22	32%	Cooling Tower	(O'mahony et al., 1990)
1999	Bovenkarspel (Netherlands)	135	16	12%	Hot Tub Sprinkler	(Den Boer et al., 2002; Lettinga et al., 2002)
2000	Melbourne (Australia)	125	4	3.2%	Cooling tower	(Greig et al., 2004)
2001	Murcia (Spain)	449	5	1.1%	Cooling tower (Hypothetical)	(García-Fulgueiras et al., 2003)
2003-2004	Pas de Calais (France)	86	18	21%	Cooling towers	(Mathieu et al., 2006)
2005	Toronto (Canada)	112	23	20%	Cooling towers	(Gilmour et al., 2007)
2007	Jastrzebiu-Zdroj (Poland)	4	3	75%	Hot Water Distribution	(Juda, 2009)
2012	Québec City	182	13	7.1%	Cooling towers	(Lévesque et al., 2014)
2015	Bronx, NYC, USA (3 outbreaks the same year)	138	16	12%	Cooling towers	(Fitzhenry et al., 2017; Weiss et al., 2017)

2014-2015	Flint, Michigan, USA	88	12	75%	Drinking Water Systems	(Rhoads et al., 2017)
2017	Lenox Hill, NYC, USA	7	1	14%	Cooling tower (Hypothetical)	(Christopher Miller, 2017)
2017	Anaheim, California, USA (Disneyland)	12	1	8.3%	Cooling tower	(Gallagher, 2017)
2019	North Carolina	141	4	2.8%	Hot tubs	(Nicole Chavez, 2019)

L. pneumophila is usually the most important cause of Legionnaires' disease, causing about 90% of cases in North America and Europe (Kozak-Muiznieks et al., 2014; Whiley and Bentham, 2011). The other 10% of cases are caused by other species such as *L. longbeachae*, *L. anisa*, and many others. It has been shown that *L. longbeachae* causes as many cases as *L. pneumophila* in Australia, New Zealand, and Japan (Whiley and Bentham, 2011). Furthermore, the prevalence of cases based on different species are dependent on the community of patients analysed. For instance, a recent study of 59 *Legionella* infections in HIV patients found that, 31.3% were due *L. anisa*, 25.0% were due to *L. bozemanii*, 12.5% were due to *L. micdadei*, and only 18.8% were due to *L. pneumophila* (Head et al., 2017). Thus, risk factors and geographic location are confounding factors that alter the prevalence of certain species or strains of *Legionella*.

The case fatality rate of Legionnaires' disease is around 12% in Europe and 8% in the USA (Phin et al., 2014). However, risk factors can skew the fatality rate, with chronic lung disease,

smoking, immunosuppression, older age, sex, diabetes, alcoholism, cancer, and cardiovascular disease being factors associated with higher incidence and mortality rates in these communities (Cunha et al., 2016; Phin et al., 2014). The hospitalisation and mortality rates of LD can be quite burdensome from an economic perspective. Indeed, LD can either result in patients taking small medical leave of absence to hospitilization, and death, which can leave a financial, emotional, and political toll to the parties involved, such as family member, compagnie managers, or governments. Lock et *al.*, analysed the financial cost of a relatively small outbreak (around 14 people) in South East London in 2005. Their findings showed that hospital treatment was the main expense of the outbreak, costing on average 27 971 GBP per patient (Lock et al., 2008). Another study by Collier et *al.*, estimated that LD is the waterborne disease with the highest cost per episode in the USA, costing around 33 366 USD per episode (Collier et al., 2012). Furthermore, total hospitalization insurance claims related to Legionnaires' disease were estimated at 434 million USD per year in the USA (Collier et al., 2012).

Urinary antigen test and culture methods are usually used as diagnosis tests for detection of *L. pneumophila* in LD cases (Sharma et al., 2017). Other tests that can be used are serological methods, PCR, and direct fluorescent assays (Sharma et al., 2017). Antibiotics are usually the first line of treatment, with fluoroquinolones and macrolides being the most commonly used (Sharma et al., 2017). In the most severe cases, specific medical procedures are used, such as the use of ventilators. Consequently, the sooner the antibiotic regiment is administered the better the outcome.

2. *LEGIONELLA ECOLOGY AND LIFE CYCLE*

Legionella are Gram negative, rod shaped, oxidase negative, urease negative, and strictly aerobic species of bacteria (Winn Jr, 2015). Most species of *Legionella* are found ubiquitously in aquatic ecosystems, such as rivers or lakes (Fliermans et al., 1981; Kwaik et al., 1998). However, several species, such as *L. pneumophila* serogroup 1 and *L. longbeachae*, have been found in soil and compost samples (Travis et al., 2012; Wallis and Robinson, 2005). Interestingly, *Legionella* species have not been found in marine environments, and research has shown a decrease in counts as salt concentration increase in river ecosystems flowing into coastal areas (Fliermans, 1983).

All known *Legionella* species are host adapted and can grow in different protozoan host cells, such as amoeba and ciliates (Amaro et al., 2015; Boamah et al., 2017). Most species are believed to be facultative intracellular parasites of these protozoa, but recent findings seem to suggest that certain species may be obligate host adapted bacteria, either parasitic or symbiotic. For instance, *Candidatus* Legionella polyplacis is an obligate symbiont of different species of lice from the genus *Polyplax* (Říhová et al., 2017). It was suggested that *L. polyplacis* produces biotin in exchange for nutrients from its host (Říhová et al., 2017). On the other hand, *L. drancourtii* is an obligate parasite of different species of amoeba, growing inside its host and causing lysis at temperatures from 25°C to 32°C (La Scola et al., 2004).

In the case of *L. pneumophila*, around 30 different species of protozoa have been experimentally confirmed as its host (Boamah et al., 2017). Some of these species can be viewed in Table 2. It is believed that many more host species have yet to be characterized. These host species span various phylogenetic groups. For instance, *Acanthamoeba castellanii*, *Naegleria fowleri*, *Tetrahymena pyriformis*, and human macrophages are host species belonging to different

eukaryotic phyla (respectively *Amoebozoa*, *Percolozoa*, *Ciliophora*, *Chordata*) and are routinely used as host models for research (Boamah et al., 2017).

Table 2: Experimentally verified host cells species of *Legionella pneumophila* (adapted from (Boamah et al., 2017).

Host Phylum	Host species	Common Name
Amoebozoa	*Acanthamoeba castellanii*	Amoeba
Amoebozoa	*Acanthamoeba lenticulata*	Amoeba
Amoebozoa	*Acanthamoeba palestinensis*	Amoeba
Amoebozoa	*Acanthamoeba polyphaga*	Amoeba
Amoebozoa	*Acanthamoeba royreba*	Amoeba
Amoebozoa	*Balamuthia mandrillaris*	Amoeba
Amoebozoa	*Dictyostelium discoideum*	Amoeba/slime mold
Amoebozoa	*Echinamoeba exudans*	Amoeba
Amoebozoa	*Hartmanella cantabrigiensis*	Amoeba
Amoebozoa	*Vermamoeba vermiformis*	Amoeba
Ciliophora	*Paramecium caudatum*	Ciliate
Ciliophora	*Tetrahymena pyriformis*	Ciliate
Ciliophora	*Tetrahymena thermophila*	Ciliate
Percolozoa	*Naegleria fowleri*	Amoeba
Percolozoa	*Naegleria gruberi*	Amoeba
Percolozoa	*Naegleria jadini*	Amoeba
Percolozoa	*Naegleria lovaniensis*	Amoeba
Percolozoa	*Tetramitus jugosus*	Amoeba
Percolozoa	*Willaertia magna*	Amoeba

Although some differences in the intracellular life cycle of *L. pneumophila* exist between different species of host cells, in general the life cycle of *L. pneumophila* can be summarized into four general stages (Eisenreich and Heuner, 2016). In the first stage, *L. pneumophila* cells must be ingested by the host cell. The type of ingestion of *L. pneumophila* cells will depend on the species of host. Amoeba will usually ingest through coiling phagocytosis, by extending their pseudopods, or by pinocytosis (Horwitz, 1984; Watarai et al., 2001). Conversely, ciliates will use their cilia to move *L. pneumophila* cells into their mouth cavity, where a vacuole will form containing the ingested bacteria. Depending on the species of host cell infected, *L. pneumophila* can use different genetic and biochemical systems during the initial infection step (Harb et al., 1998).

In the second stage, *L. pneumophila* cells are captured in a vacuole, called the phagosome, following initial engulfment. Normally, the phagosome will be used to degrade and recycle biological materials, such as prey cells, for nutrition and growth of the host cell. This is accomplished when the lysosome fuses with the phagosome (containing the bacteria). The lysosome is an organelle in eukaryotic cells, containing hydrolytic enzymes (Ballabio, 2016). The fusion of the lysosome to the phagosome will unload hydrolytic enzymes and create an acidic pH, changing the phagosome into a phagolysosome, which will degrade the biological material within the phagolysosome (Ballabio, 2016). However, in the case of *L. pneumophila*, this process will be stopped. In order to this, *L. pneumophila* will secrete a high number of different effector proteins using a type IVB secretion system (TIVBSS) into the intracellular environment of the host cell (Isberg et al., 2009). These effector proteins create a hospitable growth environment within the host cell's phagosome (Ninio and Roy, 2007), known as a *Legionella* Containing Vacuole (LCV). The effectors modify vesicular trafficking (Isberg et al., 2009), inhibiting the fusion of the lysosomes to the phagosome and recruiting vesicles from the endoplasmic reticulum, as well as

mitochondria, to the LCV (Isberg et al., 2009; Ninio and Roy, 2007). The endoplasmic reticulum vesicles will stud the LCV and be used as a source of nutrients, feeding peptides chains and proteins to the LCV. It is at this stage that *L. pneumophila* differentiates into the replicative phase (Eisenreich and Heuner, 2016).

During the third stage, *L. pneumophila* will replicate and consume the nutrients of the host cell. Once the nutrients are scarce the bacteria differentiate into a transmissive form and then to a dormant form called the Mature Infectious Form or MIF (Eisenreich and Heuner, 2016). During the replicative phase, *L. pneumophila* is characterized as non-infectious and sensitive to stress, whereas, in the transmissive phase and the MIF, the bacteria is becomes infectious to host cells, flagellated, and resistant to stress (Robertson et al., 2014).

Finally, in the fourth stage, *L. pneumophila* MIF cells are released from the LCV to the cytosol of the host cell. *L. pneumophila* will then induce host cell lysis, using its effectors and other enzymes in order to be released into the environment (Eisenreich and Heuner, 2016). These newly released *L. pneumophila* cells are then ready to infect new host cells or survive in the aquatic environment for prolonged periods of time (Mendis et al., 2015; Shaheen et al., 2019).

3. LEGIONELLA PHYLOGENY

The genus *Legionella* encompasses around 50 different species, with the type species being *L. pneumophila*. The genus is part of the *Proteobacterium* phylum and the *Legionellales* order. A characteristic of this order is that all known species are host-adapted to various degrees, from facultative to obligate intracellular lifestyles (Graells et al., 2018). This host adaptation is possible through the possession of a TIVBSS and an enormous arsenal of effector proteins, around 18,000

for the *Legionella* genus (Gomez-Valero et al., 2019; Graells et al., 2018). The *Legionellales* order is divided into two families: the *Legionellaceae* and the *Coxiellaceae* (Duron et al., 2018; Graells et al., 2018). However, with recent advances in sequencing, the diversity of the *Legionellales* order seems to be increasing as new lineages are being uncovered, such as *Nucleophilum*, and *Aquicella* (Duron et al., 2018). Furthermore, the *Legionellales* order is much more widespread around the world than previously thought (Graells et al., 2018). *Legionellales* species have been identified in marine environments, polar lakes, sediment and soil samples (Carvalho et al., 2008; Graells et al., 2018; Whiley and Bentham, 2011). The environments harbouring *Legionellales* are much more diverse than previously believed due to the bias, in the beginning, of isolating *Legionella* species from EWSs. Accordingly, the diversity of host species of *Legionellales* is much higher than previously believed, as several *Coxiellaceae* species are host adapted to invertebrates and different marine protozoa. For examples, some *Aquicella* species infect insects and crustacean (Bojko et al., 2018; Duron et al., 2018).

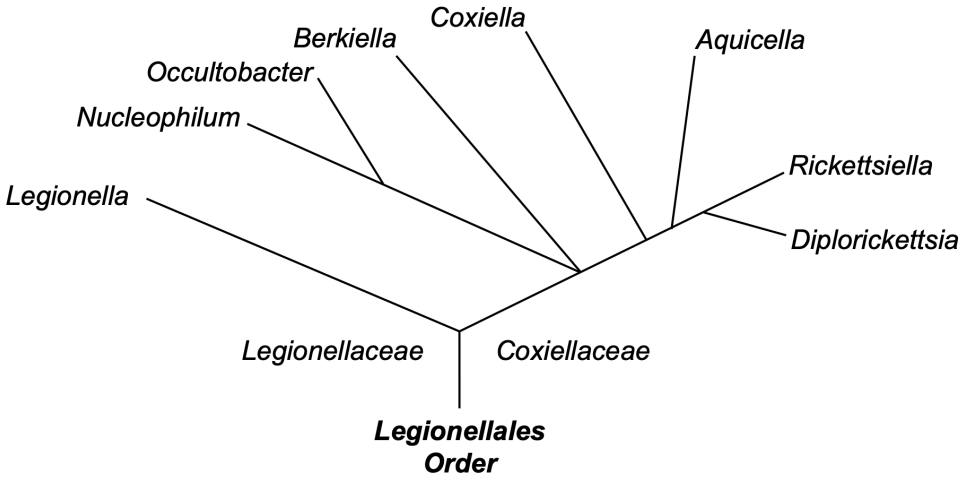

Figure 1: Phylogeny of the *Legionellales* order (adapted from *Duron et al., 2018*).

III- MICROBIOLOGY OF ENGINEERED WATER SYSTEMS

1. ENGINEERED WATER SYSTEMS

Engineered water systems (EWSs) comprise all of the infrastructures used for supplying, distributing, consuming, and evacuating water to and from an urban and industrial areas. Common examples of EWSs are water treatment plants, water distribution networks (pipes system), reservoirs, premise plumbing, sewage systems, and cooling towers. Generally, urban water systems can be divided into two groups: the supply system and the sewage systems (Wong, 2006). The supply system consists of the infrastructures that treats, holds, and distributes water designated for a specific process. Examples of water supply system infrastructures are water treatment plants, potable water distribution systems, or potable water reservoirs. In developed countries, usually raw water from a natural source is directed to a treatment plant, where it is decontaminated and disinfected to safe levels. This is done through several processes such as filtration, coagulation, flocculation, sedimentation and chlorination (Lesnik et al., 2016). The water is then distributed to a series of pipes and reservoir to urban areas for consumption for different uses (Mei and Liu, 2019). On the other hand, the sewage system consists of the infrastructures that collects and handles used water (Wong, 2006). Examples of sewage systems infrastructures are sewage pipe systems, wastewater treatment plants, and the rainwater collection systems. The used water will be collected and distributed to a wastewater treatment plant, where several steps are taken to purify the water before it is dumped into a receiving body of water (Mei and Liu, 2019).

From a microbiological perspective, the supply and sewage systems will present different patterns of microbial communities. Though generally perceived by the public to be sterile, the supply system is far from it, with several bacterial phyla found to colonize these systems. For

instance, *Proteobacteria* and *Bacteroidetes* are commonly found in drinking water systems (Vaz-Moreira et al., 2014). Furthermore, different stages and components of water systems can have vastly different microbial communities. For instance, Lin et *al* demonstrated that different eukaryotic and bacterial communities thrive in different stages of a treatment plant generating potable water (Lin et al., 2014). The authors compared the biofilms and the water of the reaction tank and the settling pond and found that microalgae (*Chlorophyta*) were more abundant in the biofilms of the reaction tanks, whereas, *Rhizaria* groups were more abundant in the biofilms of the settling ponds (Lin et al., 2014). In contrast, the water phase of these two environments were dominated by the fungal phylum *Ascomycota* (Lin et al., 2014). Consequently, EWSs are highly dynamic microbial environments. In the work presented here, we will focus on the microbiology of the supply systems, paying more attention to cooling towers, as these are the environments most associated with *Legionella* outbreaks.

A. COOLING TOWERS

Cooling towers are heat exchange devices used to cool down water for different processes. They are most commonly used for air conditioning systems but can also be used for refrigeration systems and industrial purposes requiring cooling, most notably with nuclear power plants.

Cooling towers are categorized based on several characteristics, such as by use (industrial vs non-industrial), by heat transfer methods, by air draft generation, and by air flow direction (Hensley, 2009). In terms of heat transfer methods, cooling towers can either be categorised as wet (also known as evaporative), dry, or hybrid cooling system (Hensley, 2009). In the case of wet towers, hot water intended for cooling is sprinkled on to a heat exchange device, called the fill.

The fill is usually composed of materials, such as sheets of plastic or slats, that create splashing of the sprinkled hot water (Hensley, 2009). Air vents allow ambient air to flow through the cooling tower and into the fill in order to exchange heat between the hot water and the colder air. The fill increases surface area and contact time between the water and the air. Figure 2 shows a schematic of a wet cooling tower. The cooled down water is collected in a basin and is either recirculated in the system for reuse or discarded in the sewage system. On the other hand, dry cooling towers do not use evaporation as a heat transfer method, but rather exchange heat through a surface that divides the hot water from the cold air (Hensley, 2009). An example of this method is using a coiling tube containing hot water which receives air flow to cool it down. We will focus our discussion on wet cooling towers systems, as dry systems are not believed to disseminate *Legionella* species (Hunt et al., 1991).

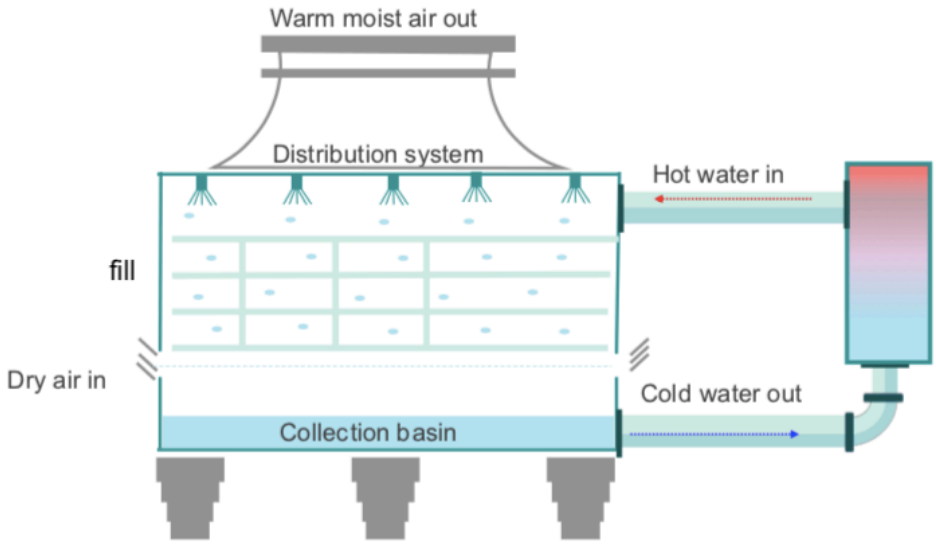

Figure 2: Diagram of cooling tower structure, adapted from Torres, 2019 (Torres Paniagua, 2019).

Due to their design, evaporative cooling towers produce high amounts of aerosols. During outbreaks of LD, it is generally believed that aerosols are contaminated with the bacteria and are disseminated in the surrounding environments, sometimes as far as 12 km (Baskerville et al., 1981; Hambleton et al., 1983; Nhu Nguyen et al., 2006). Individuals around the cooling tower inhale the bacteria and are at risk of becoming sick. Interestingly, *L. pneumophila* growth phase can have an effect on its survivability in the aerosol. Hambleton et *al* demonstrated that bacteria in the exponential growth phase survived less well than stationary phase bacteria when aerosolized (Hambleton et al., 1983).

Since cooling towers are a major source of large outbreaks of LD, several governments have enacted policies in order to measure to reduce growth of *L. pneumophila* in cooling towers. For instance, it is mandatory for cooling tower operators in Quebec to monitor *L. pneumophila* levels and to enact corrective measures if levels are between 10 000 CFU/L and 1 000 000 CFU/L, and to completely shut down and decontaminate the tower if levels are higher than 1 000 000 CFU/L (Québec, 2014). The exact mechanisms and factors that lead to the colonization, survival, and proliferation of *L. pneumophila* in the cooling tower environment are still not well understood.

Indeed, the prevalence of *Legionella* and *L. pneumophila* can vary from tower to tower, with some tower being perpetually problematic whereas other show very little colonization. In a recent study, which examined 196 wet cooling towers spread around the continental US, Llewellyn et *al* found that 84 % of towers were positive for the genus *Legionella*, but that *L. pneumophila* could only be isolated from 32 % of the positive samples (Llewellyn et al., 2017). Though the exact causes for why certain towers are contaminated with *L. pneumophila* and other are not is not well understood, several key physicochemical parameters along with several key microbial species are important factors for *L. pneumophila* colonization, survival, and proliferation. Consequently,

this has led several investigators to look into the effect of the microbiome of cooling towers and its relationship with *Legionella* ecology. We will discuss these factors and how they relate to *L. pneumophila* ecology in EWSs in the next section.

2. *Microbial Diversity of Engineered Water Systems*

As mentioned before, the supply system handles treated water that has been decontaminated and disinfected in order to reduce the presence of certain chemicals and the microbial load to safe levels. As a result, these systems are highly oligotrophic environments, but still harbour an impressive diversity and abundance of microorganisms. Next generation sequencing (NGS) has been used to characterize the microbial communities in these systems. So far, species from all three domains of life have been detected in these environments.

It is now understood that these microorganisms can influence the management of these systems due to costs associated with damage to the systems and risk to public health (Ji et al., 2015). Indeed, microorganisms are known to damage water distribution equipment through their metabolic activities. For instance, sulphur-reducing bacteria, such as *Desulfovibrio vulgaris*, can cause corrosion of pipes and metal vessels by producing hydrogen sulphide (Beech and Sunner, 2004). Other microorganisms produce biofilms, which can cause blockage by a process known as biofouling. Lastly, several eukaryotic and bacterial species inhabiting supply systems are known to cause disease in human. In the developing world, *L. pneumophila* is the leading cause of water born disease, however several other pathogens are also associated with these types of disease, such as *Pseudomonas spp.*, *Mycobacterium spp.*, *Acanthaemoba spp.*, *Naegleria spp.* (Wang et al.,

2013). Consequently, understanding the microbial diversity of water systems and the processes that shape it can be of vital importance for health management and economical purposes.

A. BACTERIAL DIVERSITY

Bacterial communities are by far the most studied communities in EWSs. More than 48 different phyla have been identified through NGS, with several operational taxonomic units (OTUs) being grouped in unclassified taxa (Proctor and Hammes, 2015). For the most part, *Proteobacteria* seem to be the dominant phylum in the supply system in most developed countries, sometimes reaching up to 90% of the community (Delafont et al., 2016; Ivone et al., 2013; Li et al., 2010; Pavissich et al., 2010; Pinto et al., 2014; Williams et al., 2004). Indeed, a study by Holinger *et al* showed that *Proteobacteria* were the dominant phylum in tap water samples collected from the Arkansas River headwaters region (Colorado) to the mouth of the Mississippi River, New Orleans (Holinger et al., 2014). In France, Poitelon *et al* demonstrated that *Proteobacteria* dominated the microbial community of Parisian water treatment plants with relative abundance level between 57.2 % and 77.4% (Poitelon et al., 2009a). Similar findings were reported in Portugal, Greece, Germany and China (Chao et al., 2013; Eichler et al., 2006; Ivone et al., 2013; Kormas et al., 2010). Though not studied as much as other EWSs, cooling towers are also dominated by *Proteobacteria*. For instance, Llewelyn et *al* found that *Proteobacteria* constituted, on average, 79.5% of the bacterial communities of 196 cooling towers from several different regions in the USA (Llewellyn et al., 2017). Similar results have been found in Europe (Di Gregorio et al., 2017; Pereira et al., 2017).

Other bacterial phyla are also identified in these systems, but at lower levels than the *Proteobacteria* phylum. *Cyanobacteria*, *Firmicutes*, *Actinobacteria, Planctomycetes, Bacteroidetes, Acidobacteria*, *Gemmatimonadetes*, *Verrucomicrobia* and *Chlamydiae* are commonly detected (Delafont et al., 2016; Holinger et al., 2014; Ji et al., 2015; Li et al., 2010). Their levels can vary depending on several factors, such as physicochemical parameters, geography, and stage of the supply system; however, they constitute usually less than 50 % of the bacterial community. For instance, Holinger et *al* found that *Actinobacteria* represented 25 % of all sequences whereas *Cyanobacteria* composed around 30 % of the community in a drinking water distribution system (Holinger et al., 2014). On the other hand, Buse et *al* found that the relative abundance of *Actinobacteria* and *Bacteroidetes* was, respectively, between 3.5 % - 9.0 % and 2.0 % - 10.8 % for tap water biofilms produced under different conditions (Buse et al., 2017). Ji *et al* found that the *Actinobacteria* community varied between 1 % - 30 %, the *Cyanobacteria* community varied between 2 % - 10 %, and the *Bacteroidetes* community varied from below 1 % to around 10 % of the microbial population from different sampled sites of a water treatment plant (Ji et al., 2015).

Interestingly, the microbial composition of natural freshwater environments (lakes and rivers) and EWSs is different. *Actinobacteria* represent a much more important group of the microbial community of natural systems. In fact, they are present in relatively equal proportion with the *Proteobacteria* population in natural aquatic environments, sometimes even dominating the community (Newton et al., 2011). This higher abundance of *Actinobacteria* seems to be a global trend as several sampling sites of freshwater systems around the world have shown similar results. For instance, Ghai et *al* found that *Actinobacteria* were the dominant phylum in the headwaters of the Amazon River, in a region 420 km upstream from Manaus, Brazil, and thus with

little impact from human activity (Ghai et al., 2011). Meanwhile, in France, a metagenomic study that examined the bacterial community of the Lac du Bourget found that *Actinobacteria* (43.5%) were the most dominant phylum followed by the *Betaproteobacteria* (22.9%) and the *Alphaproteobacteria* (14%) classes, and the *Bacteroidetes* (7%) phylum (Debroas et al., 2009). Similar findings were found for lake Tanganyika (East Africa), the White Volta basin (Burkina Faso), the Danube River (East Europe), and Tyrolean lakes, Austria (De Wever et al., 2005; Humbert et al., 2009; Savio et al., 2015; Warnecke et al., 2005).

The decrease of the Actinobacterial population in EWSs is not well understood; however, several factors may contribute to its disappearance. For instance, water treatment by chlorination was shown to select for the *Alphaproteobacteria* class in a Chinese water treatment plant, whereas, stagnation of water in piping systems was shown to increase levels of *Proteobacteria* (Chao et al., 2013; Pinto et al., 2014). Thus, the reduction in *Actinobacteria* may be due to unfavourable growth conditions present in EWSs.

Moreover, several candidate phyla have been identified in EWSs. For instance, Pinto et *al* characterized the bacterial community of a drinking water treatment plant and its water distribution system in Ann Arbor, USA. They found that the candidate phylum OD1 was the second most abundant phylum, composing 6.5 % of the sequences detected (Pinto et al., 2014). As of now, the OD1 candidate phylum contains no cultivable representative species. Members of this group seem to be distributed in anoxic environments, especially freshwater, marine and terrestrial environments around the world (Elshahed et al., 2005; Nelson and Stegen, 2015). Other candidate phyla have also been detected in EWSs through sequencing studies. Ji et *al* detected the TM6 and WPS-2 candidate phyla in water treatment plants and Lautenschlager et *al* detected several candidate phylum sequences, such as SC3, TM7, WS3, OD1, OP11, TM6, ZB2, OP3 and GN02,

in the reservoir and the effluent water of different filtration systems in water treatment plants in Zurich, Switzerland (Ji et al., 2015; Lautenschlager et al., 2014). Cooling towers can also harbour some of these candidate taxa. For instance, Pereira et *al* were the first to identify the environmental group ARKICE-90 in cooling towers (Pereira et al., 2017). This group was first detected from arctic ice pack samples in 2003, but since then, it has been detected in drinking water systems and sludge bioreactors (Pereira et al., 2017). Though these candidate phyla communities usually represent a very low portion of the bacterial community, their presence seem to add a layer of complexity to the EWSs environment.

When examining the bacterial community at lower taxonomic ranks it is much harder to discern a core community that is prevalent amongst all EWSs. Indeed, the bacterial genera identified in the different EWSs are much more variable. This variability is partly due to certain characteristics of the ecosystem studied but may also be due to random events. For instance, when comparing the biofilm phase vs water phase of different stages of a treatment plant, Lin et *al* identified higher levels of *Rhizobiales* order (*Alphaproteobacteria*) in the biofilms then in the water phase, and the opposite for the *Sphingomonadales* order (Lin et al., 2014). In contrast, the *Pseudomonadales* order showed random variations in abundance between the different samples (Lin et al., 2014).

Finally, EWSs contain an enormous diversity of bacterial genera. Genera of medical concern are usually the most studied (Falkinham et al., 2015). OPPPs are different than classic waterborne pathogens, such as *E. coli* or *Salmonella*, as they are natural inhabitants of EWSs and can grow and survive within these systems (Falkinham et al., 2015). Generally, *Legionella*, non-tuberculosis *Mycobacterium*, and *Pseudomonas* are the most important genera of OPPPs (Falkinham et al., 2015). Examples of other genera that have been identified from EWSs and

cooling towers are : *Acidovorax*, *Acinetobacter*, *Aeromonas*, *Bacillus*, *Blastomonas*, *Brevundimonas*, *Flavobacterium*, *Hydrogenophaga*, *Limnobacter*, *Methylobacterium*, *Nevskia*, Novosphingobium, *Sphingomonas*, *Sphingobium*, *Xanthobacter* (Di Gregorio et al., 2017; Wang et al., 2013). These genera are less of a concern medically, but with the era of "microbiome" studies, more and more research is examining the effect of the microbiome of EWSs on survival and proliferation of OPPPs (Wang et al., 2013). On the other hand, some of these bacteria may cause problems from an engineering perspective. Indeed, production of biofilms or metabolites can cause loss of water quality or damage the equipment (Beech and Sunner, 2004).

Thus, the bacterial diversity is much more complex at lower taxonomic levels. The dominance of a specific species or genus in a water system seems to be dependent on several factors, such as the type of system and the phase (planktonic vs biofilm) studied.

B. EUKARYOTIC DIVERSITY

Research on eukaryotic diversity in EWSs is far more limited compared to that of bacterial diversity, and even less for cooling tower environments. This is probably due to the fact that the eukaryotic community is present at much lower levels than the bacterial community in EWSs. For instance, the amoeba population of treated water of four drinking water treatment plants in France and Spain varied between 0.1 and 46 MPN (Most Probable Number)/L (Loret et al., 2008). The rotifer and nematode (micro-animals) population of GAC filtered water of a water treatment plant along the Rhine River were around 5.5 rotifer/L and below 19 nematodes/L (Schreiber et al., 1997). Bichai et *al* reported that the average zooplankton concentration of three water treatment plants in Amsterdam was around 13 organisms/L (Bichai et al., 2011). In contrast, the bacterial population of EWSs usually ranges from 10^3 to 10^9 CFU/L, depending on the system studied. These numbers

have been observed in water distribution systems (hot and cold drinking water) and cooling towers (Perrin et al., 2019; Serrano-Suárez et al., 2013; Yamamoto et al., 1992).

Regardless of this low abundance, eukaryotic diversity is quite high in EWSs. For instance, *Alveolata* (Protist), *Ascomycota* (Fungus), *Bacillariophyta* (Diatoms), *Basidiomycota* (Fungus), *Cercazoa*, *Ciliophora* (Ciliates), *Chlorophyta* (Microalgae), *Intramacronucleata* (Ciliates), *Lobosea* (Amoeba), *Myzozoa*, *Percolozoa* (Amoeba/Flagellates), *Rhizaria*, *Tubulinea* (Amoeba), and *Stramenopiles* are just a few examples of phyla or groups of microorganisms that have been identified in various EWSs, such as water distribution systems, treatment plants, drinking water taps, or reservoirs (Buse et al., 2013; Lin et al., 2014; Magnet et al., 2013; Poitelon et al., 2009b; Valster et al., 2011).

Furthermore, unlike the bacterial population, which was dominated by the *Proteobacteria* phylum, no single eukaryotic phylum seems to dominate all EWSs. Instead, different phyla will dominate different systems depending on various factors. For instance, Buse et *al* studied the eukaryotic community of hot and cold drinking water distribution system over a year and showed that the community was not dominated by a single phylum (Buse et al., 2013). Rather, this dominance was shared between several eukaryotic groups that varied and shifted across the sampling period (Buse et al., 2013). Furthermore, the cold water system was dominated by different organism than the hot water system, indicating that temperature may be a factor (Buse et al., 2013). Similarly, Lin et *al* showed that biofilms from different sites were dominated by different taxa in a water treatment plant (Lin et al., 2014). In terms of community ecology, these findings seem to indicate that the eukaryotic community are unstable and may be prone to change frequently.

In the case of cooling towers, research on the eukaryotic community is very scarce. Up to now, most research focuses on the *Legionella* host species, especially the Free-Living Amoeba (FLA) community, as they are believed to be the reservoir of *Legionella* and can cause disease in humans (Berk et al., 2006; Scheikl et al., 2016; Tsao et al., 2019; Valster et al., 2009). Additionally, other studies have focused on the FLA community but in other systems, such as different water distribution systems. Consequently, the results from those studies could also hold true in cooling towers. In terms of diversity, various species have been detected in EWSs and cooling towers. For instance, *Acanthamoeba castellanii*, *Acanthamoeba polyphaga*, *Balamuthia mandrillaris*, *Echinamoeba thermarum*, *Neoparamoeba aestuarina*, *Naeglaeria andersoni*, *Naegleria fowleri*, *Naeglaeria gruberi*, *Saccamoeba spp.*, *Vahlkampfia spp.*, *Vanella cirrifera,* and *Vermamoeba vermiformis* (previously known as *Hartmanella vermiformis*) are all species that have been detected in these systems (Hoffmann and Michel, 2001; Magnet et al., 2013; Otterholt and Charnock, 2011; Thomas and Ashbolt, 2010; Thomas et al., 2010; Valster et al., 2009). Consequently, a diverse number of amoebae are present in EWSs. These FLA are important for the ecology of the EWSs as they graze on the microbial community.

A part for the FLA population, other eukaryotic organisms are also of potential importance for the ecosystem of cooling towers. For instance, microalgae, fungal groups, ciliates, and micro-animals have been identified in cooling towers and various other EWSs (Buse et al., 2013; Lin et al., 2014; Tsao et al., 2019; Wang et al., 2014). Due to their different trophic levels these different organisms can have profound effects on the ecology of EWSs. For instance, microalgae are photoautotrophic and thus are an important primary producer. Consequently, they may be of importance for growth of the FLA community. Additionally, new research has come to show that the ciliate population may be of importance for *Legionella* outbreaks (Tsao et al., 2019). However,

very little research is done on these communities in EWSs. More research needs to be invested in these communities to better understand their role in ecosystem and potentially how it relates to *Legionella* ecology. For instance, Fungal groups are heterotrophs and thus depend on organic matter for their survival and proliferation. Some species are associated with deterioration of wooden elements of cooling towers (Savory, 1954; Udaiyan and Manian, 1991). Consequently, they can be problematic for the proper functioning of cooling towers. *Coeloanguillospora appalchiensis*, *Monodictys levis*, *Sclerotium sp.*, and *Veronaea botryose* have been discovered in the make-up water of cooling towers, whereas, *Cladosporium oxysporum*, *Coriolus versicolor*, *Curvularia pallenscens*, *Goniotrichum sp*., and *Torula caligans* are example of species that have been discovered in water basin and the inlet spray nozzle (Udaiyan and Manian, 1991).

Micro-animals, such as rotifers and nematodes, have also been discovered in the cooling tower environment. These organisms can graze on the microbial population and detritus in cooling towers environment. As a result, they are important in recycling nutrients and can affect the microbial composition of an ecosystem by their grazing choices. It is unclear how these organisms affect *L. pneumophila* ecology. They could potentially consume *L. pneumophila*, host cells, or biofilms. However, it has been shown that *L. pneumophila* can colonize the gut of certain nematode species (Brassinga et al., 2010; Rasch et al., 2016). This could be a means by which the bacteria can protect and disseminate itself (Rasch et al., 2016). Interestingly, carnivorous or "nematophagous" fungi are known predators of nematode species in soil ecosystems, causing inhibition of the nematode population (Lopez-Llorca et al., 2008). This may also be the case in cooling towers and could be a means by which the fungal groups could shape certain elements of the microbiome. Further research should be undertaken to discern if these nematophagous species are present in the cooling tower environment.

C. ARCHAEAN COMMUNITIES OF COOLING TOWERS AND OTHER ENGINEERED WATER SYSTEMS

Archaea are the third domain of life and represent the least studied group in the supply system of EWSs. Most research is focused on Ammonia Oxidizing Archaea (AOA) as these are important for water quality. Indeed, ammonium in raw water can be problematic as it is toxic, increases the demand for chlorine disinfection, and can be a precursor for trichloramine, which gives water an undesirable odour and taste (Niu et al., 2013). Granulated activated charcoal filters are used to reduce the ammonium coming from raw water as it is believed that they can harbour bacteria and archaea that can oxidize ammonium to nitrite, through nitrification (Niu et al., 2013). In addition, monochloramine is sometimes used as a disinfectant to reduce biofilm production. However, this can release ammonium in the water, which can be used by the AOA (Waak et al., 2019). Consequently, AOAs may have an important role in the nitrogen cycle in EWSs.

Little is known about the other archaeal groups. This may be due to the scarcity of reliable methods that can identify and distinguish different archaeal organisms. Additionally, the bias of associating archaea with extreme environments, such as deep-sea hydrothermal vents or geysers, is probably a reason for reduced attention of this population in EWSs, as these habitats are not considered extreme (Fischer et al., 2016).

In the case of cooling towers have been shown to contain archaea, but very little is known about their diversity or ecology in these systems. Di Gregorio et *al*, used fluorescence in situ hybridization techniques to show that archaeal cells were present in the source water feeding a cooling tower at around 10^4 cell/ml (Di Gregorio et al., 2017). Further research is required to understand their effect on the cooling tower ecosystem and if this is relevant for *Legionella* ecology.

3. *Legionella Ecology in Engineered Water Systems and Cooling Towers*

Though the exact mechanisms that lead to outbreaks or sporadic cases of Legionnaires disease are still not well understood, it is believed that *L. pneumophila* must accomplish certain steps in order to be successful. As a result, the first step is colonization of the water system, followed by survival in this environment, and then proliferation of the *L. pneumophila* population. In this regard, the ecology of the bacterium plays an important role, including the interactions with its environment and with other organisms in that environment, the results of which will dictate whether or not *L. pneumophila* will thrive.

So far, most research on *L. pneumophila* ecology focuses on a variety of different EWSs. Research in cooling tower ecology is more limited. As cooling towers are composed of various parts, this creates sections that are similar to other water systems and unique ones to cooling towers. For instance, the inlet nozzle spray and the collection basin are similar ecosystems to showerheads and water reservoirs, respectively. In contrast, the fill can be considered unique to cooling towers. Massive amounts of air from the surrounding environment are channelled through the fill, leading to colonization of these systems by airborne microorganisms. As water is sprayed on the fill, this also leads to the microbiome of the fill to be colonized by organisms from the water system. The water and air also create unique physicochemical parameters in the fill, such as creation of aerosols or rapid changes in temperature.

Finally, managerial practices, some of which are unique to cooling towers, can affect the chemistry of certain areas. Thus, the water basin usually has an alkaline pH around 8.0 in order to reduce corrosion and to increase effectiveness of chlorination (Conley et al., 1987). Additionally, the use of specific types of biocides and frequency of application are unique characteristics of cooling towers and can affect *L. pneumophila* colonisation, survival, and proliferation (Kurtz et

al., 1982). Consequently, this makes cooling towers an interesting ecosystem to study. In the next section, we will discuss the parameters and microbial interactions that are important for *L. pneumophila* ecology that affect the three steps mentioned earlier.

A. PHYSICOCHEMICAL PARAMETERS THAT AFFECT *L. PNEUMOPHILA* ECOLOGY

TEMPERATURE

One of the more important factors for *L. pneumophila* proliferation is temperature. *L. pneumophila* has an optimal growth temperature of 35°C on buffered charcoal yeast extract agar; however, its suboptimal growth range is from 25°C to 44°C (Kusnetsov et al., 1996; Rowbotham, 1980). This temperature range is commonly found in EWSs, such as cooling towers or hot water distribution systems. For instance, Yamamoto et *al* surveyed 40 cooling towers and found that the temperature of the water basin varied between 8.3°C to 35.2°C, with most (87%) of the towers having a temperature above 20°C (Yamamoto et al., 1992). This study also found that the *Legionella* counts correlated positively with temperatures.

The effects of temperature are further demonstrated by the fact that the number of LD cases increase during the summer and early autumn period (Phin et al., 2014). This spike in the number cases of LD in the summer months is seen around the world. For instance, Europe and the United States have all reported similar spikes in LD cases during that time period (ECDC, 2019; Shah et al., 2018). This increase in LD cases is hypothesized to be related to increased warm and humid weather (Fisman et al., 2005; Ricketts et al., 2011). This would suggest that climatic environments influence the risk of LD.

Moreover, natural water systems, such as lakes and rivers, harbour *Legionella* species, however, they are never the source of outbreaks of LD. Potentially, this may be due to lower temperatures which hinder *L. pneumophila* growth. This is indirectly demonstrated by the fact that natural hot springs are sources of outbreaks, mostly in eastern Asian countries (Matsumoto et al., 2004; Nakamura et al., 2003). Indeed, in these environments, temperatures can vary from 22°C to 48°C and different species of *Legionella* have been detected in Japan and Taiwan (Huang et al., 2010; Lin et al., 2006).

Below 25°C and above 44°C, *L. pneumophila* does not generally grow, but will survive in the environment (Kusnetsov et al., 1996). The length of survival can vary with temperature. Mendis et *al* showed that *L. pneumophila* could survive in water for around 50 days at temperatures lower than 37°C, however at higher temperatures, the survival decreased drastically (Mendis et al., 2015). Temperatures at 15°C also seemed to promote survival (Mendis et al., 2015).

Though survival decreased overtime at different rates for different temperatures, *L. pneumophila* infectivity was retained for various host species was throughout the incubation periods (Mendis et al., 2015). The effects of growth temperature on virulence of *L. pneumophila* have been studied previously (Edelstein et al., 1987). The results demonstrated that the same strain grown at different temperature in liquid media could have different infectivity levels when growing within host cells. As a result, it was suggested that lower growth temperatures increased the virulence of *L. pneumophila* by affecting bacterial adherence to host cells (Edelstein et al., 1987). Consequently, temperature is an important factor to consider as it affects the growth, survival, and virulence of *L. pneumophila* in water systems.

DISINFECTION TREATMENTS

Disinfection practices are commonly used for reducing microbial loads in EWS. Physical disinfection procedures, such as flocculation, filtration, or pasteurization are usually first used. These procedures are effective at reducing the microbial load. For instance, Lesnik et *al* showed that flocculation and filtration was able to reduce by 75% the total number of cells from raw water treated at a plant (Lesnik et al., 2016). However, it has been shown that the communities that pass through the filtration step and the communities present at the filters can seed and shape the microbiome of downstream EWSs (Pinto et al., 2012). This is due to these microorganisms being able to grow in oligotrophic environments, such as downstream EWSs. This is also applicable to the *Legionella* population. For instance, in the same study by Lesnik et *al*, the treatment mentioned above reduced substantially the abundance and complexity of the *Legionella* population compared to the raw water (Lesnik et al., 2016). However, the *Legionella* population was restored further downstream in the distribution system to levels equivalent to the raw water (Lesnik et al., 2016).

Moreover, chemical disinfectants are used in conjunction with the physical disinfectant measures. These disinfectants can be classified as oxidative or non-oxidative. Chlorine, monochloramine, organic biocides, copper-silver ionization, ozone, isothiazolines, and chlorine dioxide are used as disinfectant (Iervolino et al., 2017; Lin et al., 2011). Chlorine and bromine products are generally used as oxidative disinfectant in cooling towers (Rangel, 2010). In Quebec, disinfection can be applied either continuously, weekly, or daily. These treatments have different efficiencies in controlling *Legionella* growth, with continuous treatment generally believed to be the better option (Biurrun et al., 1999; Orsi et al., 2014; Paranjape et al., 2020). Indeed, periodic (also called hyperchlorination when using chlorine) treatment are at times very efficient in decreasing the microbial load, but their effects are not long lasting. Consequently, following this

type of treatment, regrowth is typically observed in EWSs and cooling towers. Furthermore, hyperchlorination can be expensive and can be inadequate when biofilms are present or if only one injection point is used (Lin et al., 2011). Thus, complicated systems, such water distribution system can have lowered efficiency in decreasing the microbial lowed when using hyperchlorination.

Furthermore, certain disinfection treatments can either promote evolution of increased resistance or can create a viable but not culturable (VBNC) state in the *Legionella* population (Kuchta et al., 1985). VBNCs are cells which are viable but cannot be grown on CYE plates (Dietersdorfer et al., 2018). Consequently, they are of great concerns since they are still infectious, but undetectable by culture methods. Several disinfection methods can induce VBNC state, such as chlorination and heat (Allegra et al., 2008; García et al., 2007).

Other Parameters Affecting *L. pneumophila* Ecology in Engineered Water Systems

Water stagnation is an import factor in *Legionella* ecology. This is especially true for cooling towers that are infrequently used, where water can accumulate and stagnate in the various pipes and reservoirs of the system. Stagnation of warm water is believed to be a major contributor to *L. pneumophila* colonization and proliferation (Bédard et al., 2015; Ricketts et al., 2010). For instance, Ciesielski et *al* found that reducing stagnation in two hospital hot water tanks decreased the *L. pneumophila* levels, whereas the bacterium persisted in the control tanks that were left to stagnate (Ciesielski et al., 1984). Stagnation is believed to increase microbial growth and biofilm production. These biofilms can then get colonized by different *L. pneumophila* strains and attract host cells.

In addition, the presence and concentration of nutrients have been associated with *Legionella* ecology, though with contradicting evidence. For instance, Stout et *al.* showed a positive relationship between the concentration of non-sterilized sediments and the survival of *L. pneumophila* in hospital hot water storage tanks (Stout et al., 1985). Furthermore, they demonstrated that the microbial community could act in combination with sediments to provide *L. pneumophila* its nutritional requirements for survival (Stout et al., 1985). On the other hand, Kusnetsov et *al* found that lower concentrations of nutrients were associated with more positive samples of *L. pneumophila* in cooling towers (Kusnetsov et al., 1993). Organic matter is an important element in aquatic systems, as it is an important source of nutrients for heterotrophic bacteria. Consequently, higher levels of organic matter can increase growth of microbial biomass in EWSs. This in turn could potentially increase biofilm production by the bacteria and the host cell population, which feeds off of the bacterial population. However, further research is required to better understand the role of nutrients in *L. pneumophila* ecology.

Finally, materials used for EWSs can significantly alter the microbiome and affect *Legionella* ecology. Piping and plumbing material of cooling tower can be made of different metals, for instance copper can be used for pipes feeding the cooling tower, whereas, the fill material can be made of plastics, metals, or even wood, and the collection basin can be made of galvanized steel (Hensley, 2009). These materials can have different effects on the bacterial community. For instance, Ji et *al* found that the microbiomes was constituted of different bacterial communities depending on the type of piping material used for building water distribution systems (Ji et al., 2015). This might partly be due to the piping material changing the water chemistry and intrinsic properties that are important for biofilm attachment. Indeed, different metals can leach into the water depending on the piping material used. For instance, in the same study by Ji et *al*,

higher concentrations of lead were found in copper/lead pipes than in the other types of pipes (Ji et al., 2015). These different metals can have different toxicity levels and thus can select for different microbes.

B. BIOTIC FACTORS AFFECTING *L. PNEUMOPHILA* ECOLOGY

L. PNEUMOPHILA HOST SPECIES ECOLOGY

As we mentioned before, *L. pneumophila* is an intracellular parasite of various protozoan species. These species are fundamental for *L. pneumophila*'s life cycle, and consequently, without the host population, it is generally believed that the bacterium cannot proliferate in nature. The host species comprise a wide variety of phylogenetic groups; however, their common characteristic is that they are microbial grazers. *L. pneumophila* uses this to its own advantage by allowing itself to be preyed upon and then growing intracellularly in the host cell. The intracellular lifestyle was first characterized in protozoa by Rowbotham in 1980 (Rowbotham, 1980). However, since then it has been discovered that protozoa are more than just a replication medium for *L. pneumophila* and offer other important functions for the bacterium's ecology.

Indeed, some protozoan species can have two forms: a trophozoite form or a cyst form (Clarke and Niederkorn, 2006). The trophozoite form is usually a motile and active form of the species, which is capable of grazing activity when conditions are suitable (Clarke and Niederkorn, 2006). *L. pneumophila* can infect the trophozoite form. However, under stressful conditions, trophozoites can enter a dormant cyst form, which *L. pneumophila* cannot infect (Clarke and Niederkorn, 2006). Cyst represent a way for the amoeba to evade harsh chemicals in EWSs. *L.*

pneumophila is frequently found within these cyst forms and it is theorized that this is a way the bacterium can evade chemical disinfection, as well as detection from humans. For instance, it was demonstrated that *A. polyphaga* cyst could protect *L. pneumophila* from at least 50 mg/l of free chlorine (Kilvington and Price, 1990). In comparison, concentrations as low as 2 mg/l have been shown to severely reduce free *L. pneumophila* populations in water systems (Muraca et al., 1987). In this scenario, *L. pneumophila* cells infect host species in the trophozoite form; however, if stressful conditions appear in the environment, the host species can quickly transform in the cyst form, entrapping the bacterium within it until better conditions appear. This capacity of *L. pneumophila* to evade environmental stressor and human detection through protozoan cyst has led to the qualification of host cells as "Trojan horses" (Barker and Brown, 1994).

Moreover, amoebic host cells have been shown to resuscitate VBNC *L. pneumophila* cells. For example, the amoeba *A. castellanii* was demonstrated to be able to resuscitate *L. pneumophila* strain JR32 in VBNC form after 125 days of incubation in water (Steinert et al., 1997). The VBNC state has either been considered a long term survival strategy for bacteria in non-permissive environments, or it has been considered as an injured state in which the bacteria finds itself when under prolonged stressful period of time (Dietersdorfer et al., 2018). If the latter is true, then the host population could be perceived as curative remedy for damaged *L. pneumophila* cells.

Furthermore, host species are an important breeding ground for evolutionary processes which mould host adaptation in bacteria. Undeniably, protozoa create a pressure of selection through their grazing activity, forcing bacterial populations to adapt to this threat. In the case of *L. pneumophila*, grazing is believed to be the cause for it to have acquired adaptations for an intracellular lifestyle (Amaro et al., 2015). As evidence, the number of different effector proteins, in the species and the genus (more than 18000), and the number of different host species it can

infect leads to believe that adaptations for intracellular lifestyle in specific host species were acquired multiple times throughout the evolution of the genus *Legionella* (Gomez-Valero et al., 2019). This is supported by the fact that the *L. pneumophila* genome contains a wide variety of eukaryotic domains and eukaryotic like proteins in its effector protein arsenal (Burstein et al., 2016; Gomez-Valero et al., 2019). These eukaryotic domains are believed to have been acquired through horizontal gene transfer. These findings are relevant as cooling towers have been hypothesized as breeding grounds for the generation of new bacterial intracellular pathogens (Berk et al., 2006). Amoeba-associated microorganisms have been detected in several cooling tower studies and it is believe that they represent a pool of potential pathogens, as these microbes have not been associated with human infection yet (Adeleke et al., 1996; Bousbia et al., 2013; Pagnier et al., 2009). More importantly, Berk et *al* revealed that cooling towers seem to increase the number of infected amoeba by 16 times compared to natural water systems, giving further validation to the argument that cooling towers are important for generation of intracellular pathogens , as more interactions between non-intracellular organisms and amoeba could increase adaptative mechanisms to the intracellular environment (Berk et al., 2006). The design of the tower (creation of stagnation spots), biocides used, and higher concentration of amoeba in cooling towers found in the data are suggested as possible cause for the increase of infected amoeba in these systems (Berk et al., 2006). In addition, amoebae were shown to increase virulence of *L. pneumophila* towards human host cells. This was shown by Cirillo et *al* who demonstrated that amoeba-grown *L. pneumophila* where less affected by the immune response and less sensitive to intracellular inhibitors of monocytes, when grown in monocytes, as opposed to the agar grown *L. pneumophila* strain (Cirillo et al., 1999). Consequently, protozoa play an integral part in the evolution of host adaptation mechanisms in *L. pneumophila* and potentially other pathogens.

Additionally, protozoa play an important role in moulding the bacterial community. Grazing can be a selective process that depends on the prey species, its morphology, prey size, the presence of certain proteins, and the physiological state of the prey and the predator (Alsam et al., 2006; Hahn and Höfle, 2001). Hahn et al demonstrated that protozoan grazing can shift bacterial communities based on size, which affects the taxonomic structure of the community (Hahn and Höfle, 2001). This can have potential consequences on the *Legionella* community, as grazing can potentially select for different species that could potentially affect the *Legionella* community, though further research is required to better understand this. Several bacterial species can inhibit *L. pneumophila*, such as certain *Pseudomonas* species (Corre et al., 2018; Guerrieri et al., 2008). Consequently, grazing may select for certain anti-*Legionella* bacteria, creating a non-permissive environment for *Legionella*.

Finally, recent research has revealed that the host cell-*L. pneumophila* interaction is far more complex in nature than previously thought. A recent study by Shaheen et *al* demonstrated that different species of hosts will, if given the choice, not necessarily consume *L. pneumophila* in a multi-species biofilms (Shaheen et al., 2019). This was observed when studying the interaction between the amoebic species *Willaertia magna* and *L. pneumophila*, which showed a time delay in feeding on *L. pneumophila* of three weeks after inoculation into the experimental system. The authors further suggest that this time delay, of host cells feeding on *L. pneumophila*, could potentially be due to a preference for other prey species. However, these results are somewhat contradictory to earlier work done by Declerck et *al,* where they observed no difference in uptake levels for the amoebic species *Acanthamoeba castellanii* and *Naegleria lovaniensis*, when fed with non-*L. pneumophila* bacteria and *L. pneumophila* bacteria (Declerck et al., 2005). The findings did, however, find significant uptake differences between the two amoeba species, with *A.*

castellanii being able to uptake 20 times more *L. pneumophila* than *N. lovaniensis* (Declerck et al., 2005). Moreover, several studies seem to indicate that numerous protozoan species can resist *L. pneumophila* infection through various mechanisms. Thus, pelletization by ciliates is an effective way of clearing the host cell of *L. pneumophila* (Berk et al., 2008). *Tetrahymena* species have been shown to ingest *L. pneumophila* cells; however, the cells are then packaged into pellets and then excreted externally (Berk et al., 2008). In contrast, some protists can altogether avoid up-taking *L. pneumophila* cells, and others can consume *L. pneumophila* in a digestive vacuole (Amaro et al., 2015). These results suggest that *L. pneumophila* has a spectrum of adaptation to different host cell species, with certain species being highly effective at growing the bacterium whereas others are less permissive for intracellular growth. This latter group may be an important source for generating new genetic mechanisms for host adaptation in different *L. pneumophila* strains. A recent study examining the evolutionary history of the *Legionella* genus demonstrated the genus gained a higher number of genes, through horizontal gene transfer, than the number of lost genes, indicating that gene acquisition is dominant in the genus (Gomez-Valero et al., 2019). Moreover, the authors stipulate that these acquisition events are still ongoing. Therefore, this could be a means to generate new adaptation mechanisms to non-host protozoan species, that are currently capable of consuming different *Legionella* species.

BIOFILMS AND *L. PNEUMOPHILA* ECOLOGY

Biofilms are an extremely important factor for the microbial ecology of EWSs. It is estimated that biofilms contain up to 95% of the microbial population in EWSs (Flemming et al., 2002). Consequently, most of the microbial interactions are occurring in the biofilms in these environments. A biofilm is a community of microorganisms which are enveloped in an

extracellular matrix of different biopolymers produced by the microorganisms themselves (Flemming and Wingender, 2010). The polymers constituting the matrix can be quite different, anywhere from proteins, nucleic acids, to carbohydrates (Flemming and Wingender, 2010). Furthermore, depending on the microbial species present in the biofilm, different microhabitats can form. For instance, the surface layer is much more oxygenated whereas the under layers can include microaerophilic or anaerobic zones (De Beer et al., 1994). Biofilms are important in *L. pneumophila* ecology, as they offer various advantages for the bacterium's survival and proliferation; however, they are also a source of antagonistic interactions.

One of the most important roles of biofilms, in terms of *L. pneumophila* ecology, is that they attract microbial grazers since most of the microbial load is found within them. As a result, host cell species can concentrate at the biofilm surface and thus allow *L. pneumophila* to proliferate (Lau and Ashbolt, 2009). Without these host cells it is generally believed that the bacterium cannot grow but will persist or survive on or within the biofilm. Murga et *al* demonstrated that *L. pneumophila* was only able to proliferate in a model bioreactor with biofilms that were inoculated with *V. vermiformis* (Murga et al., 2001).

Moreover, increased survival in biofilms is mediated by the extracellular matrix protecting from harsh chemicals, such as disinfectants (Kim et al., 2002). This is shown by several studies demonstrating that increased concentrations and contact times are required to inactivate *L. pneumophila* associated in biofilms, as opposed to planktonic *L. pneumophila* (Kim et al., 2002). For instance, planktonic *L. pneumophila* and other species were inactivated with 0.4 mg/L chlorine, whereas, more than 3 mg/L of free chlorine was required to inactivate it when *L. pneumophila* was associated with biofilms (Muraca et al., 1987; Yabuchi et al., 1995). As mentioned before, the extracellular matrix is composed of various biopolymers which confer

protection against disinfectants. Monochloramine has been promoted as a better disinfectant against biofilm than chlorination or silver-copper ionization (Lin et al., 2011).

Additionally, biofilms offer nutritional support for the bacterium's survival. Biofilms contain a multitude of different microorganism, certain of these can have positive ecological interactions with *L. pneumophila*, supplying it with some nutrients (Declerck, 2010). For instance, although *L. pneumophila* is categorized as a fastidious grower, requiring very specific nutritional requirements for its proliferation, studies have shown that several bacteria can promote its growth, such as species of *Fischerella* and *Flavobacterium* (Tison et al., 1980; Wadowsky and Yee, 1983). In addition, *L. pneumophila* can grow on heat inactivated *Pseudomonas putida*, *E. coli*, *A. castellanii*, and *Saccharomyces boulardii*, and biofilm samples (Temmerman et al., 2006). However, this growth is less substantial than growth in host cells (1.49 log increase through necrotophic growth, as opposed to, several logs increase through intracellular growth). Stout et *al* demonstrated that sediments found in EWSs could also increase survival of *L. pneumophila*, as well as, stimulate the growth of the resident microbial community, which in turn was able to stimulate the growth of *L. pneumophila* (Stout et al., 1985).

Finally, biofilms play an important role in the dissemination of *L. pneumophila* in EWS. The normal life cycle of a biofilm is characterized by the initial attachment of the bacteria to the surface, development of microcolonies, maturation of the biofilm, and finally detachment of cells or parts of the biofilm (Kierek‐Pearson and Karatan, 2005). This last stage, detachment, is an important mechanism for the dissemination of microorganisms in EWS (Stewart, 1993). The detachment of biofilm can occur through various mechanisms, such as nutrient starvation, quorum sensing, shear stress, or even bacteriophages (Stewart, 1993). Detached biofilm have been

observed to contain *L. pneumophila* and protist host species, suggesting that biofilms could be an important vehicle for dissemination of the bacterium in EWS (Hsu et al., 2011).

IV- CONCLUDING REMARKS

In conclusion, since its first discovery in 1976, intense research has been focused on understanding how *Legionella pneumophila* colonizes, survives, and proliferates in engineered water systems, and how the ecological factors implicated in its life cycle contribute to outbreaks of Legionnaires' disease from a public health perspective. Currently, *L. pneumophila* is one of the most important sources of waterborne disease in developed countries, and trends seem to indicate that the number of cases will continue to rise in the future. Though research in the past was mainly focused on understanding the bacterium's molecular processes related to its intracellular life cycle and virulence, as well as, the physicochemical parameters associated with its survival in water, the recent advances in sequencing have encouraged more research in understanding its relationship with the microbial community of EWS. Previous findings seem to indicate that certain bacterial groups are important factors for *L. pneumophila* survival and proliferation in these environments, and consequently, these groups could be targets for future research or industrial purposes for production of antimicrobials or as biomarkers for *L. pneumophila* surveillance programs. Moreover, future research should focus on examining the relationship between the entire microbial community (encompassing the eukaryotic, archaeal, and viral), along with the physicochemical parameters, with *Legionella* ecology, in order to get a more holistic view of the mechanisms and parameters which lead to outbreaks of Legionnaires' disease.

VII- REFERENCE

Adeleke, A., Pruckler, J., Benson, R., Rowbotham, T., Halablab, M. and Fields, B. 1996. Legionella-like amebal pathogens--phylogenetic status and possible role in respiratory disease. Emerging infectious diseases 2(3), 225-230.

Allegra, S., Berger, F., Berthelot, P., Grattard, F., Pozzetto, B. and Riffard, S. 2008. Use of flow cytometry to monitor Legionella viability. Appl. Environ. Microbiol. 74(24), 7813-7816.

Allen, R.C. 1997. Agriculture and the Origins of the State in Ancient Egypt. Explorations in Economic History 34(2), 135-154.

Alsam, S., Jeong, S.R., Sissons, J., Dudley, R., Kim, K.S. and Khan, N.A. 2006. Escherichia coli interactions with Acanthamoeba: a symbiosis with environmental and clinical implications. Journal of medical microbiology 55(6), 689-694.

Amaro, F., Wang, W., Gilbert, J.A., Anderson, O.R. and Shuman, H.A. 2015. Diverse protist grazers select for virulence-related traits in Legionella. The ISME journal 9(7), 1607.

Ashbolt, N.J. 2004. Microbial contamination of drinking water and disease outcomes in developing regions. Toxicology 198(1-3), 229-238.

Ballabio, A. 2016. The awesome lysosome. EMBO molecular medicine 8(2), 73-76.

Barker, J. and Brown, M. 1994. Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. Microbiology 140(6), 1253-1259.

Baskerville, A., Broster, M., Fitzgeorge, R., Hambleton, P. and Dennis, P. 1981. Experimental Transmission of Legionnaires' disease by Exposure to Aerosols of Legionella pneumophila. The Lancet 318(8260-8261), 1389-1390.

Bédard, E., Fey, S., Charron, D., Lalancette, C., Cantin, P., Dolcé, P., Laferrière, C., Déziel, E. and Prévost, M. 2015. Temperature diagnostic to identify high risk areas and optimize Legionella pneumophila surveillance in hot water distribution systems. Water research 71, 244-256.

Bédard, E., Lévesque, S., Martin, P., Pinsonneault, L., Paranjape, K., Lalancette, C., Dolcé, C.-É., Villion, M., Valiquette, L. and Faucher, S.P. 2016. Energy conservation and the promotion of Legionella pneumophila growth: the probable role of heat exchangers in a nosocomial outbreak. infection control & hospital epidemiology 37(12), 1475-1480.

Beech, I.B. and Sunner, J. 2004. Biocorrosion: towards understanding interactions between biofilms and metals. Current opinion in Biotechnology 15(3), 181-186.

Beer, K.D., Gargano, J.W., Roberts, V.A., Hill, V.R., Garrison, L.E., Kutty, P.K., Hilborn, E.D., Wade, T.J., Fullerton, K.E. and Yoder, J.S. 2015. Surveillance for waterborne disease outbreaks associated with drinking water—United States, 2011–2012. MMWR. Morbidity and mortality weekly report 64(31), 842.

Berk, S., Gunderson, J., Newsome, A., Farone, A., Hayes, B., Redding, K., Uddin, N., Williams, E., Johnson, R. and Farsian, M. 2006. Occurrence of infected amoebae in cooling towers compared with natural aquatic environments: implications for emerging pathogens. Environmental science & technology 40(23), 7440-7444.

Berk, S.G., Faulkner, G., Garduno, E., Joy, M.C., Ortiz-Jimenez, M.A. and Garduno, R.A. 2008. Packaging of live Legionella pneumophila into pellets expelled by Tetrahymena spp. does not require bacterial replication and depends on a Dot/Icm-mediated survival mechanism. Appl. Environ. Microbiol. 74(7), 2187-2199.

Bichai, F., Hijnen, W., Baars, E., Rosielle, M., Dullemont, Y. and Barbeau, B. 2011. Preliminary study on the occurrence and risk arising from bacteria internalized in zooplankton in drinking water. Water science and technology 63(1), 108-114.

Biurrun, A., Caballero, L., Pelaz, C., León, E. and Gago, A. 1999. Treatment of a Legionella pneumophila-colonized water distribution system using copper-silver ionization and continuous chlorination. Infection Control & Hospital Epidemiology 20(6), 426-428.

Boamah, D.K., Zhou, G., Ensminger, A.W. and O'Connor, T.J. 2017. From many hosts, one accidental pathogen: the diverse protozoan hosts of Legionella. Frontiers in cellular and infection microbiology 7, 477.

Bojko, J., Dunn, A.M., Stebbing, P.D., van Aerle, R., Bacela-Spychalska, K., Bean, T.P., Urrutia, A. and Stentiford, G.D. 2018. 'Candidatus Aquirickettsiella gammari'(Gammaproteobacteria: Legionellales: Coxiellaceae): A bacterial pathogen of the freshwater crustacean Gammarus fossarum (Malacostraca: Amphipoda). Journal of invertebrate pathology 156, 41-53.

Borges, V., Nunes, A., Sampaio, D.A., Vieira, L., Machado, J., Simões, M.J., Gonçalves, P. and Gomes, J.P. 2016. Legionella pneumophila strain associated with the first evidence of person-to-person transmission of Legionnaires' disease: a unique mosaic genetic backbone. Scientific reports 6, 26261.

Bousbia, S., Papazian, L., Saux, P., Forel, J.-M., Auffray, J.-P., Martin, C., Raoult, D. and La Scola, B. 2013. Serologic prevalence of amoeba-associated microorganisms in intensive care unit pneumonia patients. PLoS One 8(3), e58111.

Brassinga, A.K.C., Kinchen, J.M., Cupp, M.E., Day, S.R., Hoffman, P.S. and Sifri, C.D. 2010. Caenorhabditis is a metazoan host for Legionella. Cellular microbiology 12(3), 343-361.

Brunkard, J.M.A., Elizabeth; Roberts, Virginia A.; Hill, Vincent; Hilborn, Elizabeth D.; Craun, Gunther F. et al 2011. Surveillance for Waterborne Disease Outbreaks Associated with Drinking Water — United States, 2007—2008. Morbidity and Mortality Weekly Report 60(SS12), 38-68.

Burstein, D., Amaro, F., Zusman, T., Lifshitz, Z., Cohen, O., Gilbert, J.A., Pupko, T., Shuman, H.A. and Segal, G. 2016. Genomic analysis of 38 Legionella species identifies large and diverse effector repertoires. Nature genetics 48(2), 167.

Buse, H.Y., Ji, P., Gomez-Alvarez, V., Pruden, A., Edwards, M.A. and Ashbolt, N.J. 2017. Effect of temperature and colonization of Legionella pneumophila and Vermamoeba vermiformis on bacterial community composition of copper drinking water biofilms. Microbial biotechnology 10(4), 773-788.

Buse, H.Y., Lu, J., Struewing, I.T. and Ashbolt, N.J. 2013. Eukaryotic diversity in premise drinking water using 18S rDNA sequencing: implications for health risks. Environmental Science and Pollution Research 20(9), 6351-6366.

Carvalho, F.R., Nastasi, F.R., Gamba, R.C., Foronda, A.S. and Pellizari, V.H. 2008. Occurrence and diversity of Legionellaceae in polar lakes of the Antarctic peninsula. Current microbiology 57(4), 294-300.

CDC 1999. A Century of US Water Chlorination and Treatment: One of the Ten Greatest Public Health Achievements of the 20th Century. Morbidity and Mortality Weekly Report 48(29), 621.

Cech, T.V. (2009) Principles of water resources: history, development, management, and policy, John Wiley & Sons.

Chao, Y., Ma, L., Yang, Y., Ju, F., Zhang, X.-X., Wu, W.-M. and Zhang, T. 2013. Metagenomic analysis reveals significant changes of microbial compositions and protective functions during drinking water treatment. Scientific reports 3, 3550.

Christopher Miller, J.M. 2017. Health Department Investigatng Community Cluster of Legionnaires' Disease in Section of Lenox Hill, Manhattan. NYC Health.

Ciesielski, C., Blaser, M. and Wang, W. 1984. Role of stagnation and obstruction of water flow in isolation of Legionella pneumophila from hospital plumbing. Appl. Environ. Microbiol. 48(5), 984-987.

Cirillo, J.D., Cirillo, S.L., Yan, L., Bermudez, L.E., Falkow, S. and Tompkins, L.S. 1999. Intracellular growth in Acanthamoeba castellanii affects monocyte entry mechanisms and enhances virulence of Legionella pneumophila. Infection and immunity 67(9), 4427-4434.

Clarke, D.W. and Niederkorn, J.Y. 2006. The pathophysiology of Acanthamoeba keratitis. Trends in parasitology 22(4), 175-180.

Collier, S.A., Stockman, L.J., Hicks, L.A., Garrison, L.E., Zhou, F. and Beach, M.J. 2012. Direct healthcare costs of selected diseases primarily or partially transmitted by water. Epidemiology & Infection 140(11), 2003-2013.

Conley, L., Towner, S., Wolford, R., Stephenson, T., McNamara, A., Wadowsky, R. and Yee, R. 1987. An alkaline approach to treating cooling towers for control of Legionella pneumophila. Appl. Environ. Microbiol. 53(8), 1775-1779.

Corre, M.-H., Delafont, V., Legrand, A., Berjeaud, J.-M. and Verdon, J. 2018. Exploiting the richness of environmental waterborne bacterial species to find natural anti-Legionella active biomolecules. Frontiers in microbiology 9, 3360.

Correia, A.M., Ferreira, J.S., Borges, V., Nunes, A., Gomes, B., Capucho, R., Gonçalves, J., Antunes, D.M., Almeida, S. and Mendes, A. 2016. Probable person-to-person transmission of Legionnaires' disease. New England Journal of Medicine 374, 497-498.

Cunha, B.A., Burillo, A. and Bouza, E. 2016. Legionnaires' disease. The Lancet 387(10016), 376-385.

Dalfes, H.N., Kukla, G. and Weiss, H. (2013) Third millennium BC climate change and old world collapse, Springer Science & Business Media.

De Beer, D., Stoodley, P., Roe, F. and Lewandowski, Z. 1994. Effects of biofilm structures on oxygen distribution and mass transport. Biotechnology and bioengineering 43(11), 1131-1138.

De Wever, A., Muylaert, K., Van der Gucht, K., Pirlot, S., Cocquyt, C., Descy, J.-P., Plisnier, P.-D. and Vyverman, W. 2005. Bacterial community composition in Lake Tanganyika: vertical and horizontal heterogeneity. Appl. Environ. Microbiol. 71(9), 5029-5037.

Debroas, D., Humbert, J.F., Enault, F., Bronner, G., Faubladier, M. and Cornillot, E. 2009. Metagenomic approach studying the taxonomic and functional diversity of the bacterial community in a mesotrophic lake (Lac du Bourget–France). Environmental microbiology 11(9), 2412-2424.

Declerck, P. 2010. Biofilms: the environmental playground of Legionella pneumophila. Environmental microbiology 12(3), 557-566.

Declerck, P., Behets, J., Delaedt, Y., Margineanu, A., Lammertyn, E. and Ollevier, F. 2005. Impact of non-Legionella bacteria on the uptake and intracellular replication of Legionella pneumophila in Acanthamoeba castellanii and Naegleria lovaniensis. Microbial ecology 50(4), 536-549.

Delafont, V., Bouchon, D., Héchard, Y. and Moulin, L. 2016. Environmental factors shaping cultured free-living amoebae and their associated bacterial community within drinking water network. Water research 100, 382-392.

Den Boer, J.W., Yzerman, P., Schellekens, J., Lettinga, K.D., Boshuizen, H.C., Van Steenbergen, J.E., Bosman, A., Van den Hof, S., Van Vliet, H.A. and Peeters, M.F. 2002. A large outbreak of Legionnaires' disease at a flower show, the Netherlands, 1999. Emerging infectious diseases 8(1), 37.

Di Gregorio, L., Tandoi, V., Congestri, R., Rossetti, S. and Di Pippo, F. 2017. Unravelling the core microbiome of biofilms in cooling tower systems. Biofouling 33(10), 793-806.

Dietersdorfer, E., Kirschner, A., Schrammel, B., Ohradanova-Repic, A., Stockinger, H., Sommer, R., Walochnik, J. and Cervero-Aragó, S. 2018. Starved viable but non-culturable (VBNC) Legionella strains can infect and replicate in amoebae and human macrophages. Water research 141, 428-438.

Dooling, K.L., Toews, K.-A., Hicks, L.A., Garrison, L.E., Bachaus, B., Zansky, S., Carpenter, L.R., Schaffner, B., Parker, E. and Petit, S. 2015. Active bacterial core surveillance for legionellosis—United States, 2011–2013. Morbidity and Mortality Weekly Report 64(42), 1190-1193.

Duron, O., Doublet, P., Vavre, F. and Bouchon, D. 2018. The importance of revisiting Legionellales diversity. Trends in parasitology 34(12), 1027-1037.

ECDC 2019. Legionnaires' Disease. ECDC Annual epdimilogical report for 2017.

Edelstein, P., Beer, K. and DeBoynton, E. 1987. Influence of growth temperature on virulence of Legionella pneumophila. Infection and immunity 55(11), 2701-2705.

Edelstein, P.H. 2008. Legionnaires' disease: history and clinical findings. Legionella: Molecular Microbiology 1, 1-19.

Eichler, S., Christen, R., Höltje, C., Westphal, P., Bötel, J., Brettar, I., Mehling, A. and Höfle, M.G. 2006. Composition and dynamics of bacterial communities of a drinking water supply system as assessed by RNA-and DNA-based 16S rRNA gene fingerprinting. Appl. Environ. Microbiol. 72(3), 1858-1872.

Eisenreich, W. and Heuner, K. 2016. The life stage-specific pathometabolism of Legionella pneumophila. FEBS letters 590(21), 3868-3886.

Elshahed, M.S., Najar, F.Z., Aycock, M., Qu, C., Roe, B.A. and Krumholz, L.R. 2005. Metagenomic analysis of the microbial community at Zodletone Spring (Oklahoma): insights into the genome of a member of the novel candidate division OD1. Appl. Environ. Microbiol. 71(11), 7598-7602.

Falkinham III, J.O., Hilborn, E.D., Arduino, M.J., Pruden, A. and Edwards, M.A. 2015. Epidemiology and ecology of opportunistic premise plumbing pathogens: Legionella pneumophila, Mycobacterium avium, and Pseudomonas aeruginosa. Environmental health perspectives 123(8), 749-758.

Falkinham, J.O., Pruden, A. and Edwards, M. 2015. Opportunistic premise plumbing pathogens: increasingly important pathogens in drinking water. Pathogens 4(2), 373-386.

Fischer, M.A., Guellert, S., Neulinger, S.C., Streit, W.R. and Schmitz, R.A. 2016. Evaluation of 16S rRNA gene primer pairs for monitoring microbial community structures showed high reproducibility within and low comparability between datasets generated with multiple archaeal and bacterial primer pairs. Frontiers in microbiology 7, 1297.

Fisman, D.N., Lim, S., Wellenius, G.A., Johnson, C., Britz, P., Gaskins, M., Maher, J., Mittleman, M.A., Victor Spain, C. and Haas, C.N. 2005. It's not the heat, it's the humidity: wet weather increases legionellosis risk in the greater Philadelphia metropolitan area. The Journal of infectious diseases 192(12), 2066-2073.

Fitzhenry, R., Weiss, D., Cimini, D., Balter, S., Boyd, C., Alleyne, L., Stewart, R., McIntosh, N., Econome, A. and Lin, Y. 2017. Legionnaires' disease outbreaks and cooling towers, New York City, New York, USA. Emerging infectious diseases 23(11), 1769.

Flemming, H.-C., Percival, S. and Walker, J. 2002. Contamination potential of biofilms in water distribution systems. Water science and technology: water supply 2(1), 271-280.

Flemming, H.-C. and Wingender, J. 2010. The biofilm matrix. Nature reviews microbiology 8(9), 623.

Fliermans, C., Cherry, W., Orrison, L., Smith, S., Tison, D. and Pope, D. 1981. Ecological distribution of Legionella pneumophila. Appl. Environ. Microbiol. 41(1), 9-16.

Fliermans, C.B. 1983. Autecology of Legionella pneumophila. Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene. 1. Abt. Originale. A, Medizinische Mikrobiologie, Infektionskrankheiten und Parasitologie 255(1), 58-63.

Fraser, D.W., Tsai, T.R., Orenstein, W., Parkin, W.E., Beecham, H.J., Sharrar, R.G., Harris, J., Mallison, G.F., Martin, S.M. and McDade, J.E. 1977. Legionnaires' disease: description of an epidemic of pneumonia. New England Journal of Medicine 297(22), 1189-1197.

Gallagher, G. 2017. Disneyland cooling towers linked to Legionnaires' disease outbreak. Infectious Diseases in Children 30(12), 16-16.

García, M.T., Jones, S., Pelaz, C., Millar, R.D. and Abu Kwaik, Y. 2007. Acanthamoeba polyphaga resuscitates viable non-culturable Legionella pneumophila after disinfection. Environmental microbiology 9(5), 1267-1277.

García-Fulgueiras, A., Navarro, C., Fenoll, D., García, J., González-Diego, P., Jiménez-Buñuales, T., Rodriguez, M., Lopez, R., Pacheco, F. and Ruiz, J. 2003. Legionnaires' disease outbreak in Murcia, Spain. Emerging infectious diseases 9(8), 915.

Ghai, R., Rodŕíguez-Valera, F., McMahon, K.D., Toyama, D., Rinke, R., de Oliveira, T.C.S., Garcia, J.W., de Miranda, F.P. and Henrique-Silva, F. 2011. Metagenomics of the water column in the pristine upper course of the Amazon river. PloS one 6(8), e23785.

Gilmour, M.W., Bernard, K., Tracz, D.M., Olson, A.B., Corbett, C.R., Burdz, T., Ng, B., Wiebe, D., Broukhanski, G. and Boleszczuk, P. 2007. Molecular typing of a Legionella pneumophila outbreak in Ontario, Canada. Journal of Medical Microbiology 56(Pt 3), 336.

Gomez-Valero, L., Rusniok, C., Carson, D., Mondino, S., Pérez-Cobas, A.E., Rolando, M., Pasricha, S., Reuter, S., Demirtas, J. and Crumbach, J. 2019. More than 18,000 effectors in the Legionella genus genome provide multiple, independent combinations for

replication in human cells. Proceedings of the National Academy of Sciences 116(6), 2265-2273.

Graells, T., Ishak, H., Larsson, M. and Guy, L. 2018. The all-intracellular order Legionellales is unexpectedly diverse, globally distributed and lowly abundant. FEMS microbiology ecology 94(12), fiy185.

Greig, J.E., Carnie, J.A., Tallis, G.F., Zwolak, B., Hart, W.G., Guest, C.S., Ryan, N.J., Leydon, J.A., Tan, A.G. and Gordon, I.R. 2004. An outbreak of Legionnaires' disease at the Melbourne Aquarium, April 2000: investigation and case–control studies. Medical Journal of Australia 180(11), 566-572.

Guerrieri, E., Bondi, M., Sabia, C., de Niederhäusern, S., Borella, P. and Messi, P. 2008. Effect of Bacterial Interference on Biofilm Development by Legionella pneumophila. Current Microbiology 57(6), 532-536.

Hahn, M.W. and Höfle, M.G. 2001. Grazing of protozoa and its effect on populations of aquatic bacteria. FEMS microbiology ecology 35(2), 113-121.

Hambleton, P., Broster, M., Dennis, P., Henstridge, R., Fitzgeorge, R. and Conlan, J. 1983. Survival of virulent Legionella pneumophila in aerosols. Epidemiology & Infection 90(3), 451-460.

Harb, O.S., Venkataraman, C., Haack, B.J., Gao, L.-Y. and Kwaik, Y.A. 1998. Heterogeneity in the attachment and uptake mechanisms of the Legionnaires' disease bacterium, Legionella pneumophila, by protozoan hosts. Appl. Environ. Microbiol. 64(1), 126-132.

Haupt, T.E., Heffernan, R.T., Kazmierczak, J.J., Nehls-Lowe, H., Rheineck, B., Powell, C., Leonhardt, K.K., Chitnis, A.S. and Davis, J.P. 2012. An outbreak of Legionnaires disease associated with a decorative water wall fountain in a hospital. Infection Control & Hospital Epidemiology 33(2), 185-191.

Head, B., Trajtman, A., Bernard, K., Burdz, T., Vélez, L., Rueda, Z. and Keynan, Y. 2017 The Prevalence of Legionella Species as a Co-pathogen in HIV-Associated Community-Acquired Pneumonia, p. S211, Oxford University Press.

Hensley, J.C. 2009. Cooling tower fundamentals, SPX cooling technologies. Overland, KS.

Hoffmann, R. and Michel, R. 2001. Distribution of free-living amoebae (FLA) during preparation and supply of drinking water. International journal of hygiene and environmental health 203(3), 215-219.

Holinger, E.P., Ross, K.A., Robertson, C.E., Stevens, M.J., Harris, J.K. and Pace, N.R. 2014. Molecular analysis of point-of-use municipal drinking water microbiology. Water research 49, 225-235.

Horwitz, M.A. 1984. Phagocytosis of the Legionnaires' disease bacterium (Legionella pneumophila) occurs by a novel mechanism: engulfment within a pseudopod coil. Cell 36(1), 27-33.

Hsu, B.-M., Huang, C.-C., Chen, J.-S., Chen, N.-H. and Huang, J.-T. 2011. Comparison of potentially pathogenic free-living amoeba hosts by Legionella spp. in substrate-associated biofilms and floating biofilms from spring environments. Water research 45(16), 5171-5183.

Huang, S.-W., Hsu, B.-M., Wu, S.-F., Fan, C.-W., Shih, F.-C., Lin, Y.-C. and Ji, D.-D. 2010. Water quality parameters associated with prevalence of Legionella in hot spring facility water bodies. Water research 44(16), 4805-4811.

Humbert, J.F., Dorigo, U., Cecchi, P., Le Berre, B., Debroas, D. and Bouvy, M. 2009. Comparison of the structure and composition of bacterial communities from temperate and tropical freshwater ecosystems. Environmental microbiology 11(9), 2339-2350.

Hunt, D., Cartwright, K., Smith, M., Middleton, J., Bartlett, C., Lee, J., Dennis, P. and Harper, D. 1991. An outbreak of Legionnaires' disease in Gloucester. Epidemiology & Infection 107(1), 133-141.

Iervolino, M., Mancini, B. and Cristino, S. 2017. Industrial Cooling Tower Disinfection Treatment to Prevent Legionella spp. International journal of environmental research and public health 14(10), 1125.

Isberg, R.R., O'connor, T.J. and Heidtman, M. 2009. The Legionella pneumophila replication vacuole: making a cosy niche inside host cells. Nature Reviews Microbiology 7(1), 13.

Ivone, V.-M., Conceição, E., Olga C, N. and Célia M, M. 2013. Bacterial diversity from the source to the tap: a comparative study based on 16S rRNA gene-DGGE and culture-dependent methods. FEMS Microbiology Ecology 83(2), 361-374.

Jansen, M. 1989. Water supply and sewage disposal at Mohenjo-Daro. World Archaeology 21(2), 177-192.

Jernigan, D.B., Hofmann, J., Cetron, M.S., Nuorti, J., Fields, B., Benson, R., Breiman, R., Lipman, H., Carter, R. and Genese, C. 1996. Outbreak of Legionnaires' disease among cruise ship passengers exposed to a contaminated whirlpool spa. The Lancet 347(9000), 494-499.

Ji, P., Parks, J., Edwards, M.A. and Pruden, A. 2015. Impact of water chemistry, pipe material and stagnation on the building plumbing microbiome. PLoS One 10(10), e0141087.

Juda, T. 2009. Legionella-experience of the Provincial Specialist Hospital in Jastrzębiu Zdrój-clinical cases. Nowa Medycyna 16(1).

Kierek-Pearson, K. and Karatan, E. 2005. Biofilm development in bacteria. Advances in applied microbiology 57, 79-111.

Kilvington, S. and Price, J. 1990. Survival of Legionella pneumophila within cysts of Acanthamoeba polyphaga following chlorine exposure. Journal of applied bacteriology 68(5), 519-525.

Kim, B., Anderson, J., Mueller, S., Gaines, W. and Kendall, A. 2002. Literature review—efficacy of various disinfectants against Legionella in water systems. Water Research 36(18), 4433-4444.

Kormas, K.A., Neofitou, C., Pachiadaki, M. and Koufostathi, E. 2010. Changes of the bacterial assemblages throughout an urban drinking water distribution system. Environmental monitoring and assessment 165(1-4), 27-38.

Kozak-Muiznieks, N.A., Lucas, C.E., Brown, E., Pondo, T., Taylor, T.H., Frace, M., Miskowski, D. and Winchell, J.M. 2014. Prevalence of sequence types among clinical and environmental isolates of Legionella pneumophila serogroup 1 in the United States from 1982 to 2012. Journal of clinical microbiology 52(1), 201-211.

Kuchta, J., McGlaughlin, J., Overmeyer, J., Wadowsky, R., McNamara, A., Wolford, R. and Yee, R. 1985. Enhanced chlorine resistance of tap water-adapted Legionella pneumophila as compared with agar medium-passaged strains. Appl. Environ. Microbiol. 50(1), 21-26.

Kurtz, J., Bartlett, C., Newton, U., White, R. and Jones, N. 1982. Legionella pneumophila in cooling water systems: report of a survey of cooling towers in London and a pilot trial of selected biocides. Epidemiology & Infection 88(3), 369-381.

Kusnetsov, J., Ottoila, E. and Martikainen, P. 1996. Growth, respiration and survival of Legionella pneumophila at high temperatures. Journal of Applied Bacteriology 81(4), 341-347.

Kusnetsov, J.M., Martikainen, P.J., Jousimies-Somer, H.R., Väisänen, M.-L., Tulkki, A.I., Ahonen, H.E. and Nevalainen, A.I. 1993. Physical, chemical and microbiological water characteristics associated with the occurrence of Legionella in cooling tower systems. Water Research 27(1), 85-90.

Kwaik, Y.A., Gao, L.-Y., Stone, B.J., Venkataraman, C. and Harb, O.S. 1998. Invasion of protozoa by Legionella pneumophila and its role in bacterial ecology and pathogenesis. Appl. Environ. Microbiol. 64(9), 3127-3133.

La Scola, B., Birtles, R.J., Greub, G., Harrison, T.J., Ratcliff, R.M. and Raoult, D. 2004. Legionella drancourtii sp. nov., a strictly intracellular amoebal pathogen. International Journal of Systematic and Evolutionary Microbiology 54(3), 699-703.

Lau, H. and Ashbolt, N. 2009. The role of biofilms and protozoa in Legionella pathogenesis: implications for drinking water. Journal of applied microbiology 107(2), 368-378.

Lautenschlager, K., Hwang, C., Ling, F., Liu, W.-T., Boon, N., Köster, O., Egli, T. and Hammes, F. 2014. Abundance and composition of indigenous bacterial communities in a multi-step biofiltration-based drinking water treatment plant. Water research 62, 40-52.

Lesnik, R., Brettar, I. and Höfle, M.G. 2016. Legionella species diversity and dynamics from surface reservoir to tap water: from cold adaptation to thermophily. The ISME journal 10(5), 1064.

Lettinga, K.D., Verbon, A., Weverling, G.-J., Schellekens, J.F., Den Boer, J.W., Yzerman, P., Prins, J., Boersma, W.G., van Ketel, R.J. and Prins, J.M. 2002. Legionnaires' disease at a Dutch flower show: prognostic factors and impact of therapy. Emerging infectious diseases 8(12), 1448.

Lévesque, S., Plante, P.-L., Mendis, N., Cantin, P., Marchand, G., Charest, H., Raymond, F., Huot, C., Goupil-Sormany, I. and Desbiens, F. 2014. Genomic characterization of a large outbreak of Legionella pneumophila serogroup 1 strains in Quebec City, 2012. PLoS One 9(8), e103852.

Li, D., Li, Z., Yu, J., Cao, N., Liu, R. and Yang, M. 2010. Characterization of bacterial community structure in a drinking water distribution system during an occurrence of red water. Appl. Environ. Microbiol. 76(21), 7171-7180.

Lin, W., Yu, Z., Zhang, H. and Thompson, I.P. 2014. Diversity and dynamics of microbial communities at each step of treatment plant for potable water generation. Water research 52, 218-230.

Lin, Y.E., Lu, W.-m., Huang, H.-I. and Huang, W.-k. 2006. Environmental Survey of Legionella pneumophila in Hot Springs in Taiwan. Journal of Toxicology and Environmental Health, Part A 70(1), 84-87.

Lin, Y.E., Stout, J.E. and Victor, L.Y. 2011. Controlling Legionella in hospital drinking water: an evidence-based review of disinfection methods. Infection Control & Hospital Epidemiology 32(2), 166-173.

Llewellyn, A.C., Lucas, C.E., Roberts, S.E., Brown, E.W., Nayak, B.S., Raphael, B.H. and Winchell, J.M. 2017. Distribution of Legionella and bacterial community composition among regionally diverse US cooling towers. PloS one 12(12), e0189937.

Lock, K., Millett, C., Heathcock, R., Joseph, C., Harrison, T., Lee, J., Rao, G., Surman-Lee, S. and Team, O.C. 2008. Public health and economic costs of investigating a suspected outbreak of Legionnaires' disease. Epidemiology & Infection 136(10), 1306-1314.

Loomis, D. and Wing, S. 1990. Is molecular epidemiology a germ theory for the end of the twentieth century? International journal of epidemiology 19(1), 1-3.

Lopez-Llorca, L., Maciá-Vicente, J. and Jansson, H.-B. (2008) Integrated management and biocontrol of vegetable and grain crops nematodes, pp. 51-76, Springer.

Loret, J., Jousset, M., Robert, S., Saucedo, G., Ribas, F., Thomas, V. and Greub, G. 2008. Amoebae-resisting bacteria in drinking water: risk assessment and management. Water Science and Technology 58(3), 571-577.

Magnet, A., Fenoy, S., Galván, A., Izquierdo, F., Rueda, C., Vadillo, C.F. and Del Aguila, C. 2013. A year long study of the presence of free living amoeba in Spain. Water research 47(19), 6966-6972.

Mahoney, F.J., Hoge, C.W., Farley, T.A., Barbaree, J.M., Breiman, R.F., Benson, R.F. and McFarland, L.M. 1992. Communitywide outbreak of Legionnaires' disease associated with a grocery store mist machine. Journal of Infectious Diseases 165(4), 736-739.

Mathieu, L., Robine, E., Deloge-Abarkan, M., Ritoux, S., Pauly, D., Hartemann, P. and Zmirou-Navier, D. 2006. Legionella bacteria in aerosols: sampling and analytical approaches used during the legionnaires disease outbreak in Pas-de-Calais. The Journal of infectious diseases 193(9), 1333-1335.

Matsumoto, N., Matsumoto, Y., Ashitani, J., Katoh, S. and Nakazato, M. 2004. An outbreak of legionnaires' disease associated with a circulating bath water system at a public bathhouse. Nihon Kokyuki Gakkai zasshi= the journal of the Japanese Respiratory Society 42(1), 75-79.

Mays, L.W. (2010) Ancient water technologies, pp. 1-28, Springer.

McGuire, M.J. 2006. Eight revolutions in the history of US drinking water disinfection. Journal-American Water Works Association 98(3), 123-149.

Mei, R. and Liu, W.-T. 2019. Quantifying the contribution of microbial immigration in engineered water systems. Microbiome 7(1), 144.

Mendis, N., McBride, P. and Faucher, S.P. 2015. Short-term and long-term survival and virulence of Legionella pneumophila in the defined freshwater medium Fraquil. PloS one 10(9), e0139277.

Muraca, P., Stout, J.E. and Yu, V.L. 1987. Comparative assessment of chlorine, heat, ozone, and UV light for killing Legionella pneumophila within a model plumbing system. Appl. Environ. Microbiol. 53(2), 447-453.

Murga, R., Forster, T.S., Brown, E., Pruckler, J.M., Fields, B.S. and Donlan, R.M. 2001. Role of biofilms in the survival of Legionella pneumophila in a model potable-water system. Microbiology 147(11), 3121-3126.

Nakamura, H., Yagyu, H., Tsuchida, F., Sudou, A., Watanabe, O., Kioi, K., Kishi, K., Oh-ishi, S., Kiguchi, T. and Yamaguchi, K. 2003. A major outbreak of Legionnaire's disease due to a public bathhouse: clinical examination. Nihon Kokyuki Gakkai zasshi= the journal of the Japanese Respiratory Society 41(5), 325-330.

Nelson, W.C. and Stegen, J.C. 2015. The reduced genomes of Parcubacteria (OD1) contain signatures of a symbiotic lifestyle. Frontiers in microbiology 6, 713-713.

Newton, R.J., Jones, S.E., Eiler, A., McMahon, K.D. and Bertilsson, S. 2011. A guide to the natural history of freshwater lake bacteria. Microbiol. Mol. Biol. Rev. 75(1), 14-49.

Nhu Nguyen, T.M., Ilef, D., Jarraud, S., Rouil, L., Campese, C., Che, D., Haeghebaert, S., Ganiayre, F., Marcel, F. and Etienne, J. 2006. A community-wide outbreak of legionnaires disease linked to industrial cooling towers—how far can contaminated aerosols spread? The Journal of infectious diseases 193(1), 102-111.

Nicole Chavez, J.L. 2019. Death toll rises after a Legionnaires' disease outbreak in North Carolina. CNN.

Ninio, S. and Roy, C.R. 2007. Effector proteins translocated by Legionella pneumophila: strength in numbers. Trends in microbiology 15(8), 372-380.

Niu, J., Kasuga, I., Kurisu, F., Furumai, H. and Shigeeda, T. 2013. Evaluation of autotrophic growth of ammonia-oxidizers associated with granular activated carbon used for drinking water purification by DNA-stable isotope probing. Water Research 47(19), 7053-7065.

O'mahony, M., Stanwell-Smith, R., Tillett, H., Harper, D., Hutchison, J., Farrell, I., Hutchinson, D., Lee, J., Dennis, P. and Duggal, H. 1990. The Stafford outbreak of Legionnaires' disease. Epidemiology & Infection 104(3), 361-380.

Orsi, G.B., Vitali, M., Marinelli, L., Ciorba, V., Tufi, D., Del Cimmuto, A., Ursillo, P., Fabiani, M., De Santis, S. and Protano, C. 2014. Legionella control in the water system of antiquated hospital buildings by shock and continuous hyperchlorination: 5 years experience. BMC infectious diseases 14(1), 394.

Otterholt, E. and Charnock, C. 2011. Identification and phylogeny of the small eukaryote population of raw and drinking waters. Water research 45(8), 2527-2538.

Pagnier, I., Merchat, M. and La Scola, B. 2009. Potentially pathogenic amoeba-associated microorganisms in cooling towers and their control. Future microbiology 4(5), 615-629.

Palmore, T.N., Stock, F., White, M., Bordner, M., Michelin, A., Bennett, J.E., Murray, P.R. and Henderson, D.K. 2009. A cluster of cases of nosocomial legionnaires disease linked to a contaminated hospital decorative water fountain. Infection Control & Hospital Epidemiology 30(8), 764-768.

Paranjape, K., Bédard, É., Whyte, L.G., Ronholm, J., Prévost, M. and Faucher, S.P. 2020. Presence of Legionella spp. in cooling towers: the role of microbial diversity, Pseudomonas, and continuous chlorine application. Water Research 169, 115252.

Pavissich, J., Vargas, I., González, B., Pastén, P. and Pizarro, G. 2010. Culture dependent and independent analyses of bacterial communities involved in copper plumbing corrosion. Journal of applied microbiology 109(3), 771-782.

Pereira, R.P.A., Peplies, J., Höfle, M.G. and Brettar, I. 2017. Bacterial community dynamics in a cooling tower with emphasis on pathogenic bacteria and Legionella species using universal and genus-specific deep sequencing. Water Research 122, 363-376.

Perrin, Y., Bouchon, D., Delafont, V., Moulin, L. and Héchard, Y. 2019. Microbiome of drinking water: A full-scale spatio-temporal study to monitor water quality in the Paris distribution system. Water Research 149, 375-385.

Phin, N., Parry-Ford, F., Harrison, T., Stagg, H.R., Zhang, N., Kumar, K., Lortholary, O., Zumla, A. and Abubakar, I. 2014. Epidemiology and clinical management of Legionnaires' disease. The Lancet infectious diseases 14(10), 1011-1021.

Pinto, A.J., Schroeder, J., Lunn, M., Sloan, W. and Raskin, L. 2014. Spatial-temporal survey and occupancy-abundance modeling to predict bacterial community dynamics in the drinking water microbiome. MBio 5(3), e01135-01114.

Pinto, A.J., Xi, C. and Raskin, L. 2012. Bacterial community structure in the drinking water microbiome is governed by filtration processes. Environmental science & technology 46(16), 8851-8859.

Poitelon, J.-B., Joyeux, M., Welté, B., Duguet, J.-P., Prestel, E., Lespinet, O. and DuBow, M.S. 2009a. Assessment of phylogenetic diversity of bacterial microflora in drinking water using serial analysis of ribosomal sequence tags. Water research 43(17), 4197-4206.

Poitelon, J.B., Joyeux, M., Welte, B., Duguet, J.P., Peplies, J. and DuBow, M. 2009b. Identification and phylogeny of eukaryotic 18S rDNA phylotypes detected in chlorinated finished drinking water samples from three Parisian surface water treatment plants. Letters in applied microbiology 49(5), 589-595.

Proctor, C.R. and Hammes, F. 2015. Drinking water microbiology—from measurement to management. Current Opinion in Biotechnology 33, 87-94.

Québec, G.d. 2014. Décret 454-2014 Loi sur le Bâtiment. Gazette Officielle du Québec 146(22), 1923-1927.

Rangel, K. 2010. A systematic review of biocides used in cooling tower for prevention and control of Legionella spp. contamination. Journal of the Cooling Tower Institute 31(1), 72.

Rasch, J., Krüger, S., Fontvieille, D., Ünal, C.M., Michel, R., Labrosse, A. and Steinert, M. 2016. Legionella-protozoa-nematode interactions in aquatic biofilms and influence of Mip on Caenorhabditis elegans colonization. International Journal of Medical Microbiology 306(6), 443-451.

Rhoads, W.J., Garner, E., Ji, P., Zhu, N., Parks, J., Schwake, D.O., Pruden, A. and Edwards, M.A. 2017. Distribution system operational deficiencies coincide with reported legionnaires' disease clusters in flint, michigan. Environmental science & technology 51(20), 11986-11995.

Ricketts, K., Charlett, A., Gelb, D., Lane, C., Lee, J. and Joseph, C. 2011. Weather patterns and Legionnaires' disease: a meteorological Study: CORRIGENDUM. Epidemiology and infection 139(9).

Ricketts, K., Yadav, R., Rota, M., Joseph, C. and Infections, E.W.G.f.L. 2010. Characteristics of reoffending accommodation sites in Europe with clusters of Legionnaires' disease, 2003–2007. Eurosurveillance 15(40), 19680.

Říhová, J., Nováková, E., Husník, F. and Hypša, V. 2017. Legionella becoming a mutualist: adaptive processes shaping the genome of symbiont in the louse Polyplax serrata. Genome biology and evolution 9(11), 2946-2957.

Robertson, P., Abdelhady, H. and Garduño, R.A. 2014. The many forms of a pleomorphic bacterial pathogen—the developmental network of Legionella pneumophila. Frontiers in microbiology 5, 670.

Rowbotham, T.J. 1980. Preliminary report on the pathogenicity of Legionella pneumophila for freshwater and soil amoebae. Journal of clinical pathology 33(12), 1179-1183.

Savio, D., Sinclair, L., Ijaz, U.Z., Parajka, J., Reischer, G.H., Stadler, P., Blaschke, A.P., Blöschl, G., Mach, R.L. and Kirschner, A.K. 2015. Bacterial diversity along a 2600 km river continuum. Environmental microbiology 17(12), 4994-5007.

Savory, J. 1954. Breakdown of timber by ascomycetes and fungi imperfecti. Annals of Applied Biology 41(2), 336-347.

Scheikl, U., Tsao, H.-F., Horn, M., Indra, A. and Walochnik, J. 2016. Free-living amoebae and their associated bacteria in Austrian cooling towers: a 1-year routine screening. Parasitology research 115(9), 3365-3374.

Schreiber, H., Schoenen, D. and Traunspurger, W. 1997. Invertebrate colonization of granular activated carbon filters. Water Research 31(4), 743-748.

Serrano-Suárez, A., Dellundé, J., Salvadó, H., Cervero-Aragó, S., Méndez, J., Canals, O., Blanco, S., Arcas, A. and Araujo, R. 2013. Microbial and physicochemical parameters associated with Legionella contamination in hot water recirculation systems. Environmental Science and Pollution Research 20(8), 5534-5544.

Shah, P.P., Barskey, A.E., Binder, A.M., Edens, C., Lee, S., Smith, J.C., Schrag, S., Whitney, C.G. and Cooley, L.A. 2018. Legionnaires' disease surveillance summary report, United States: 2014-1015.

Shaheen, M., Scott, C. and Ashbolt, N.J. 2019. Long-term persistence of infectious Legionella with free-living amoebae in drinking water biofilms. International journal of hygiene and environmental health 222(4), 678-686.

Sharma, L., Losier, A., Tolbert, T., Cruz, C.S.D. and Marion, C.R. 2017. Pneumonia updates on Legionella, Chlamydophila, and Mycoplasma pneumonia. Clinics in chest medicine 38(1), 45.

Steinert, M., Emödy, L., Amann, R. and Hacker, J. 1997. Resuscitation of viable but nonculturable Legionella pneumophila Philadelphia JR32 by Acanthamoeba castellanii. Appl. Environ. Microbiol. 63(5), 2047-2053.

Stewart, P.S. 1993. A model of biofilm detachment. Biotechnology and bioengineering 41(1), 111-117.

Stout, J., Yu, V. and Best, M. 1985. Ecology of Legionella pneumophila within water distribution systems. Appl. Environ. Microbiol. 49(1), 221-228.

Temmerman, R., Vervaeren, H., Noseda, B., Boon, N. and Verstraete, W. 2006. Necrotrophic growth of Legionella pneumophila. Appl. Environ. Microbiol. 72(6), 4323-4328.

Thomas, J.M. and Ashbolt, N.J. 2010. Do free-living amoebae in treated drinking water systems present an emerging health risk? Environmental science & technology 45(3), 860-869.

Thomas, V., McDonnell, G., Denyer, S.P. and Maillard, J.-Y. 2010. Free-living amoebae and their intracellular pathogenic microorganisms: risks for water quality. FEMS microbiology reviews 34(3), 231-259.

Tison, D., Pope, D., Cherry, W. and Fliermans, C. 1980. Growth of Legionella pneumophila in association with blue-green algae (cyanobacteria). Appl. Environ. Microbiol. 39(2), 456-459.

Torres Paniagua, A. (2019) Study of the microbiome of a model cooling tower harboring Legionella pneumophila. Master thesis, McGill University, Montreal, Canada.

Travis, T.C., Brown, E.W., Peruski, L.F., Siludjai, D., Jorakate, P., Salika, P., Yang, G., Kozak, N.A., Kodani, M. and Warner, A.K. 2012. Survey of Legionella species found in Thai soil. International journal of microbiology 2012.

Tsao, H.-F., Scheikl, U., Herbold, C., Indra, A., Walochnik, J. and Horn, M. 2019. The cooling tower water microbiota: Seasonal dynamics and co-occurrence of bacterial and protist phylotypes. Water research 159, 464-479.

Udaiyan, K. and Manian, S. 1991. Fungi colonising wood in the cooling tower water system at the Madras Fertilizer Company, Madras, India. International biodeterioration 27(4), 351-371.

Valster, R.M., Wullings, B.A., Bakker, G., Smidt, H. and van der Kooij, D. 2009. Free-living protozoa in two unchlorinated drinking water supplies, identified by phylogenic analysis of 18S rRNA gene sequences. Appl. Environ. Microbiol. 75(14), 4736-4746.

Valster, R.M., Wullings, B.A., van den Berg, R. and van der Kooij, D. 2011. Relationships between free-living protozoa, cultivable Legionella spp., and water quality characteristics in three drinking water supplies in the Caribbean. Appl. Environ. Microbiol. 77(20), 7321-7328.

van Heijnsbergen, E., Schalk, J.A., Euser, S.M., Brandsema, P.S., den Boer, J.W. and de Roda Husman, A.M. 2015. Confirmed and potential sources of Legionella reviewed. Environmental science & technology 49(8), 4797-4815.

Vaz-Moreira, I., Nunes, O.C. and Manaia, C.M. 2014. Bacterial diversity and antibiotic resistance in water habitats: searching the links with the human microbiome. FEMS microbiology reviews 38(4), 761-778.

Waak, M.B., Hozalski, R.M., Hallé, C. and LaPara, T.M. 2019. Comparison of the microbiomes of two drinking water distribution systems—with and without residual chloramine disinfection. Microbiome 7(1), 87.

Wadowsky, R.M. and Yee, R.B. 1983. Satellite growth of Legionella pneumophila with an environmental isolate of Flavobacterium breve. Appl. Environ. Microbiol. 46(6), 1447-1449.

Wallis, L. and Robinson, P. 2005. Soil as a source of Legionella pneumophila serogroup 1 (Lp1). Australian and New Zealand journal of public health 29(6), 518-520.

Wang, H., Edwards, M.A., Falkinham III, J.O. and Pruden, A. 2013. Probiotic approach to pathogen control in premise plumbing systems? A review. Environmental science & technology 47(18), 10117-10128.

Wang, H., Masters, S., Edwards, M.A., Falkinham III, J.O. and Pruden, A. 2014. Effect of disinfectant, water age, and pipe materials on bacterial and eukaryotic community structure in drinking water biofilm. Environmental science & technology 48(3), 1426-1435.

Warnecke, F., Sommaruga, R., Sekar, R., Hofer, J.S. and Pernthaler, J. 2005. Abundances, identity, and growth state of actinobacteria in mountain lakes of different UV transparency. Appl. Environ. Microbiol. 71(9), 5551-5559.

Watarai, M., Derre, I., Kirby, J., Growney, J.D., Dietrich, W.F. and Isberg, R.R. 2001. Legionella pneumophila Is Internalized by a Macropinocytotic Uptake Pathway Controlled by the Dot/Icm System and the Mouse Lgn1 Locus. Journal of Experimental Medicine 194(8), 1081-1096.

Weiss, D., Boyd, C., Rakeman, J.L., Greene, S.K., Fitzhenry, R., McProud, T., Musser, K., Huang, L., Kornblum, J. and Nazarian, E.J. 2017. A large community outbreak of Legionnaires' disease associated with a cooling tower in New York City, 2015. Public health reports 132(2), 241-250.

Whiley, H. and Bentham, R. 2011. Legionella longbeachae and legionellosis. Emerging infectious diseases 17(4), 579.

Williams, M., Domingo, J., Meckes, M., Kelty, C. and Rochon, H. 2004. Phylogenetic diversity of drinking water bacteria in a distribution system simulator. Journal of applied microbiology 96(5), 954-964.

Winn Jr, W.C. 2015. Legionella. Bergey's Manual of Systematics of Archaea and Bacteria, 1-44.

Winn, W.C. 1988. Legionnaires disease: historical perspective. Clinical Microbiology Reviews 1(1), 60-81.

Wong, T.H. 2006. Water sensitive urban design-the journey thus far. Australasian Journal of Water Resources 10(3), 213-222.

Yabuchi, E., Wang, L., Yamayoshi, T., Arakawa, M. and Yano, I. 1995. Bactericidal effect of chlorine on strains of Legionella species. Kansenshogaku zasshi. The Journal of the Japanese Association for Infectious Diseases 69(2), 151-157.

Yamamoto, H., Sugiura, M., Kusunoki, S., Ezaki, T., Ikedo, M. and Yabuuchi, E. 1992. Factors stimulating propagation of legionellae in cooling tower water. Appl. Environ. Microbiol. 58(4), 1394-1397.

CONNECTING TEXT

The next chapter is the manuscript for my first published article entitled: "Presence of *Legionella spp.* in Cooling Towers: The Role of Microbial Diversity, *Pseudomonas*, and Continuous Chlorine Application". This article was published in the peer-reviewed journal *Water Research*. This article examined the bacterial community of cooling towers, in Quebec, and its relationship with *L. pneumophila* ecology. The results showed that several physicochemical parameters were responsible for moulding the bacterial community. Geographic location, water source, and chlorine were examples of major physicochemical factors that could mould the community. Continuous application of chlorine was of particular interest as it was associated with the establishment of *Pseudomonas* population in the towers. This *Pseudomonas* population was negatively correlated with most other bacterial taxa in the cooling tower, including *Legionella* OTUs. Consequently, continuous application of chlorine could be a method to reduce levels of *L. pneumophila* in cooling towers. The research identified several bacterial taxa predictors of the presence or absence of *Legionella* in the cooling towers.

Contribution of authors: I am the lead author of the article. I contributed to the experimental design and I was involved with the preparation of the genomic DNA library samples for next generation sequencing. I processed the samples, analyzed the data, and wrote the draft of the article. Émilie Bédard was responsible for the organising the sampling campaign, managing some of the physicochemical tests, contributed to the design of the experiment, and reviewed the draft manuscript. Lyle G. White provided some of the primers for the sequencing run and reviewed the draft manuscript. Jennifer Ronholm provided some of the primers for sequencing, and helped with

creating the DNA library for sequencing, running the samples on the sequencing platform, and reviewing the draft manuscript. Michèle Prévost and Sébastien P. Faucher contributed to the experimental design, writing and editing of the manuscript.

CHAPTER 3. PRESENCE OF *LEGIONELLA SPP.* IN COOLING TOWERS: THE ROLE OF MICROBIAL DIVERSITY, *PSEUDOMONAS*, AND CONTINUOUS CHLORINE APPLICATION.

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Running title: Microbiome of cooling towers.

HIGHLIGHTS

- The bacterial community profiles of 18 cooling towers were characterized.
- The bacterial communities are influenced by the source of the makeup water.
- The presence of *Legionella* is positively correlated with alpha diversity.
- *Legionella* is negatively correlated with *Pseudomonas*.
- Continuous chlorine treatment restricts *Legionella* but promotes *Pseudomonas*

ABSTRACT

Legionnaire's disease (LD) is a severe pneumonia caused by several species of the genus *Legionella*, most frequently by *Legionella pneumophila*. Cooling towers are the most common source for large community-associated outbreaks. Colonization, survival, and proliferation of *L. pneumophila* in cooling towers are necessary for outbreaks to occur. These steps are affected by the chemical and physical parameters of the cooling tower environment. We hypothesize that the bacterial community residing in the cooling tower could also affect the presence of *L. pneumophila*. A *16S rRNA* gene targeted amplicon sequencing approach was used to study the bacterial community of cooling towers and its relationship with the *Legionella spp.* and *L. pneumophila* communities. The results indicated that the water source shaped the bacterial community of cooling towers. Several taxa were enriched and positively correlated with *Legionella spp.* and *L. pneumophila*. In contrast, *Pseudomonas* showed a strong negative correlation with *Legionella spp.* and several other genera. Most importantly, continuous chlorine application reduced microbial diversity and promoted the presence of *Pseudomonas* creating a non-permissive environment for *Legionella spp*. This suggests that disinfection strategies as well as the resident microbial population influences the ability of *Legionella spp.* to colonize cooling towers.

Keywords: Microbiome, *16S rRNA* Gene Targeted Amplicon Sequencing, Cooling Towers, *Legionella pneumophila*, *Pseudomonas*, Chlorine

I- INTRODUCTION

Legionnaires' disease (LD) is a severe and potentially fatal pneumonia caused by several bacterial species of the genus *Legionella*. More than 90% of cases are caused by the species *Legionella pneumophila* (Kozak-Muiznieks et al., 2014) . The remaining 10% of cases are caused by other species, such as *L. longbeachae*, *L. bozemanii*, and *L. dumoffii* (Brenner et al., 1980; McKinney et al., 1981; Phin et al., 2014). LD is usually contracted through the inhalation of contaminated aerosols. Consequently, Engineered Water Systems (EWS), such as hot water distribution systems, cooling towers, water fountains, misters, and whirlpool spas are sources of dissemination of the bacterium (Bédard et al., 2016; Haupt et al., 2012; Jernigan et al., 1996; Mahoney et al., 1992; Palmore et al., 2009; van Heijnsbergen et al., 2015). Potable water systems are the most common source of LD outbreaks, however, cooling towers are a major source for large community-associated outbreaks, and up to 28% of all sporadic cases(Fitzhenry et al., 2017; Llewellyn et al., 2017).

In recent years, the number of cases of LD has increased both in Europe and North America (Control and Prevention, 2011; ECDC, 2019). From 2000 to 2014, the CDC reported an increase of 286% in cases of legionellosis (LD and Pontiac fever) in the USA (Garrison et al., 2016). This increase is likely due to increasing population in urban areas, improvements in surveillance methods, aging populations, and climate change (ECDC, 2019). *Legionella* is now the main cause of death due to waterborne diseases in the US (McClung et al., 2017).

Several steps are needed for a cooling tower to become the source of an outbreak of LD. First, the tower must be seeded with *Legionella* species causing LD. During operation, the water lost through evaporation is replenished either with municipal water, on-site well water or available surface water, which may be the source of *L. pneumophila* (Donohue et al., 2014; Lesnik et al.,

2016). Next, *L. pneumophila* must survive and proliferate in the cooling tower environment. Encountered stresses include low quantity of nutrients, disinfectants, and competing microbes (Berjeaud et al., 2016; Lau and Ashbolt, 2009). *L. pneumophila* can survive up to several months in oligotrophic water while retaining infectivity (Mendis et al., 2015). Multiple factors may affect the prevalence of *Legionella* and its hosts in cooling towers including operational factors, temperature, water quality, the age of the equipment, the use of biocides (dosage, type, application schedule, and residual concentration), and elevated bacterial indicators such as heterotrophic plate counts (HPC) (Bédard et al., 2015; Canals et al., 2015; Mouchtouri et al., 2010; Yamamoto et al., 1992). In addition, biofilms offer protection against disinfectants, while also providing nutrients and host cells (Declerck et al., 2009; Donlan et al., 2002; Lau and Ashbolt, 2009; Murga et al., 2001; Stewart et al., 2012). While it is not clear if *L. pneumophila* can grow in biofilms independently of protozoan host cells, several studies indicate that this might be possible (Murga et al., 2001; Surman et al., 2002; Temmerman et al., 2006). Moreover, the ability of *L. pneumophila* to colonize biofilms may depend on the microbial community composition of these biofilms (Gião et al., 2011; Stewart et al., 2012). For example, *L. pneumophila* persists in *Klebsiella pneumoniae* biofilms but not in *Pseudomonas aeruginosa* biofilms (Stewart et al., 2012). In addition, the surface material on which biofilms grow influence *L. pneumophila* survivability (Buse et al., 2014b; Rogers et al., 1994; Türetgen and Cotuk, 2007; Van der Kooij et al., 2005). Finally, some microorganisms present in cooling towers can prey on *L. pneumophila* and reduce its population. For instance, protozoa, such as *Solumitrus palustris*, and bacteria, such as *Bdellovibrio spp.*, can feed on *L. pneumophila* in experimental settings (Amaro et al., 2015; Richardson, 1990; Tomov et al., 1982). Consequently, the presence of these species may restrict *L. pneumophila*'s colonization of cooling towers.

Following this initial colonisation, the *L. pneumophila* population must grow to sufficient number to be dispersed effectively and cause an LD outbreak. *L. pneumophila* is an intracellular parasite of amoeba and ciliates, such as *Acanthamoeba castellanii*, *Vermamoeba vermiformis* and *Tetrahymena pyriformis* (Fields et al., 2002; Gao et al., 1997; Rowbotham, 1980). Consequently, the cooling tower must harbour a large number of host cells in order for *L. pneumophila* to grow sufficiently to contaminate the aerosols produced. The host cell population is also affected by the chemical and physical parameters of the cooling tower environment (Buse et al., 2014a; Lu et al., 2014; Wang et al., 2014). As these host cells graze on the bacterial community of cooling towers, microbial interactions necessarily impact their growth. For instance, some host cells may require specific prey in order to grow (Amaro et al., 2015). Conversely, certain species of bacteria are able to resist predation and even grow intracellularly, effectively competing against *L. pneumophila* (Abd et al., 2008). For instance, *Mycobacterium* is frequently found in water systems alongside *Legionella* and can have ecological associations with certain protozoan species (Falkinham et al., 2015; Steinert et al., 1998). *Pseudomonas* may also play an important role in the ecology of *Legionella* through direct or indirect interactions (Corre et al., 2018; Matz et al., 2008). Of note, some members of *Mycobacterium* and *Pseudomonas* are opportunistic pathogen associated with EWS (Falkinham III et al., 2015). In contrast, the bacterial community may have a direct beneficial effect on the proliferation of *Legionella*. For instance, *Fischerella spp.* (*Cyanobacteria*) and *Flavobacterium* promote the growth of *L. pneumophila* in experimental setups (Tison et al., 1980; Wadowsky and Yee, 1983).

The majority of cooling towers seem to contain a core *Legionella spp.* community (Llewellyn et al., 2017; Pereira et al., 2017; Wéry et al., 2008). However, the stability of this community is still not well understood and *L. pneumophila* seems able to proliferate to the

detriment of other *Legionella* species (Pereira et al., 2017; Wéry et al., 2008). Chemical disinfection is a disruptor to the *Legionella* community but *L. pneumophila* seems quicker to recover after chlorine treatment and can dominate the *Legionella* community (Pereira et al., 2017; Wéry et al., 2008). Moreover, relative abundance of the family *Legionellaceae* is positively correlated with alpha diversity (Llewellyn et al., 2017) suggesting that microbial interactions are essential for the growth of *L. pneumophila* in cooling towers.

Thus, outbreaks of LD are driven by chemical and physical properties, as well as microbial interactions. Nevertheless, the ecology of *L. pneumophila* in cooling towers is still poorly understood and potential interactions with resident microbes need to be clarified. Consequently, we used a *16S rRNA* gene targeted amplicon sequencing approach to characterize the bacterial community of cooling towers, along with the chemical and physical characteristics, and investigate their relationship with *L. pneumophila*. We hypothesize that the presence of *L. pneumophila* depends on certain groups of bacteria, whose presence is influenced by other factors such as disinfectants or water characteristics.

II- MATERIALS AND METHODS

1. SAMPLING OF COOLING TOWERS

A total of 18 cooling towers were sampled from six different regions in Quebec, Canada, between the 10th and 21st of July 2017. Location of towers, total and residual chlorine levels, and disinfection regimes are listed in Table 1. Water was sampled with sterile polypropylene bottles from the basin of the cooling towers or from a sampling port when the basin was inaccessible. All

towers were sampled in triplicate in volumes of one litre to perform heterotrophic plate counts and *16S rRNA* gene targeted amplicon sequencing. Sodium thiosulfate was added at a final concentration of 0.01% to the sampled water as soon as the sample was collected. The water samples were processed based on the standard method 9215-D for the examination of water and wastewater (American Public Health et al., 2005). An additional two litres were collected to analyze chemical and physical parameters. Samples were brought back to the lab stored at room temperature and processed within 48 hours.

2. *HETEROTROPHIC PLATE COUNTS, PHYSICAL AND CHEMICAL PARAMETER MEASUREMENTS*

Heterotrophic plate counts (HPC) were performed on R2A agar medium, which were incubated at 30°C for 24 hours. Turbidity, pH, temperature, total chlorine, residual chlorine, conductivity and dissolved oxygen were measured on-site. Residual and total chlorine were measured using a Pocket Colorimeter™ II (Hach, Loveland, CO, USA), conductivity, turbidity with a Hach 2100Q (Hach, Loveland, CO, USA) while pH and dissolved oxygen were measured using a Hach Multi-Parameter HQ40d tool (Hach, Loveland, CO, USA). Water samples were further analysed for the following chemical parameters: total suspended solids (TSS) and suspended volatile solids (VSS, Standard Methods 2540D, E), dissolved organic carbon (DOC, Standard Methods 5310C with 0.45 um filtration), biodegradable dissolved organic carbon (Servais et al., 1987), dissolved and total iron (Inductively Coupled Plasma). Nitrite, nitrate, ammonia, phosphorus, sulphide, and sulphate were measured using colorimetric kits (CHEMetrics, Midland, VA, USA) according to the manufacturer's instruction.

3. *Filtration of Biomass and DNA Extraction*

Water samples were filtered through 0.45 µm pore size mixed cellulose ester membrane filters (Millipore, Burlington, MA, USA). Each replicate was filtered and processed separately. The DNeasy PowerWater Kit from Qiagen (Cat. No. 14900-100-NF, Germantown, MD, USA) was used to extract DNA from the filters. The manufacturer's protocol was followed, except that nuclease-free water was used for the final elution step. The extracted DNA was quantified using a Nanodrop (Thermofisher, MA, USA) and the purified DNA was stored at -20°C.

4. *Bacterial Profiling of CoolingTowers Using 16S rRNA Gene Targeted Amplicon Sequencing*

16S rRNA gene targeted amplicon sequencing was performed on the Illumina MiSeq platform (Illumina, inc) using a sequencing strategy developed by *Kozich et al*, which uses a dual index sequencing strategy using the F548 and R806 primers which amplify the V4 region of the bacterial *16S rRNA* gene (Kozich et al., 2013). Briefly, the V4 region of the bacterial *16S rRNA* gene was amplified using the Hot Start Taq Plus Master Mix (Qiagen, Germantown, MD, USA) and indexed primers (Kozich et al., 2013). The cycling program consisted of an initial denaturation step of 95°C for 2min, followed by 25 cycles of 95°C for 20 seconds, 55°C for 15 seconds, and 72°C for 5 minutes, and a final elongation of 10 minutes at 72°C. The PCR products were then purified using Ampure XP beads (Beckman Coulter, Indianapolis, IN, USA) according to the manufacturer's instruction. The purified DNA was quantified using the Quant-iT PicoGreen dsDNA assay kit (Thermofisher, MA, USA). The DNA samples were then normalized to a concentration of 1.5 ng/µl, pooled together, mixed with 10% PhiX sequencing control (Illumina, inc), diluted to 4.0 pM, and denatured with a final concentration of 0.0002N NaOH. The

sequencing run was performed on the MiSeq platform with the MiSeq Reagent kit V2, according to the manufacturer's instruction. Raw sequence reads were deposited in Sequence Read Archive under accession number PRJNA507738.

Sequencing data was processed using the Mothur pipeline (Kozich et al., 2013). Briefly, the paired reads were assembled into contigs, and any contig with ambiguous bases or longer than 275bp were culled. Sequences were aligned to the bacterial Silva reference database release 132. Sequences that did not align to the reference database were removed. The ends and gaps from the sequence alignment were trimmed so that all sequences had the same alignment coordinates. The sequences were further denoised using a pre-cluster algorithm implemented in Mothur. The resulting unique sequences were purged of chimeras using the VSEARCH algorithm. Additionally, any undesirable sequences remaining, such as *Eukaryota*, *Archaea*, chloroplasts, and mitochondria, were removed using a Bayesian classifier algorithm in Mothur. Next, sequences were assigned into de novo OTUs by clustering the sequences with a 97% similarity threshold. The MicrobiomeAnalyst web-based tool was used to analyse the OTU data and perform LEfSe analysis (http://www.microbiomeanalyst.ca/faces/home.xhtml) (Dhariwal et al., 2017). OTUs with low counts were filtered out using the default parameters (at least 20% of the samples contain 2 counts or more). One of the replicates for tower CN4 had significantly lower read levels than all the other samples. Thus, this replicate was omitted from the analysis, and the remaining samples were rarefied to the next lowest read count sample (20 712 sequences). Only duplicates were analysed for tower CN4. GraphPad Prism 7.03 and the R package tidyverse were used to produce the graphs along with some statistical analysis (Team, 2019; Wickham and Wickham, 2017).

5. *Quantification of L. pneumophila*

L. pneumophila was quantified from the DNA extract using the iQ-check *L. pneumophila* quantification kit (Bio-Rad), according to the manufacturer's instruction. The qPCR was run with a BioRad CFX Connect Real Time system thermocycler. The data was analyzed with the CFX manager 3.1 and GraphPad Prism 7.03. The results are expressed as genome unit per litre (GU/L).

III- RESULTS

1. *Characteristics of Cooling Towers included in this Study*

Eighteen cooling towers were sampled between the 10th and 21st of July 2017. Characteristics of each cooling tower, as well as, water profiles are described in Table 1 and Supplementary Table S1 (see appendix 1. On average, the water of the cooling towers sampled had the following characteristics: temperature, 25.2 ± 2.4 °C; pH, 8.7 ± 0.2; conductivity, 881 ± 275 µS/cm; dissolved oxygen, 8.0 ± 0.5 mg/L; dissolved organic carbon, 17 ± 10 mg/L. As seen in Figure 1, HPC were highly variable, ranging from 10^5 CFU/L for tower MTL3 to 10^9 CFU/L for tower Out1. Only five towers (CdQ1, CN2, CN3, MTL5 and Est2) showed detectable level of *L. pneumophila* ranging from 300 to 1300 GU/L (Figure 1), below the regulatory standards (Québec, 2014). Of note, *L. pneumophila*-positive towers were not restricted to a particular region.

Table 1: Disinfection schedule and location of cooling towers

Tower name	Total chlorine (mg Cl_2/L)	Free chlorine residual (mg Cl_2/L)	Disinfection schedule*	Administrative regions	Source of water
CdQ1	0.11	0.04	Weekly	Centre du Québec	Nicolet River
CN1	0.33	0.06	Daily	Capitale Nationale	St-Lawrence river
CN2	0.43	0.15	Continuous	Capitale Nationale	St-Charles lake, St-Lawrence river
CN3	1.72	0.32	Daily	Capitale Nationale	St-Charles lake, St-Lawrence river
CN4	3.66	0.47	Weekly	Capitale Nationale	St-Charles lake, St-Lawrence river
Out1	0.44	0.07	Continuous	Outaouais	Ottawa river
Out2	0.95	0.33	Continuous	Outaouais	Ottawa river
MTL1	0.35	0.13	Continuous	Montréal	St-Lawrence river
MTL2	0.16	0.07	NA	Montréal	St-Lawrence river

MTL3	0.93	0.27	Continuous	Montréal	St-Lawrence river
MTL4	0.34	0.08	Continuous	Montréal	St-Lawrence river
MTL5	0.11	0.06	Weekly	Montréal	St-Lawrence river
MTL6	0.14	0.05	Daily	Montréal	St-Lawrence river
Est1	0.15	0.12	Weekly	Estrie	St-François river
Est2	0.00	0.00	Weekly	Estrie	St-François river
Mont1	0.48	0.07	Continuous	Montérégie	St-Lawrence river
MTL7	4.11	0.27	Weekly	Montréal	St-Lawrence river
MTL8	3.85	0.83	Weekly	Montréal	St-Lawrence river

NA: not available.

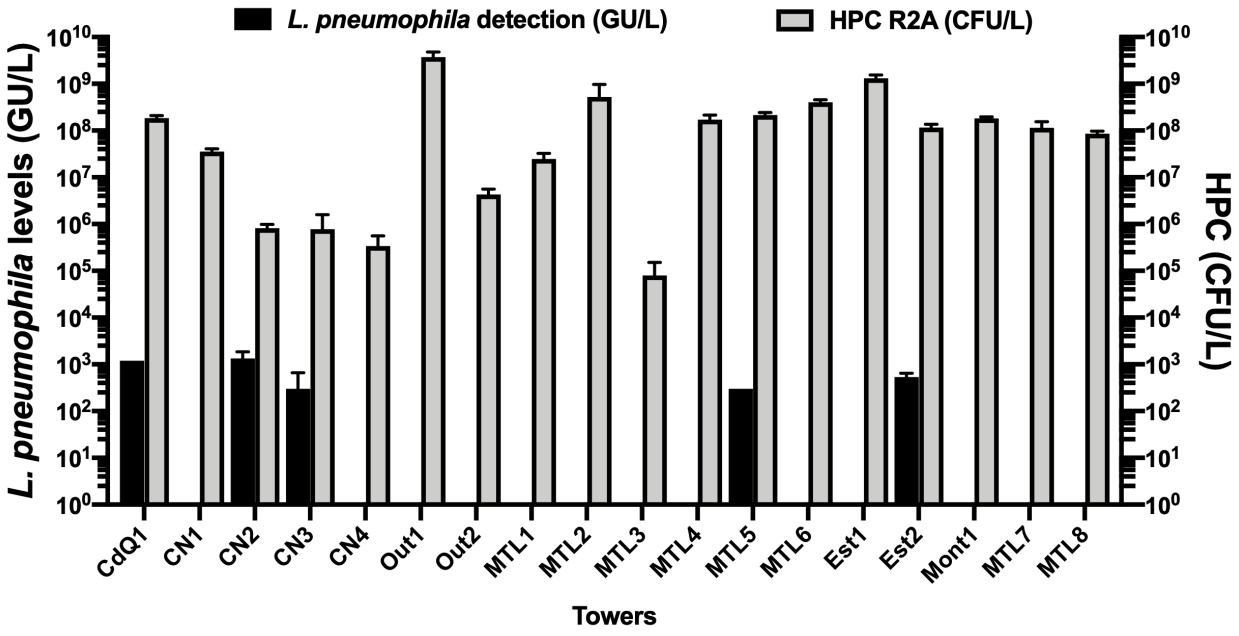

Figure 1: Levels of *L. pneumophila* in genomic units per litre (GU/L) detected by qPCR (black), and HPC measured on R2A agar (light grey). The data presented are the average and standard deviation of three sampling replicate. See table 1 for tower name and location details.

2. Characterisation of the Bacterial Community of Cooling Towers

16S rRNA gene targeted amplicon sequencing was performed on sampling triplicates (except for tower CN4, which was analyzed in duplicate since one of the replicates had a significant lower number of reads than the others) to study the bacterial makeup of the cooling towers. *Proteobacteria* dominated the bacterial population of all towers at the phylum level (Figure 2A). *Cyanobacteria* and *Bacteroidetes* were the second and third most abundant phyla. Seven towers showed a *Cyanobacteria* population above 1%, which in some cases reached up to around 30% of the entire population (tower CN3). In all cases, the *Cyanobacteria* population consisted of non-photosynthetic candidate phylum *Melainabacteria* (Di Rienzi et al., 2013). In the case of

Bacteroidetes, only five towers had a population above 1% reaching 10% for towers CdQ1 and MTL6. On average, the other phyla, such as *Actinobacteria* or *Firmicute*, constituted less than 1% of the population.

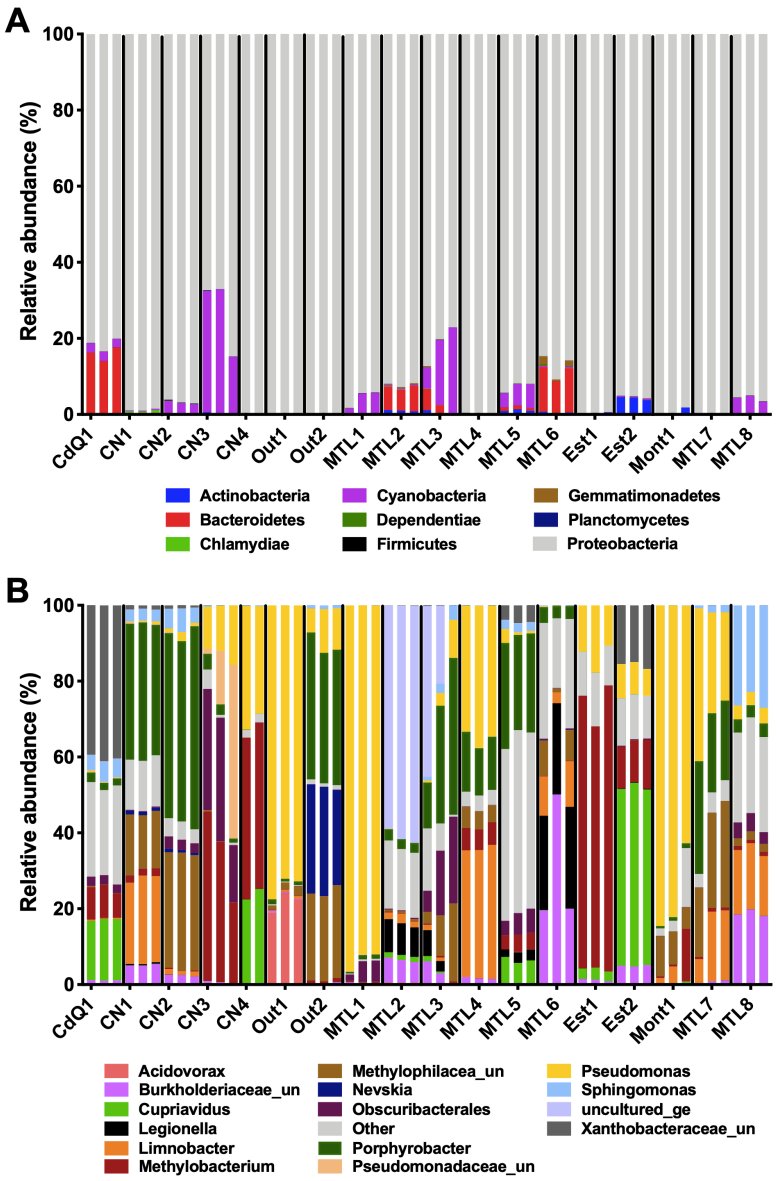

Figure 2: Relative abundance of bacterial OTU classified at the phylum level (A) and at the genus level (B) of the different cooling towers sampled in Quebec, Canada during the summer of 2017. See table 1 for name and location details.

The bacterial populations were also examined at lower taxonomic levels (Figure 2B). Overall, a total of 72 genera passed the low count filter described in the materials and methods section. The relative abundance patterns were similar between replicates but varied greatly between towers (Figure 2B). Several genera commonly found in other water systems were identified, such as *Pseudomonas*, *Limnobacter*, *Porphyrobacter*, *Legionella*, *Cupriavidus* and *Mycobacterium*. Interestingly, rare and uncharacterized genera were also identified, such as *Yonghaparkia* and Tra3-20 (Edwards et al., 1999; Nicomrat et al., 2008; Yoon et al., 2006). Methylotrophs were found in all towers, with groups such as *Methylobacterium* or unclassified *Methylophilaceae* being highly abundant in some. For instance, more than 70% of the bacterial population of tower Est1 belonged to the *Methylobacterium* genus.

3. Effect of Water Chemistry on Alpha Diversity of Cooling Towers

The Shannon diversity index was used to measure alpha diversity. The average Shannon index of the towers showed significant differences (Kruskal-Wallis, $P < 0.0001$; H=47.612; Supplementary Figure S1, see appendix 1). A multiple comparison test using Dunn's uncorrected test revealed that 41 pairs of towers out of a total of 153 pairs showed statistically different Shannon indexes (Supplementary Table S2, see appendix 1). TSS, VSS, DOC, total iron, and dissolved iron negatively affected alpha diversity (Supplementary Figure S2, see appendix 1). High conductivity was associated with higher alpha diversity (Supplementary Figure S2, see appendix 1). Next, the effect of chlorine concentration on alpha diversity was investigated. A threshold of 0.3 mg Cl_2/L was used to categorize the towers into two chlorine groups, as this is the required residual chlorine concentration in potable water leaving the municipal water treatment

plant in Quebec, which supplied the towers sampled (Québec, 2014). Measured total and residual chlorine had no effect on alpha diversity (Figure 3A and B). The frequency of application of chlorine had a significant effect on alpha diversity: continuous chlorination reduced alpha diversity compared to periodic application (daily and weekly, $P < 0.004$, Figure 3C). This suggests that the frequency of application of chlorine has a stronger impact on the microbial diversity of cooling towers than the concentrations of chlorine at the time of sampling.

Finally, the effect of alpha diversity on *Legionella*, *Mycobacterium* and *Pseudomonas* was investigated. The relationship between *Legionella* and alpha diversity was investigated by plotting the *Legionella* reads of each tower against their respective Shannon index (Figure 3C). Using a non-linear regression model, a positive correlation was identified, which followed an exponential curve (R^2=0.55). *Legionella pneumophila* and *Mycobacterium* were not present in all samples and, consequently, samples were partitioned into samples containing or not containing these genera. The presence of *Mycobacterium* was also associated with a higher alpha diversity ($P = 0.036$, Supplementary Figure S3, see appendix 1). No significant differences in alpha diversity were observed between *L. pneumophila*-positive towers and negative towers ($P > 0.05$, Figure 3E), indicating that alpha diversity is not correlated with *L. pneumophila*; however, the low number of positive towers could hide a relationship. Finally, the relation between *Pseudomonas* and alpha diversity was investigated. The data followed a non-linear regression model and indicated that alpha diversity of the towers decreased exponentially as *Pseudomonas* read counts increased (R^2=0.71 Figure 3F).

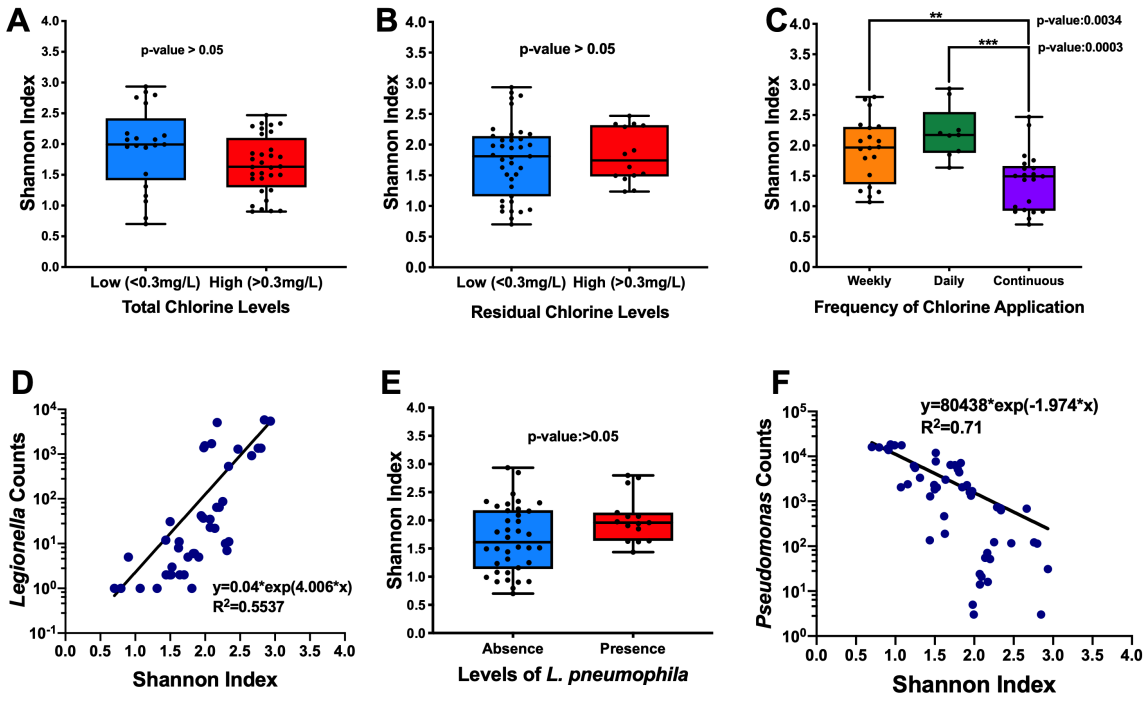

Figure 3: Alpha diversity of cooling towers categorized by levels of total chlorine (A), free residual chlorine (B), and frequency of application (C). The relationship between alpha diversity and *Legionella* and *Pseudomonas* was also investigated. The cooling towers categorized by (D) the presence of *Legionella spp.* according to 16S rRNA gene amplicon sequencing and (E) *L. pneumophila* detected by qPCR. In A, B, and E, a Mann-Whitney test was used to assess statistical significance. In C, a Kruskall-Wallis test followed by Dunn's test for pairwise comparison of samples was used to test statistical significance. In D and F, a non-linear regression model was used to fit the point to a curve.

4. Effect of Geographic Location on the Microbiome

Beta diversity was calculated to analyse differences between towers. The Bray-Curtis dissimilarity index was used to create a dissimilarity matrix and non-metric multidimensional

scaling (NMDS) was used to visualize the data. The data points were then clustered according to the physical, chemical, and biological parameters. ANOSIM was used to test the statistical significance and strength of clustering correlation. The source of the treated water feeding the cooling towers was the only parameter that created significantly different clusters (Figure 4A) in agreement with hydrological basin (Figure 4B). The towers fed by the Ottawa River (located in Gatineau) and the ones fed by the St-François River (located in Sherbrooke) had the highest dissimilarity (pairwise test: R = 1, *P* = 0.005). The towers fed from the St-Lawrence River (located in Montreal, Monteregie, and Quebec) and the towers fed with a mixture of water from St-Charles Lake and the St-Lawrence River (Quebec) clustered together (pairwise test: R = -0.07, P = 0.7).

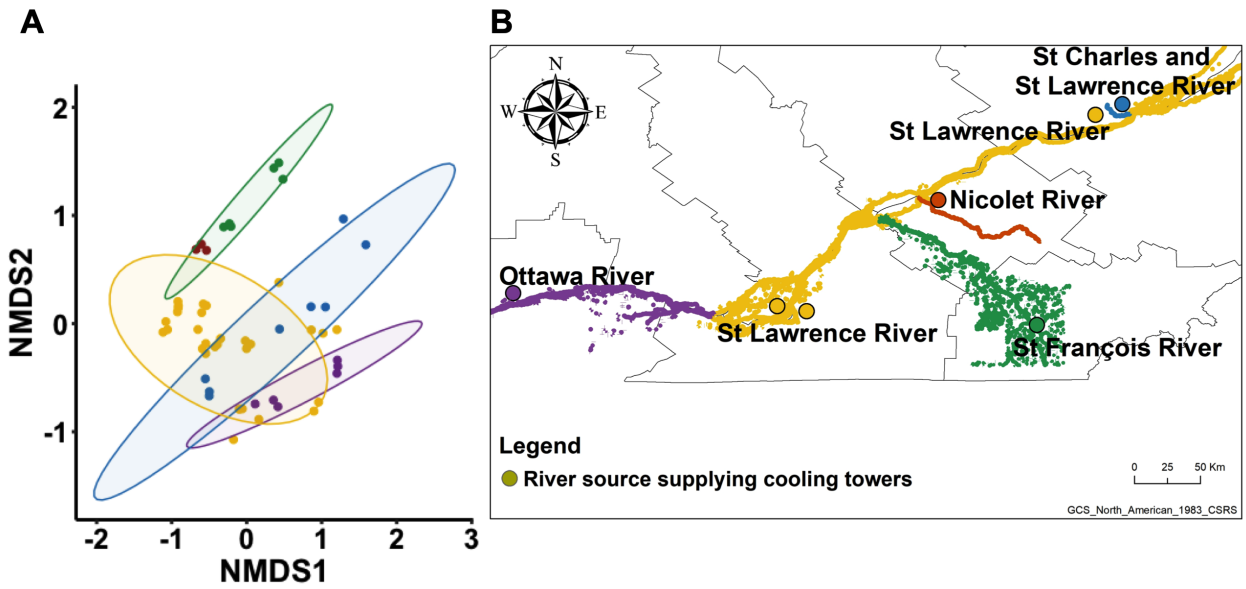

Figure 4: (A) Non-metric Multidimensional Scaling plot of tower microbiomes grouped by source of the water (stress = 0.1866). ANOSIM was used for statistical testing (R = 0.3927, P < 0.001). (B) Locations of the cooling towers sampled are indicated by circles colored according to the source of the water: Ottawa river (purple), St-Lawrence river (yellow), a mixture of water from St-Charles lake and St-Lawrence river (blue), St-François river (green), and Nicolet river (red).

5. *CORRELATION BETWEEN THE MICROBIOME AND KEY GENERA*

Next, the prevalence of different microorganisms in the cooling towers was investigated to determine the core community of cooling towers. Seven out of the 72 genera showed prevalence above 80%, including *Pseudomonas*, *Porphyrobacter*, *Methylobacterium*, *Blastomonas*, and unclassified genera from the *Methylophilaceae*, the *Burkholderiaceae*, and the *Sphingomonadaceae* families (Supplementary Figure S4, see appendix 1). *Pseudomonas* and *Methylobacterium* have near 100% prevalence in all towers at a relative abundance of 0.001; however, as relative abundance levels increased, prevalence decreased, indicating that these organisms are prevalent in most towers but at different abundance levels. Six other genera had prevalence between 50% and 80%, including *Limnobacter*, *Obscuribacteriales*, *Sphingomonas*, *Sphingopyxis*, *Novosphingobium*, and *Bosea*.

LEfSe was used to identify genera of importance for the different conditions studied. LEfSe is a machine-learning algorithm that uses a mix of statistical testing, linear discriminant analysis (LDA), and effect size to find the taxa that most likely explain the difference between specific parameters (Segata et al., 2011). The algorithm was able to find significant taxa for most conditions; however, we decided to focus on the conditions where *Legionella* or *Pseudomonas* were distinguishing features. *Legionella* is enriched in conditions with low levels of total chlorine, medium levels of conductivity, and in towers with daily application of chlorine (Figure 5A-B and Supplementary Figure S5A, see appendix 1). Conversely, *Pseudomonas* is enriched in towers with high levels of total chlorine, high levels of suspended solids, and with continuous application of chlorine (Figure 5A-B and Supplementary Figure S5B, see appendix 1).

LEfSe was then used to identify genera enriched in towers with *Legionella* and with *L. pneumophila* (Figure 5C-D). Fifteen taxa were enriched in *Legionella*-positive towers and *Pseudomonas* was the only taxon enriched in towers without *Legionella* (Figure 5C). This analysis is in good agreement with a Spearman's correlation analysis (Supplementary Figure S6, see appendix 1). Several of the bacterial groups enriched in the *Legionella*-positive towers are unclassified or poorly studied. Seven genera were enriched in *L. pneumophila*-positive towers (Figure 5D), including *Xanthobacteraceae* family, *Obscuribacterales* order, and the *Qipengyuania* genera. On the other hand, *Sphingobium* was the only genus enriched in towers that tested negative for *L. pneumophila* (LDA of 4.59).

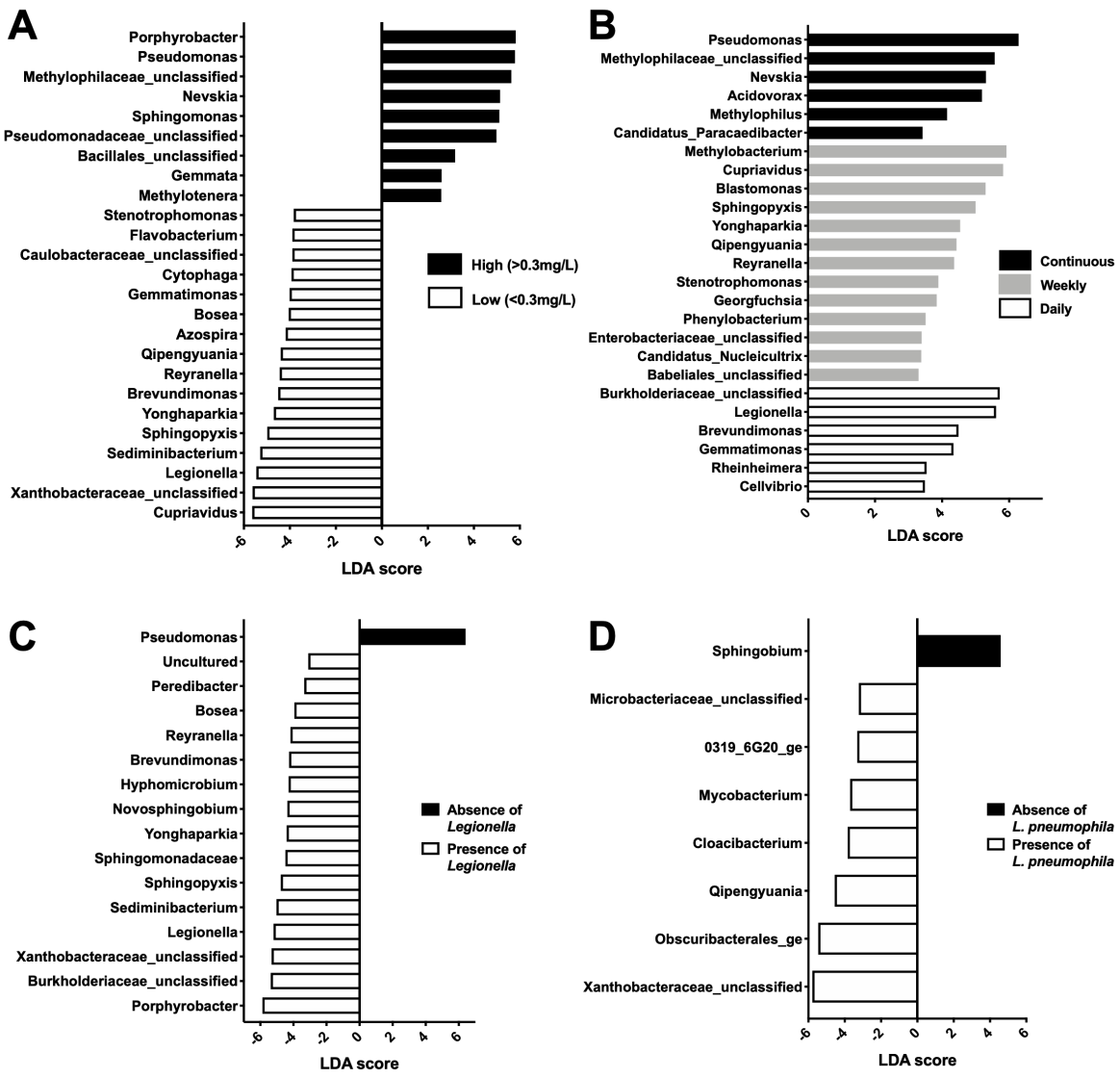

Figure 5: The machine learning algorithm LEfSe was used to identify significant taxa associated with chlorine concentrations (A), with daily, weekly, and continuous application of chlorine (B) as well as taxa enriched in towers with and without *Legionella spp.* (C), and in towers with and without *L. pneumophila* (D). The LDA score is an effect size that measures the importance of the taxa in the condition studied.

IV- DISCUSSION

This study provides a snapshot of the ecology of the bacterial community of cooling towers in Southern Quebec. We hypothesized that the resident microbial population influences the colonization, survival, and proliferation of *L. pneumophila* in cooling towers. The source of the water was the main factor explaining the difference in the microbial composition of the cooling towers in our study (Figure 4A). The St-Francois River and the Ottawa River are distinct hydrological basin resulting in distinct microbiomes in the cooling towers respectively fed by these sources. The towers fed by the St-Lawrence River showed similar microbiomes and overlapped with the towers fed with a mixture of water from the St-Lawrence River and the St-Charles lake (CN 2, 3 and 4). The Ottawa River feeds into the northern shore of the St-Lawrence River at the west of Montreal and the St-François River is a tributary joining the St-Lawrence river about 160 km downstream of Montreal, (Figure 4B). Both rivers probably have minimal impact on the St-Lawrence River microbiome. Taken together, our results suggest that the microbial composition of the source water dictates the microbial population of the cooling towers; however, other parameters associated with geographic location are likely to play a role. For example, the airborne microbiome could be a confounding factor as cooling towers intake great volumes of air due to their function and design (Milosavljevic and Heikkilä, 2001). To our knowledge there are no studies that have tested this possibility. Other parameters did not create significantly different clusters. Although chlorination schedule clearly affects the microbial diversity in cooling towers (Figure 3), its effect is non-specific.

Generally, the bacterial community were dominated by species from the *Proteobacteria* phylum. The use of 0.45 µm pore sized filters may have affected the populations, as some of the smaller bacteria might have passed through. Water from the cooling towers contained too much suspended particles, clogging 0.22 µm pore size filters after less than 100 mL. The use of 0.45 µm pore size filters probably had a minimal effect on the microbial profiles since most bacteria have a low passage rate (0.15% to 3.48%) through 0.45 µm pore size filters (Wang et al., 2008). Furthermore, our results were in agreement with several other studies that looked at cooling towers and other EWS (Delafont et al., 2016; Ivone et al., 2013; Ling et al., 2018; Llewellyn et al., 2017; Pavissich et al., 2010; Pereira et al., 2017; Pinto et al., 2014). While *Actinobacteria* and *Proteobacteria* dominate in equal proportions freshwater sources feeding EWS, the *Actinobacteria* population is greatly and significantly reduced in EWS, leaving the *Proteobacteria* as the dominant phylum of these environments (Debroas et al., 2009; Ghai et al., 2011; Linz et al., 2017; Newton et al., 2011; Pereira et al., 2017; Pinto et al., 2014). Water treatment increases levels of certain groups of *Alphaproteobacteria*, such as *Sphingomonadaceae*, *Beijerinckiaceae*, and *Rhizobiaceae* (Chao et al., 2013). Stagnation of water in pipes also contributes to increase levels of *Proteobacteria* (Pinto et al., 2014). Since all cooling towers in our study are fed with treated municipal water, the dominance of *Proteobacteria* was expected.

Some genera present in the cooling towers are frequently observed in other EWS, whereas others are less frequently identified. *Pseudomonas*, *Blastomonas*, *Methylobacterium*, and unclassified genera from the *Bukholderiaceae* family constituted the core microbiome of the cooling towers. The high prevalence of *Methylobacterium* indicates that methylotrophy could be an important ecological function in cooling towers. *Limnobacter*, *Sphingopyxis*, *Novosphingobium*, *Bosea* were only found in 50 to 60% of towers indicating that these taxa could

be part of a transient community or may depend on specific physical and chemical parameters only found in a subset of cooling towers (Supplementary Figure S4, see appendix 1). Our results differ somewhat compared to other studies. For instance, a two-year study of a German cooling tower showed high abundance of the environmental *Proteobacteria* ARKICE-90, *Nevskia* genus, *Methilophilus*, and uncultured bacteria from the family *Cytophagaceae*, but relatively low abundance of *Pseudomonadales* and absence of *Methylobacterium* (Pereira et al., 2017). Another study that looked at cooling towers of pharmaceutical plants and oil refinery in Italy and Eastern Europe also found high levels of *Proteobacteria*, such as *Rhodobacteraceae*, *Sphingomonadaceae*, *Bradyrhizobiaceae*, as well as *Cyanobacteria*, but no *Pseudomonas* (Di Gregorio et al., 2017). Thus, the community composition seems influenced by the intrinsic properties of a cooling tower and its geographic location. For instance, piping material, disinfection strategies, water sources, nitrate concentrations, iron concentrations, water treatment, dissolved organic carbon, and seasonality are all factors that have been shown to shape the bacterial population of different EWS (Gomez-Alvarez et al., 2012; Holinger et al., 2014; Ji et al., 2015; Li et al., 2010; Pinto et al., 2014; Poitelon et al., 2009; Van der Wielen et al., 2009).

In our case, several physico-chemical parameters affected the microbiome of cooling towers (Supplementary Figure S2, see appendix 1). *Legionella* was enriched in towers with low levels of total chlorine (<0.3mg/L) and with daily applications of chlorine whereas *Pseudomonas* was enriched in towers with high levels of chlorine and continuous application (Figure 5). These findings suggest that continuous application and maintenance of a free chlorine residual greater than 0.3 mg/L is key to prevent the colonization of cooling towers by *Legionella*. From the data, three possible mechanisms may explain this phenomenon. First, the most obvious explanation is that these parameters could ensure sufficient concentration and contact time to inactivate

Legionella, which is supported by previous studies showing that a free chlorine concentration superior to 0.1 mg/L is sufficient to kill *L. pneumophila* in vitro (Chang et al., 2008; Kuchta et al., 1983; Yabuchi et al., 1995). It is not clear, however, if this chlorine concentration is sufficient to kill *L. pneumophila* in a real cooling tower. In the natural environment, the resistance of *L. pneumophila* to chlorine may be improved by integration within biofilm or during intracellular growth (Barker and Brown, 1994; Lau and Ashbolt, 2009). The second explanation is linked to the decrease in alpha diversity caused by a continuous application of chlorine (Figure 3C), potentially restricting the growth of species beneficial for *Legionella spp*. A positive correlation between alpha diversity and *Legionella* is clear in the present study and was previously reported for cooling towers located in the United States (Llewellyn et al., 2017). The continuous presence of residual chlorine reduces the concentrations and diversity of host cells and the biofilm mass (Canals et al., 2015; Thomas et al., 2004), thus limiting the possibility for increased resistance of *L. pneumophila* through integration into the biofilm and inside protozoan hosts (Barker and Brown, 1994; Lau and Ashbolt, 2009). As seen in Figure 5C, *Legionella* was positively correlated with many genera, which could promote its survival and proliferation. For instance, *Reyranella*, *Brevundimonas*, *Sphingopyxis*, and *Yonghparkia* are enriched in *Legionella*-postitive towers with low level of chlorine and treated by periodic application. These genera may either directly or indirectly promote the growth of *Legionella spp*. For instance, *Brevundimonas vesicularis* improve growth of *Legionella* species in nutrient poor conditions (Koide et al., 2014). Alternatively, these taxa may be indicators of environmental condition permissive for the presence of *Legionella*. The third possible explanation for the lack of *Legionella spp.* in towers with high levels of chlorine and continuous application of chlorine may be linked with the presence of *Pseudomonas spp.* in these towers. This was demonstrated by using LEfSe and Spearman's correlation, which showed that

these two genera were the most negatively correlated to one another (Figure 5C and Supplementary Figure S6, see appendix 1). In contrast, the relation between the genus *Pseudomonas* and the species *L. pneumophila*, detected by qPCR, was less clear, since *Pseudomonas* is not a significant taxon in towers without *L. pneumophila* (Figure 7B). However, the average number of *Pseudomonas* reads was significantly lower (Mann-Whitney, $P < 0.05$) in *L. pneumophila* positive towers (920 reads) than in negative towers (5476 reads). Similarly, *Llewenlyn et al.* reported higher abundance of *Pseudomonadaceae* in *Legionella*-negative towers (Llewellyn et al., 2017). Thus, continuous chlorination may promote the establishment of a *Pseudomonas* community, which in turn would lower alpha diversity and lower levels of *Legionella spp*. This is substantiated by the fact that a positive correlation between chlorine and *Pseudomonas* was previously reported (Bertelli et al., 2018; Gomez-Alvarez et al., 2012; Holinger et al., 2014). Thus, positive correlation may be due to a higher tolerance to chlorine levels. Indeed, *Pseudomonas aeruginosa* has a higher tolerance to chlorine than other water-borne bacteria, which is attributed in part to biofilm formation (Bédard et al., 2014; Grobe et al., 2001; Seyfried and Fraser, 1980; Shrivastava et al., 2004; Wang et al., 2018). Consequently, higher chlorine levels may establish a *Pseudomonas* community in the cooling tower environment. Many species of *Pseudomonas* are highly competitive and possess many mechanisms to outcompete other bacteria, such as type VI secretion systems, pyoverdine, phenazine, and metabolic flexibility (Basler et al., 2013; Butaitė et al., 2017; Ho et al., 2013; Koehorst et al., 2016; Mazurier et al., 2009). The fact that *Pseudomonas spp*. is negatively correlated with alpha diversity further adds evidence to the competitive nature of *Pseudomonas* (Figure 3F). Many species of *Pseudomonas* inhibit the growth of *L. pneumophila* on CYE agar by producing antagonistic diffusible compound (Corre et al., 2018; Guerrieri et al., 2008). Furthermore, *L. pneumophila* is unable to persist in biofilm produced by *P. aeruginosa*

(Stewart et al., 2012). Consequently, *Pseudomonas spp.* may directly restrict the presence and growth of *Legionella spp* in water system. In addition, *Pseudomonas* could indirectly act on *Legionella spp*. *P. aeruginosa* is known to kill the amoeba *A. castellanii*, a host cell of *L. pneumophila*, by secreting toxic effector proteins using the type III secretion system (Matz et al., 2008) and could therefore reduce the pool of host cells. *Pseudomonas* may also inhibit the growth of certain bacterial species that promote the growth of *Legionella spp.* or that are preys for host cells. Thus, the data suggest that a concentration of chlorine superior to 0.3 mg/L applied continuously inhibit the colonization and proliferation of *Legionella spp.* but promote the establishment of a *Pseudomonas* community. This may be of concern for tower maintenance, as *P. aeruginosa* is an opportunistic pathogen of great concern (Pollack, 1983).

Finally, our results seemed to indicate that *Legionella spp.* and *L. pneumophila* are associated with several other genera. Spearman's correlation and LEfSe analysis showed that several taxa were positively correlated and enriched in towers containing a population of *Legionella* (Figure 7 and Supplementary Figure S6, see appendix 1). Of note, the family *Xanthobacteraceae* was positively correlated with both *Legionella spp.* and *L. pneumophila*. Many members of this family are chemolithoautotrophs and some are able to fix nitrogen (Oren, 2014). Therefore, they are likely at the bottom of the food chain and could feed *L. pneumophila* host cells. In addition, several isolates are able to degrade chlorinated and brominated compounds (Oren, 2014). An uncultured *Xanthobacteraceae* was identified as a component of biofilm growing in a model hot water system colonized by *L. pneumophila* (Van der Kooij et al., 2005). It is tempting to speculate that *Xanthobacteraceae* could help the development of healthy biofilms by producing organic molecules and reducing local concentration of disinfectant or toxic by-product, which in turn could promote *L. pneumophila* colonization. The genus *Sphingobium* was the only one

negatively correlated with *L. pneumophila*. Species from this genus may be associated with free-living amoeba (Liu et al., 2012). Although it is not clear if this genus contains species that can grow within amoeba, it can be hypothesized that *Sphingobium* could compete with *L. pneumophila* for host cells, which would result in lower *L. pneumophila* growth. So far and to the best of our knowledge, none of these taxa have been documented to interact with *Legionella* species. Furthermore, several of these taxa are unclassified or uncultured organisms and thus their life cycle and ecological interactions are poorly understood. Potentially, the interaction of these different taxa and *Legionella* could be indirect as they may be prey for host cells. This would support the hypothesis that *Legionella* colonization of towers depends on the establishment of bacterial community that feeds the host cell population. Our findings support the notion that the risk of Legionnaires' disease outbreaks may depend on a network of uncharacterized microbial interactions between *L. pneumophila* and the bacterial community, along with an optimal range of physical and chemical parameters that promote its colonization, survival, and proliferation in cooling towers. Since these interactions are speculative, it is important to note that further research is required to unravel their importance in *Legionella* ecology and colonization of cooling towers.

V- CONCLUSION

The aim of this study was to clarify the relationship between *Legionella*, the chemical parameters and the resident microbiota in cooling towers. Three main conclusions emerge from this work:

- The source of the water is the main factor affecting the bacterial community of cooling towers. Additional studies would be required to investigate if and how the water source

increases the likelihood of *Legionella* and *L. pneumophila* presence in cooling towers and how it can be controlled to reduce the associated risk of LD.

- The *Legionella* population itself is largely affected by alpha diversity, the level of *Pseudomonas*, levels of chlorine, and most importantly, the frequency of chlorine application. Of note, continuous chlorination seems to promote microbial conditions that protect the cooling towers against *Legionella* colonization.
- The presence of *Legionella* and *L. pneumophila* is associated with several taxa. Therefore, controlling the composition of the resident microbiota could be an alternative strategy to help reduce the presence of *Legionella* and *L. pneumophila* in cooling tower and mitigate the risk of LD outbreaks. Interestingly, several taxa are uncultured or unclassified suggesting that colonization of towers and likelihood of outbreaks could be potentiated by as of yet uncharacterized interactions between *L. pneumophila* and several bacterial species. This warrants additional research of the microbial relationships in water systems.

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VII- REFERENCE

Abd, H., Wretlind, B., Saeed, A., Idsund, E., Hultenby, K. and SANDSTRÖM, G. 2008. Pseudomonas aeruginosa utilises its type III secretion system to kill the free-living amoeba Acanthamoeba castellanii. Journal of eukaryotic microbiology 55(3), 235-243.

Amaro, F., Wang, W., Gilbert, J.A., Anderson, O.R. and Shuman, H.A. 2015. Diverse protist grazers select for virulence-related traits in Legionella. The ISME journal 9(7), 1607.

American Public Health, A., Eaton, A.D., American Water Works, A. and Water Environment, F. (2005) Standard methods for the examination of water and wastewater, APHA-AWWA-WEF, Washington, D.C.

Barker, J. and Brown, M. 1994. Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. Microbiology 140(6), 1253-1259.

Basler, M., Ho, B. and Mekalanos, J. 2013. Tit-for-tat: type VI secretion system counterattack during bacterial cell-cell interactions. Cell 152(4), 884-894.

Bédard, E., Charron, D., Lalancette, C., Déziel, E. and Prévost, M. 2014. Recovery of Pseudomonas aeruginosa culturability following copper-and chlorine-induced stress. FEMS microbiology letters 356(2), 226-234.

Bédard, E., Fey, S., Charron, D., Lalancette, C., Cantin, P., Dolcé, P., Laferrière, C., Déziel, E. and Prévost, M. 2015. Temperature diagnostic to identify high risk areas and optimize Legionella pneumophila surveillance in hot water distribution systems. Water research 71, 244-256.

Bédard, E., Lévesque, S., Martin, P., Pinsonneault, L., Paranjape, K., Lalancette, C., Dolcé, C.-É., Villion, M., Valiquette, L. and Faucher, S.P. 2016. Energy conservation and the promotion of Legionella pneumophila growth: the probable role of heat exchangers in a nosocomial outbreak. infection control & hospital epidemiology 37(12), 1475-1480.

Berjeaud, J.-M., Chevalier, S., Schlusselhuber, M., Portier, E., Loiseau, C., Aucher, W., Lesouhaitier, O. and Verdon, J. 2016. Legionella pneumophila: the paradox of a highly sensitive opportunistic waterborne pathogen able to persist in the environment. Frontiers in microbiology 7, 486.

Bertelli, C., Courtois, S., Rosikiewicz, M., Piriou, P., Aeby, S., Robert, S., Loret, J.-F. and Greub, G. 2018. Reduced chlorine in drinking water distribution systems impacts bacterial biodiversity in biofilms. Frontiers in microbiology 9, 2520.

Brenner, D.J., Steigerwalt, A.G., Gorman, G., Weaver, R.E., Feeley, J.C., Cordes, L., Wilkinson, H.W., Patton, C., Thomason, B.M. and Sasseville, K.R.L. 1980. Legionella bozemanii sp. nov. andLegionella dumoffii sp. nov.: classification of two additional species ofLegionella associated with human pneumonia. Current Microbiology 4(2), 111-116.

Buse, H.Y., Lu, J., Lu, X., Mou, X. and Ashbolt, N.J. 2014a. Microbial diversities (16S and 18S rRNA gene pyrosequencing) and environmental pathogens within drinking water biofilms grown on the common premise plumbing materials unplasticized polyvinylchloride and copper. FEMS microbiology ecology 88(2), 280-295.

Buse, H.Y., Lu, J., Struewing, I.T. and Ashbolt, N.J. 2014b. Preferential colonization and release of Legionella pneumophila from mature drinking water biofilms grown on copper versus unplasticized polyvinylchloride coupons. International journal of hygiene and environmental health 217(2-3), 219-225.

Butaitė, E., Baumgartner, M., Wyder, S. and Kümmerli, R. 2017. Siderophore cheating and cheating resistance shape competition for iron in soil and freshwater Pseudomonas communities. Nature communications 8(1), 1-12.

Canals, O., Serrano-Suárez, A., Salvadó, H., Méndez, J., Cervero-Aragó, S., De Porras, V.R., Dellundé, J. and Araujo, R. 2015. Effect of chlorine and temperature on free-living protozoa in operational man-made water systems (cooling towers and hot sanitary water systems) in Catalonia. Environmental Science and Pollution Research 22(9), 6610-6618.

Chang, C.-W., Chang, W.-L., Chang, S.-T. and Cheng, S.-S. 2008. Antibacterial activities of plant essential oils against Legionella pneumophila. Water research 42(1-2), 278-286.

Chao, Y., Ma, L., Yang, Y., Ju, F., Zhang, X.-X., Wu, W.-M. and Zhang, T. 2013. Metagenomic analysis reveals significant changes of microbial compositions and protective functions during drinking water treatment. Scientific reports 3, 3550.

Control, C.f.D. and Prevention 2011. Legionellosis---United States, 2000-2009. MMWR. Morbidity and mortality weekly report 60(32), 1083.

Corre, M.-H., Delafont, V., Legrand, A., Berjeaud, J.-M. and Verdon, J. 2018. Exploiting the richness of environmental waterborne bacterial species to find natural anti-Legionella active biomolecules. Frontiers in microbiology 9, 3360.

Debroas, D., Humbert, J.F., Enault, F., Bronner, G., Faubladier, M. and Cornillot, E. 2009. Metagenomic approach studying the taxonomic and functional diversity of the bacterial community in a mesotrophic lake (Lac du Bourget–France). Environmental microbiology 11(9), 2412-2424.

Declerck, P., Behets, J., Margineanu, A., van Hoef, V., De Keersmaecker, B. and Ollevier, F. 2009. Replication of Legionella pneumophila in biofilms of water distribution pipes. Microbiological research 164(6), 593-603.

Delafont, V., Bouchon, D., Héchard, Y. and Moulin, L. 2016. Environmental factors shaping cultured free-living amoebae and their associated bacterial community within drinking water network. Water research 100, 382-392.

Dhariwal, A., Chong, J., Habib, S., King, I.L., Agellon, L.B. and Xia, J. 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic acids research 45(W1), W180-W188.

Di Gregorio, L., Tandoi, V., Congestri, R., Rossetti, S. and Di Pippo, F. 2017. Unravelling the core microbiome of biofilms in cooling tower systems. Biofouling 33(10), 793-806.

Di Rienzi, S.C., Sharon, I., Wrighton, K.C., Koren, O., Hug, L.A., Thomas, B.C., Goodrich, J.K., Bell, J.T., Spector, T.D. and Banfield, J.F. 2013. The human gut and groundwater harbor non-photosynthetic bacteria belonging to a new candidate phylum sibling to Cyanobacteria. elife 2, e01102.

Donlan, R., Murga, R., Carpenter, J., Brown, E., Besser, R. and Fields, B. (2002) Legionella, pp. 406-410, American Society of Microbiology.

Donohue, M.J., O'Connell, K., Vesper, S.J., Mistry, J.H., King, D., Kostich, M. and Pfaller, S. 2014. Widespread molecular detection of Legionella pneumophila serogroup 1 in cold water taps across the United States. Environmental science & technology 48(6), 3145-3152.

ECDC 2019. Legionnaires' Disease. ECDC Annual epdimilogical report for 2017.

Edwards, K.J., Goebel, B.M., Rodgers, T.M., Schrenk, M.O., Gihring, T.M., Cardona, M.M., Mcguire, M.M., Hamers, R.J., Pace, N.R. and Banfield, J.F. 1999. Geomicrobiology of pyrite (FeS2) dissolution: case study at Iron Mountain, California. Geomicrobiology Journal 16(2), 155-179.

Falkinham III, J.O., Hilborn, E.D., Arduino, M.J., Pruden, A. and Edwards, M.A. 2015. Epidemiology and ecology of opportunistic premise plumbing pathogens: Legionella pneumophila, Mycobacterium avium, and Pseudomonas aeruginosa. Environmental health perspectives 123(8), 749-758.

Falkinham, J.O., Pruden, A. and Edwards, M. 2015. Opportunistic premise plumbing pathogens: increasingly important pathogens in drinking water. Pathogens 4(2), 373-386.

Fields, B.S., Benson, R.F. and Besser, R.E. 2002. Legionella and Legionnaires' disease: 25 years of investigation. Clinical microbiology reviews 15(3), 506-526.

Fitzhenry, R., Weiss, D., Cimini, D., Balter, S., Boyd, C., Alleyne, L., Stewart, R., McIntosh, N., Econome, A. and Lin, Y. 2017. Legionnaires' disease outbreaks and cooling towers, New York City, New York, USA. Emerging infectious diseases 23(11), 1769.

Gao, L.-Y., Harb, O.S. and Kwaik, Y.A. 1997. Utilization of similar mechanisms by Legionella pneumophila to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa. Infection and immunity 65(11), 4738-4746.

Garrison, L.E., Kunz, J.M., Cooley, L.A., Moore, M.R., Lucas, C., Schrag, S., Sarisky, J. and Whitney, C.G. 2016. Vital signs: deficiencies in environmental control identified in

outbreaks of Legionnaires' disease—North America, 2000–2014. American Journal of Transplantation 16(10), 3049-3058.

Ghai, R., Rodŕíguez-Valera, F., McMahon, K.D., Toyama, D., Rinke, R., de Oliveira, T.C.S., Garcia, J.W., de Miranda, F.P. and Henrique-Silva, F. 2011. Metagenomics of the water column in the pristine upper course of the Amazon river. PloS one 6(8), e23785.

Gião, M.S., Azevedo, N.F., Wilks, S.A., Vieira, M.J. and Keevil, C.W. 2011. Interaction of Legionella pneumophila and Helicobacter pylori with bacterial species isolated from drinking water biofilms. BMC microbiology 11(1), 57.

Gomez-Alvarez, V., Revetta, R.P. and Santo Domingo, J.W. 2012. Metagenomic analyses of drinking water receiving different disinfection treatments. Appl. Environ. Microbiol. 78(17), 6095-6102.

Grobe, S., Wingender, J. and Flemming, H.-C. 2001. Capability of mucoid Pseudomonas aeruginosa to survive in chlorinated water. International journal of hygiene and environmental health 204(2-3), 139-142.

Guerrieri, E., Bondi, M., Sabia, C., de Niederhäusern, S., Borella, P. and Messi, P. 2008. Effect of Bacterial Interference on Biofilm Development by Legionella pneumophila. Current Microbiology 57(6), 532-536.

Haupt, T.E., Heffernan, R.T., Kazmierczak, J.J., Nehls-Lowe, H., Rheineck, B., Powell, C., Leonhardt, K.K., Chitnis, A.S. and Davis, J.P. 2012. An outbreak of Legionnaires disease associated with a decorative water wall fountain in a hospital. Infection Control & Hospital Epidemiology 33(2), 185-191.

Ho, B.T., Basler, M. and Mekalanos, J.J. 2013. Type 6 secretion system–mediated immunity to Type 4 secretion system–mediated gene transfer. Science 342(6155), 250-253.

Holinger, E.P., Ross, K.A., Robertson, C.E., Stevens, M.J., Harris, J.K. and Pace, N.R. 2014. Molecular analysis of point-of-use municipal drinking water microbiology. Water research 49, 225-235.

Ivone, V.-M., Conceição, E., Olga C, N. and Célia M, M. 2013. Bacterial diversity from the source to the tap: a comparative study based on 16S rRNA gene-DGGE and culture-dependent methods. FEMS Microbiology Ecology 83(2), 361-374.

Jernigan, D.B., Hofmann, J., Cetron, M.S., Nuorti, J., Fields, B., Benson, R., Breiman, R., Lipman, H., Carter, R. and Genese, C. 1996. Outbreak of Legionnaires' disease among cruise ship passengers exposed to a contaminated whirlpool spa. The Lancet 347(9000), 494-499.

Ji, P., Parks, J., Edwards, M.A. and Pruden, A. 2015. Impact of water chemistry, pipe material and stagnation on the building plumbing microbiome. PLoS One 10(10), e0141087.

Koehorst, J.J., Van Dam, J.C., Van Heck, R.G., Saccenti, E., Dos Santos, V.A.M., Suarez-Diez, M. and Schaap, P.J. 2016. Comparison of 432 Pseudomonas strains through integration of genomic, functional, metabolic and expression data. Scientific reports 6(1), 1-13.

Koide, M., Higa, F., Tateyama, M., Cash, H.L., Hokama, A. and Fujita, J. 2014. Role of Brevundimonas vesicularis in supporting the growth of Legionella in nutrient-poor environments. The new microbiologica 37(1), 33-39.

Kozak-Muiznieks, N.A., Lucas, C.E., Brown, E., Pondo, T., Taylor, T.H., Frace, M., Miskowski, D. and Winchell, J.M. 2014. Prevalence of sequence types among clinical and environmental isolates of Legionella pneumophila serogroup 1 in the United States from 1982 to 2012. Journal of clinical microbiology 52(1), 201-211.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K. and Schloss, P.D. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl. Environ. Microbiol. 79(17), 5112-5120.

Kuchta, J.M., McNamara, A., Wadowsky, R. and Yee, R. 1983. Susceptibility of Legionella pneumophila to chlorine in tap water. Appl. Environ. Microbiol. 46(5), 1134-1139.

Lau, H. and Ashbolt, N. 2009. The role of biofilms and protozoa in Legionella pathogenesis: implications for drinking water. Journal of applied microbiology 107(2), 368-378.

Lesnik, R., Brettar, I. and Höfle, M.G. 2016. Legionella species diversity and dynamics from surface reservoir to tap water: from cold adaptation to thermophily. The ISME journal 10(5), 1064.

Li, D., Li, Z., Yu, J., Cao, N., Liu, R. and Yang, M. 2010. Characterization of bacterial community structure in a drinking water distribution system during an occurrence of red water. Appl. Environ. Microbiol. 76(21), 7171-7180.

Ling, F., Whitaker, R., LeChevallier, M.W. and Liu, W.-T. 2018. Drinking water microbiome assembly induced by water stagnation. The ISME journal 12(6), 1520.

Linz, A.M., Crary, B.C., Shade, A., Owens, S., Gilbert, J.A., Knight, R. and McMahon, K.D. 2017. Bacterial community composition and dynamics spanning five years in freshwater bog lakes. MSphere 2(3), e00169-00117.

Liu, R., Yu, Z., Guo, H., Liu, M., Zhang, H. and Yang, M. 2012. Pyrosequencing analysis of eukaryotic and bacterial communities in faucet biofilms. Science of the Total Environment 435, 124-131.

Llewellyn, A.C., Lucas, C.E., Roberts, S.E., Brown, E.W., Nayak, B.S., Raphael, B.H. and Winchell, J.M. 2017. Distribution of Legionella and bacterial community composition among regionally diverse US cooling towers. PloS one 12(12), e0189937.

Lu, J., Buse, H., Gomez-Alvarez, V., Struewing, I., Santo Domingo, J. and Ashbolt, N. 2014. Impact of drinking water conditions and copper materials on downstream biofilm microbial communities and L egionella pneumophila colonization. Journal of applied microbiology 117(3), 905-918.

Mahoney, F.J., Hoge, C.W., Farley, T.A., Barbaree, J.M., Breiman, R.F., Benson, R.F. and McFarland, L.M. 1992. Communitywide outbreak of Legionnaires' disease associated with a grocery store mist machine. Journal of Infectious Diseases 165(4), 736-739.

Matz, C., Moreno, A.M., Alhede, M., Manefield, M., Hauser, A.R., Givskov, M. and Kjelleberg, S. 2008. Pseudomonas aeruginosa uses type III secretion system to kill biofilm-associated amoebae. The ISME journal 2(8), 843.

Mazurier, S., Corberand, T., Lemanceau, P. and Raaijmakers, J.M. 2009. Phenazine antibiotics produced by fluorescent pseudomonads contribute to natural soil suppressiveness to Fusarium wilt. The ISME journal 3(8), 977-991.

McClung, R.P., Roth, D.M., Vigar, M., Roberts, V.A., Kahler, A.M., Cooley, L.A., Hilborn, E.D., Wade, T.J., Fullerton, K.E. and Yoder, J.S. 2017. Waterborne disease outbreaks associated with environmental and undetermined exposures to water—United States, 2013–2014. MMWR. Morbidity and mortality weekly report 66(44), 1222.

McKinney, R.M., Porschen, R.K., Edelstein, P.H., Bissett, M.L., Harris, P.P., Bondell, S.P., Steigerwalt, A.G., Weaver, R.E., Ein, M.E. and Lindquist, D.S. 1981. Legionella longbeachae species nova, another etiologic agent of human pneumonia. Annals of Internal Medicine 94(6), 739-743.

Mendis, N., McBride, P. and Faucher, S.P. 2015. Short-term and long-term survival and virulence of Legionella pneumophila in the defined freshwater medium Fraquil. PloS one 10(9), e0139277.

Milosavljevic, N. and Heikkilä, P. 2001. A comprehensive approach to cooling tower design. Applied thermal engineering 21(9), 899-915.

Mouchtouri, V.A., Goutziana, G., Kremastinou, J. and Hadjichristodoulou, C. 2010. Legionella species colonization in cooling towers: risk factors and assessment of control measures. American journal of infection control 38(1), 50-55.

Murga, R., Forster, T.S., Brown, E., Pruckler, J.M., Fields, B.S. and Donlan, R.M. 2001. Role of biofilms in the survival of Legionella pneumophila in a model potable-water system. Microbiology 147(11), 3121-3126.

Newton, R.J., Jones, S.E., Eiler, A., McMahon, K.D. and Bertilsson, S. 2011. A guide to the natural history of freshwater lake bacteria. Microbiol. Mol. Biol. Rev. 75(1), 14-49.

Nicomrat, D., Dick, W.A., Dopson, M. and Tuovinen, O.H. 2008. Bacterial phylogenetic diversity in a constructed wetland system treating acid coal mine drainage. Soil Biology and Biochemistry 40(2), 312-321.

Oren, A. (2014) The Prokaryotes Alphaproteobacteria and Betaproteobacteria. Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E. and Thompson, F. (eds), pp. 709-726, Springer, Berlin.

Palmore, T.N., Stock, F., White, M., Bordner, M., Michelin, A., Bennett, J.E., Murray, P.R. and Henderson, D.K. 2009. A cluster of cases of nosocomial legionnaires disease linked to a contaminated hospital decorative water fountain. Infection Control & Hospital Epidemiology 30(8), 764-768.

Pavissich, J., Vargas, I., González, B., Pastén, P. and Pizarro, G. 2010. Culture dependent and independent analyses of bacterial communities involved in copper plumbing corrosion. Journal of applied microbiology 109(3), 771-782.

Pereira, R.P.A., Peplies, J., Höfle, M.G. and Brettar, I. 2017. Bacterial community dynamics in a cooling tower with emphasis on pathogenic bacteria and Legionella species using universal and genus-specific deep sequencing. Water Research 122, 363-376.

Phin, N., Parry-Ford, F., Harrison, T., Stagg, H.R., Zhang, N., Kumar, K., Lortholary, O., Zumla, A. and Abubakar, I. 2014. Epidemiology and clinical management of Legionnaires' disease. The Lancet infectious diseases 14(10), 1011-1021.

Pinto, A.J., Schroeder, J., Lunn, M., Sloan, W. and Raskin, L. 2014. Spatial-temporal survey and occupancy-abundance modeling to predict bacterial community dynamics in the drinking water microbiome. MBio 5(3), e01135-01114.

Poitelon, J.-B., Joyeux, M., Welté, B., Duguet, J.-P., Prestel, E., Lespinet, O. and DuBow, M.S. 2009. Assessment of phylogenetic diversity of bacterial microflora in drinking water using serial analysis of ribosomal sequence tags. Water research 43(17), 4197-4206.

Pollack, M. 1983. The role of exotoxin A in Pseudomonas disease and immunity. Reviews of infectious diseases 5(Supplement_5), S979-S984.

Québec, G.d. 2014. Décret 454-2014 Loi sur le Bâtiment. Gazette Officielle du Québec 146(22), 1923-1927.

Richardson, I. 1990. The incidence of Bdellovibrio spp. in man-made water systems: coexistence with legionellas. Journal of Applied Bacteriology 69(1), 134-140.

Rogers, J., Dowsett, A., Dennis, P., Lee, J. and Keevil, C. 1994. Influence of temperature and plumbing material selection on biofilm formation and growth of Legionella pneumophila in a model potable water system containing complex microbial flora. Appl. Environ. Microbiol. 60(5), 1585-1592.

Rowbotham, T.J. 1980. Preliminary report on the pathogenicity of Legionella pneumophila for freshwater and soil amoebae. Journal of clinical pathology 33(12), 1179-1183.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S. and Huttenhower, C. 2011. Metagenomic biomarker discovery and explanation. Genome biology 12(6), R60.

Servais, P., Billen, G. and Hascoët, M.-C. 1987. Determination of the biodegradable fraction of dissolved organic matter in waters. Water research 21(4), 445-450.

Seyfried, P.L. and Fraser, D.J. 1980. Persistence of Pseudomonas aeruginosa in chlorinated swimming pools. Canadian journal of microbiology 26(3), 350-355.

Shrivastava, R., Upreti, R., Jain, S., Prasad, K., Seth, P. and Chaturvedi, U. 2004. Suboptimal chlorine treatment of drinking water leads to selection of multidrug-resistant Pseudomonas aeruginosa. Ecotoxicology and environmental safety 58(2), 277-283.

Steinert, M., Birkness, K., White, E., Fields, B. and Quinn, F. 1998. Mycobacterium avium bacilli grow saprozoically in coculture with Acanthamoeba polyphaga and survive within cyst walls. Appl. Environ. Microbiol. 64(6), 2256-2261.

Stewart, C.R., Muthye, V. and Cianciotto, N.P. 2012. Legionella pneumophila persists within biofilms formed by Klebsiella pneumoniae, Flavobacterium sp., and Pseudomonas fluorescens under dynamic flow conditions. PloS one 7(11), e50560.

Surman, S., Morton, G., Keevil, B. and Fitzgeorge, R. (2002) Legionella, pp. 86-89, American Society of Microbiology.

Team, R.C. 2019. A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2012. URL https://www. R-project.org.

Temmerman, R., Vervaeren, H., Noseda, B., Boon, N. and Verstraete, W. 2006. Necrotrophic growth of Legionella pneumophila. Appl. Environ. Microbiol. 72(6), 4323-4328.

Thomas, V., Bouchez, T., Nicolas, V., Robert, S., Loret, J. and Levi, Y. 2004. Amoebae in domestic water systems: resistance to disinfection treatments and implication in Legionella persistence. Journal of applied microbiology 97(5), 950-963.

Tison, D., Pope, D., Cherry, W. and Fliermans, C. 1980. Growth of Legionella pneumophila in association with blue-green algae (cyanobacteria). Appl. Environ. Microbiol. 39(2), 456-459.

Tomov, A., Kassovsky, V., Chorbadjiiska, L., Tsvetkova, E., Tsanev, N. and Vencheva, Z. 1982. Lytic activity of Bdellovibrio bacteriovorus against bacteria of the family Legionellaceae. Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene. 1. Abt. Originale. A, Medizinische Mikrobiologie, Infektionskrankheiten und Parasitologie 252(1), 96-100.

Türetgen, I. and Cotuk, A. 2007. Monitoring of biofilm-associated Legionella pneumophila on different substrata in model cooling tower system. Environmental monitoring and assessment 125(1-3), 271-279.

Van der Kooij, D., Veenendaal, H.R. and Scheffer, W.J. 2005. Biofilm formation and multiplication of Legionella in a model warm water system with pipes of copper, stainless steel and cross-linked polyethylene. Water research 39(13), 2789-2798.

Van der Wielen, P.W., Voost, S. and van der Kooij, D. 2009. Ammonia-oxidizing bacteria and archaea in groundwater treatment and drinking water distribution systems. Appl. Environ. Microbiol. 75(14), 4687-4695.

van Heijnsbergen, E., Schalk, J.A., Euser, S.M., Brandsema, P.S., den Boer, J.W. and de Roda Husman, A.M. 2015. Confirmed and potential sources of Legionella reviewed. Environmental science & technology 49(8), 4797-4815.

Wadowsky, R.M. and Yee, R.B. 1983. Satellite growth of Legionella pneumophila with an environmental isolate of Flavobacterium breve. Appl. Environ. Microbiol. 46(6), 1447-1449.

Wang, H., Cai, L., Li, Y., Xu, X. and Zhou, G. 2018. Biofilm formation by meat-borne Pseudomonas fluorescens on stainless steel and its resistance to disinfectants. Food Control 91, 397-403.

Wang, H., Masters, S., Edwards, M.A., Falkinham III, J.O. and Pruden, A. 2014. Effect of disinfectant, water age, and pipe materials on bacterial and eukaryotic community structure in drinking water biofilm. Environmental science & technology 48(3), 1426-1435.

Wang, Y., Hammes, F., Düggelin, M. and Egli, T. 2008. Influence of size, shape, and flexibility on bacterial passage through micropore membrane filters. Environmental science & technology 42(17), 6749-6754.

Wéry, N., Bru-Adan, V., Minervini, C., Delgénes, J.-P., Garrelly, L. and Godon, J.-J. 2008. Dynamics of Legionella spp. and bacterial populations during the proliferation of L. pneumophila in a cooling tower facility. Appl. Environ. Microbiol. 74(10), 3030-3037.

Wickham, H. and Wickham, M.H. 2017. Package tidyverse. Easily Install and Load the 'Tidyverse.

Yabuchi, E., Wang, L., Yamayoshi, T., Arakawa, M. and Yano, I. 1995. Bactericidal effect of chlorine on strains of Legionella species. Kansenshogaku zasshi. The Journal of the Japanese Association for Infectious Diseases 69(2), 151-157.

Yamamoto, H., Sugiura, M., Kusunoki, S., Ezaki, T., Ikedo, M. and Yabuuchi, E. 1992. Factors stimulating propagation of legionellae in cooling tower water. Appl. Environ. Microbiol. 58(4), 1394-1397.

Yoon, J.-H., Kang, S.-J., Schumann, P. and Oh, T.-K. 2006. Yonghaparkia alkaliphila gen. nov., sp. nov., a novel member of the family Microbacteriaceae isolated from an alkaline soil. International journal of systematic and evolutionary microbiology 56(10), 2415-2420.

CONNECTING TEXT

The next chapter is the manuscript for my second article entitled: "Unravelling the Importance of the Eukaryotic and Bacterial Communities and their Relationship with *Legionella* spp. Ecology in Cooling Towers: A Complex Network". This manuscript was submitted to the journal *Microbiome* and is still in review as of the 10th of January 2020. The "materials and methods" section was placed at the end of the text due to the format of the journal. This article characterised the eukaryotic community of the cooling towers sampled in Chapter 3. Very few articles have so far characterized this community, especially in cooling towers. Since, protozoan host are an important factor in *L. pneumophila* ecology and exert a pressure of selection on the bacterial community through grazing, our rationale was to characterize the eukaryotic community in relationship to the bacterial community and *Legionella* community. A microbial network based on cooccurrence was constructed between the bacterial and eukaryotic taxa of the cooling towers. This network revealed potential ecological interactions between the different organisms that could theoretically affect *L. pneumophila* ecology. More specifically, the bacterial genus *Brevundimonas* and the ciliates of the *Oligohymenophorea* class could positively affect survival and growth of *L. pneumophila* through direct and indirect mechanisms. Moreover, the network revealed a number of potentially unknown interaction between several bacterial organisms and *Legionella*. Finally, the eukaryotic community was shaped by the concentration of dissolved organic carbon in the cooling tower environment. A significant amount of carbon was believed to have been brought by contaminating organisms from the ambient air. This dissolved organic carbon could be an important factor in *L. pneumophila* ecology by affecting the protozoan host community through the microbial loop.

Contribution of authors: Emilie Bédard, Michèle Prévost, Sébastien P. Faucher, and I contributed to the design of the study. I performed the *18S rRNA* gene amplicon sequencing, whole genome sequencing and the ciliate co-culture experiments. I also processed and analysed the sequencing data. MengQi Hue isolated the *Brevundimonas* isolate. Deeksha Shetty and Fiona Chan Pak Choon performed the *Brevundimonas* stimulation assay. I analysed the data and wrote the first draft of the manuscript. Sébastien P. Faucher, Émilie Bédard, Michèle Prévost, and I edited the manuscript.

CHAPTER 4. UNRAVELLING THE IMPORTANCE OF THE EUKARYOTIC AND BACTERIAL COMMUNITIES AND THEIR RELATIONSHIP WITH *LEGIONELLA SPP.* ECOLOGY IN COOLING TOWERS: A COMPLEX NETWORK

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Running title: Eukaryotic community of cooling towers.

Key words: *18S rRNA* gene amplicon sequencing, eukaryotic community, *Legionella pneumophila*, *Brevundimonas sp.*, dissolved organic carbon, network analysis, Whole Genome Sequencing

ABSTRACT

Background: Cooling towers are a major source of large community-associated outbreaks of Legionnaires' Disease, a severe pneumonia. This disease is due to the inhalation of several bacterial species from the genus *Legionella*. As *Legionella* species are intracellular parasites of protozoa, it is assumed that protozoan community in cooling towers play an important role in *Legionella* ecology and outbreaks. However, the exact mechanisms of how the eukaryotic community contributes to *Legionella* ecology are still not well understood. Therefore, an *18S rRNA* gene amplicon sequencing approach was used to characterize the eukaryotic communities of 18 different cooling towers. The data from the eukaryotic community was then analysed with the bacterial community of the same towers in order to understand how each community could affect *Legionella* spp. ecology in cooling towers.

Results: We identified several microbial groups in the cooling tower ecosystem that suggest the presence of a microbial loop in these systems. Dissolved organic carbon was shown to be a major factor for shaping the eukaryotic community and may be an important factor for *Legionella* ecology. Network analysis, based on co-occurrence, revealed that *Legionella* was correlated to a number of different organisms. Out of these, the bacterial genus *Brevundimonas* and the ciliate class *Oligohymenophorea* were demonstrated, through *in vitro* experiments, to stimulate the growth of *L. pneumophila* through direct and indirect mechanisms.

Conclusion: Our results suggest that *Legionella* ecology is not only dependent on the host community, but that several groups of organisms may contribute to its promotion in the cooling tower ecosystem. These findings further support the idea that *Legionella* survival and growth is dependent on the microbiomes of the environment it is in.

I- BACKGROUND

Legionnaires disease is a severe pneumonia caused by several bacterial species of the genus *Legionella,* with the specie *Legionella pneumophila* being the most prevalent. The disease is mainly contracted through the inhalation of contaminated aerosols with the bacteria (Cunha et al., 2016). Once inhaled, the bacteria find themselves in the lungs where they will infect alveolar macrophages (Isberg et al., 2009). The infection will activate an immune response, which may then lead to pneumonia.

Since the first documented outbreak of Legionnaires disease, in 1976, cases have been on the rise, and the increase has been more pronounced in the last two decades. Indeed, the Centre for Disease Control in the United States and the European Centre for Disease Prevention and Control have both reported increasing trends of the disease in North America and Europe (Control and Prevention, 2011; Dooling et al., 2015; ECDC, 2019; National Academies of Sciences and Medicine, 2020). For instance, the incidence rate in the USA increased from around 0.4 cases per 100,000 in 2000 to around 2.3 cases per 100,000 in 2017, that is to say around a 5 fold increase (National Academies of Sciences and Medicine, 2020; Shah et al., 2018). In the European Union, the incidence of reported cases increased by 50 % from 2013 to 2017, with a reported incidence of 1.2 per 100,000 in 2013 to an incidence of 1.8 per 100,000 in 2017 (ECDC, 2019). Increases in surveillance, urbanisation, ageing population, and climatic changes may all be factors that will aggravate the situation in the future (ECDC, 2019). Consequently, factors contributing to outbreaks of Legionnaires' disease are important issue that requires further investigation.

Engineered water systems, such water distribution systems, fountains, misters, premise plumbing systems, hot tubs, and water treatment plants have all been identified as sources of

Legionnaires' disease, due to the aquatic nature of *Legionella* species (van Heijnsbergen et al., 2015). However, cooling towers are by far one of the most important sources of large outbreaks, with several notable ones caused by these infrastructures, such as the 1976 Philadelphia outbreak, the 1985 Stafford outbreak, 2001 Murcia outbreak, the 2012 Quebec City outbreak, and the 2015 New York city outbreak (Fitzhenry et al., 2017; García-Fulgueiras et al., 2003; Lévesque et al., 2014; O'mahony et al., 1990; Paschke et al., 2019; van Heijnsbergen et al., 2015; Weiss et al., 2017; Winn, 1988). These outbreaks infected hundreds of people (almost 500 in the case of Murcian outbreak) and killed in the dozens. Additionally, cooling towers may also be an important source of sporadic cases of Legionnaires' Disease (Bhopal et al., 1991; Orkis et al., 2018). A study from 1978 to 1986, in the city of Glasgow, Scotland, revealed that around 28% of sporadic Legionnaires' disease cases were associated with cooling towers (Bhopal et al., 1991). The reasons for cooling towers being an important source of the disease is due to their function and design. These towers are water systems, usually found on roof tops, used for cooling water used for air-conditioning, refrigeration, and other industrial systems (Paschke et al., 2019). As a result, high levels of aerosols are produced from the cooling process and are evacuated into the surrounding environment. People in the vicinity of the tower may inhale the aerosols and become sick if contaminated with the bacteria. Contaminated aerosols have been observed to spread as far as 12 km in certain cases (Nhu Nguyen et al., 2006). Thus, cooling towers are important source of Legionnaires' disease, however, the exact mechanism that allow *Legionella* species to grow within these ecosystems is still not well understood.

The species *Legionella pneumophila* is the most significant cause of the disease, with around 90 % of cases due to it (Kozak-Muiznieks et al., 2014). In order for an outbreak to occur, this species must colonize, survive, and proliferate in the cooling tower ecosystem. In this last step,

the *L. pneumophila* population must increase to sufficient levels so that the bacteria can readily be disseminated in the aerosols and the surrounding environment. Certain models have estimated that, in certain cases, as low as 35 colony forming units may pose a severe clinical risk (Armstrong and Haas, 2008; Buse et al., 2012). Though these levels are low, the cooling tower ecosystem is still a hostile environment and fraught with difficulties. Low levels of nutrients, harsh chemicals, such as chlorine disinfectants, and negatively interacting organisms are all hurdles *L. pneumophila* must overcome in order to survive and proliferate in these environments (Iervolino et al., 2017; Mouchtouri et al., 2010; Richardson, 1990; Türetgen, 2004). One way the bacterium avoids these difficulties as by being an intracellular parasite of various protozoan host species.

It is generally believed that the growth of *L. pneumophila* and other *Legionella* species can only happen in nature, and therefore in cooling towers, if there is the presence of a host population. This assumption is based on the fact that several host species have been discovered, as well as, the fastidious growth nature of *L. pneumophila* on laboratory growth media. Indeed, the number of known host species is quite expansive, spanning several distant phylogenetic groups. For instance, *Acanthamoeba castellanii*, *Naegleria fowleri*, *Tetrahymena pyriformis*, and human macrophages are host species belonging to different eukaryotic groups (respectively *Amoebozoa*, *Percolozoa*, *Ciliophora*, *Chordata*) and are routinely used as host models for research (Boamah et al., 2017; Fields et al., 1984; Lau and Ashbolt, 2009; Murga et al., 2001; Rowbotham, 1980). On the other hand, Buffered Charcoal Yeast Extract medium supplemented with iron and L-cysteine and buffered to a pH of 6.90 is the only medium capable of growing *L. pneumophila* cultures in laboratory settings (Kilvington and Price, 1990). This would suggest highly specialized growth requirements that may not necessarily be found in cooling tower environment but may be present in host cells. Moreover, host species offer protection from harsh chemicals and other stresses by

forming cysts that can entrap *L. pneumophila* cells in addition to serving as growth medium for the bacteria's proliferation (Kilvington and Price, 1990). These cysts can also play the role of disseminating agents as they can passively move through various environments that would normally kill *L. pneumophila* cells. This characteristic has qualified host cells as "trojan horses" (Barker and Brown, 1994). Consequently, the host community is important factor for Legionnaires' disease outbreaks as it allows a way for *L. pneumophila* to survive and proliferate in the cooling tower environment.

For all the data that supports the requirement of a host community for outbreaks to occur, there is some evidence that seems to suggest otherwise. Indeed, certain water systems have been found to contain high levels of *L. pneumophila* without the detection of host species (Bédard et al., 2016; Stout et al., 1985). This would suggest that other methods are used by the bacterium to proliferate than the use of host cells. Furthermore, the bacterium has been demonstrated to grow without the presence of host cells in experimental settings. For instance, growth was observed when *L. pneumophila* cultures were incubated with heat inactivated biofilms and bacteria (Temmerman et al., 2006). Additionally, several other experiments have shown that certain bacterial species have mutualistic interactions that allow *L. pneumophila* to survive and proliferate in conditions that would otherwise not allow it (Stout et al., 1985; Tison et al., 1980; Wadowsky and Yee, 1983). These findings suggest that the host community is not the only factor that is responsible for *L. pneumophila* survival and proliferation and that other biotic components may be import for survival and growth of the bacterium.

In following with this idea, it is obvious to state that certain groups of organisms may have direct or indirect effect on the colonization, survival, and growth of *L. pneumophila*. For instance, *Legionella* host are usually protozoan species that feed on various microbes found in the

ecosystem, such as bacteria, fungi, algae and other protozoan species (Rodríguez-Zaragoza, 1994; Samba-Louaka et al., 2019). This grazing behaviour is usually a selective process that depends on several factors, such as prey species, prey morphology, prey size, and physiological state of the prey or the predator (Alsam et al., 2006; Hahn and Höfle, 2001; Shaheen et al., 2019). Consequently, the presence of the correct prey community is an important factor for the establishment and growth of the *Legionella* host community and would therefore have an indirect effect on *L. pneumophila* ecology. So far very little research has gone into studying the prey community and its effect on the *L. pneumophila* population, in water systems. This is especially true in the case of cooling towers. Another example of a community that can affect *L. pneumophila* ecology is the presence of negatively interacting organisms (Corre et al., 2019; Guerrieri et al., 2008). Over the past few decades, it has been shown that a number species can negatively interact through direct mechanisms, such as predation or competition, or through indirect mechanisms by affecting the positively interacting species with *L. pneumophila*. For instance, bacterial species of *Bdellovibrio* genus and certain eukaryotic organisms, such *S. palustris*, have been shown to prey on *L. pneumophila* (Amaro et al., 2015; Richardson, 1990). On the other hand, certain strains of *Pseudomonas aeruginosa* are known to kill host cells, such as *A. castellanii*, using a type III secretion system and several toxins (Abd et al., 2008). This would have the indirect effect of lowering the pool of hosts available for *L. pneumophila,* reducing its chances of proliferating. As a result, these other communities reinforce the idea that host cells are not the only element that would lead to proliferation of *L. pneumophila*. More likely, it would seem that certain microbiomes, that is to say the set of different organisms in an ecosystem, may be more permissive to *L. pneumophila* than other.

Building on the concept that specific microbiomes are necessary for the colonization, survival, and growth of *L. pneumophila* population, it would seem plausible that certain groups of microorganisms are crucial for outbreaks to occur. Consequently, we hypothesize that the presence of certain microbial groups would increase permissiveness of the microbiome to *L. pneumophila* colonization, survival, and proliferation. These microbial groups would consist of species that positively interact with *L. pneumophila*, such as host species and the aforementioned mutualistic species. Conversely, microbiomes that would be less permissive would contain very few host cells and species that would negatively interact with *L. pneumophila*. So far, research on identifying and characterizing groups of microbes that could promote or inhibit *L. pneumophila* in cooling towers is lacking. To date only one study has looked into this in cooling towers. Tsao et *al.* evaluated the relationship of the *Legionella* host community and the bacterial community, using co-occurrence networks, of three cooling towers over a year (Tsao et al., 2019). This study found several interesting correlations between various groups of organisms in the cooling towers, as well as, factor affecting the microbiome of cooling towers. However, in terms of the eukaryotic community analysis, the study focused primarily on the protist host community, leaving out other potential important eukaryotic groups. Furthermore, the analysis of how these various groups may affect *Legionella* ecology was limited.

In light of our hypothesis, we have conducted an exploratory investigation of the eukaryotic community of 18 different cooling towers using *18S rRNA* targeted amplicon sequencing. The bacterial communities of these same towers were previously characterized using *16S rRNA* targeted amplicon sequencing (see (Paranjape et al., 2020)). The analysis of both these communities using various techniques, such as co-occurrence networks, enabled us to investigate the relationship between the eukaryotic and bacterial community and how certain groups within

these two communities may affect *L. pneumophila* ecology. Abiotic factors were also taken into consideration in this analysis. The results enabled us to identify novel groups of organisms that may be important for proliferation of *L. pneumophila* in cooling towers and therefore important for outbreaks of Legionnaires' disease.

II- RESULTS

1. SEQUENCING RESULTS

A total of 4 280 578 paired reads were generated from the MiSeq run. The Mothur MiSeq SOP was followed for processing the sequencing data (Kozich et al., 2013). Quality filtering, denoising, and chimera removal of the sequences removed a total of 350 988 low quality sequences, keeping 3 939 401 sequences (Table 1 and supplemental table S1, Appendix 1 part II). The resulting sequences were then classified using the Bayesian classifier implemented in Mothur and the Silva ribosomal RNA reference database (Glöckner et al., 2017; Kozich et al., 2013; Quast et al., 2012; Yilmaz et al., 2013). From the classification, 1 891 109 sequences were identified as bacterial sequences, 1 029 sequences as Archean, and 260 506 sequences as "unknown". Most bacterial sequences were identified as *Proteobacteria*, mainly *gamma-* and *alpha-proteobacteria.* The first three most abundant bacterial sequences were identified as an unclassified *Gammaproteobacteria* sequence, *Porphyrobacter*, and an unclassified *Beijerinckiaceae* sequence. These three most abundant sequences constituted around 60 % of the total bacterial sequences. The bacterial, archaeal, and unknown sequences were removed from the data, leaving 1 786 757 eukaryotic sequences.

Table 1: Sequence count following the Mothur MiSeq SOP

Number of raw sequences	**4 280 578**
Number of sequences after quality filtering, denoising, chimera removal	3 939 401
Number of Sequences after classification and removal of non-eukaryotic sequences	1 786 757
Number OTU read counts after clustering sequences	1 752 739

The eukaryotic sequences were then clustered into a total 44 183 different OTUs. The counts for each processing step and for each replicate of every sample can be viewed in the supplementary table S1 (Appendix 1 part II). Since we sequenced each cooling tower sample three times, the OTU count of a cooling tower samples was created by averaging the OTU read count of the three replicates of each sample. As a result, the averaged OTU counts ranged from 4 088 counts to 106 167 counts for the different cooling tower sample (Table S2, Appendix 1 part II). Good's coverage estimator was used to evaluate if sequencing depth was adequate enough for diversity analysis. The estimator averaged 98.01 %, ranging from a minimum of 93.84 % to a maximum of 99.82 % depending on the sample. Following this, various methods were used to filter, rarefy, and normalize the data to create an OTU table for analysis (see materials and methods). The OTU table had 3440 read counts per sample. The Good's coverage for this rarefied dataset was around also 97.86 % but ranged from 96.54 % to 99.59 % depending on the sample (Table S2, Appendix 1 part II).

Furthermore, a blank sample was sequenced separately in order to determine the presence of any contaminating sequences coming from any of the sequencing library preparation kits used. This blank consisted in running a sterile filter, from the same lot used for the cooling tower samples, through the same DNA extraction protocol and *18S rRNA* DNA library protocol as mentioned in the materials and methods section. After sequencing and processing the raw sequence data, the blank contained 45 sequences, which could be clustered into a total of 15 OTUs. These OTUs were classified as "Unclassified *Embryophyta*" (plants), when using the Silva ribosomal reference database.

2. EUKARYOTIC PROFILE OF COOLING TOWERS

The *18S rRNA* gene targeted amplicon sequencing revealed a diverse community of eukaryotes inhabiting the cooling tower environment (Fig. 1A). The characteristics of each tower can be seen in supplemental table S3 (Appendix 1 part II). Overall, the community could be divided into 20 different phyla and classes. Fungal groups were the most abundant and prevalent taxa in the cooling tower samples, with the *Basidiomycota* and *Ascomycota* classes being the most dominant (Fig. 1A). For instance, the *Basidiomycota* class dominated (more than 50% of the community) the eukaryotic community in eight out of the 18 towers (Fig. 1A). Several other fungal groups, such as *Zoopagomycota* (*Zygomycota*), *Chytridiomycota*, and *Mucoromycota* were detected but at abundances of less than 1% across all towers sampled. Several taxa comprising known photosynthetic organisms were also detected, such as the *Chlorophyta* (Microalgae), *Dinoflagellates*, and *Ochrophyta*. Towers MTL4, MTL5, and MTL6 contained notably high numbers of *Orchrophyta*. Additionally, micro-animals belonging to the *Nematoda* and *Rotifera* taxa were identified in many samples, such as in tower CN3 where nematodes constituted over

80% of the eukaryotic population. However, this high abundance may be due to their multicellular nature. Macro-eukaryotes were also identified with towers containing sequences related to insects (*Arthropoda*) and plants (*Phragmoplastophyta*). For instance, plant-related sequences reached around 10 % of the community in tower Mont1.

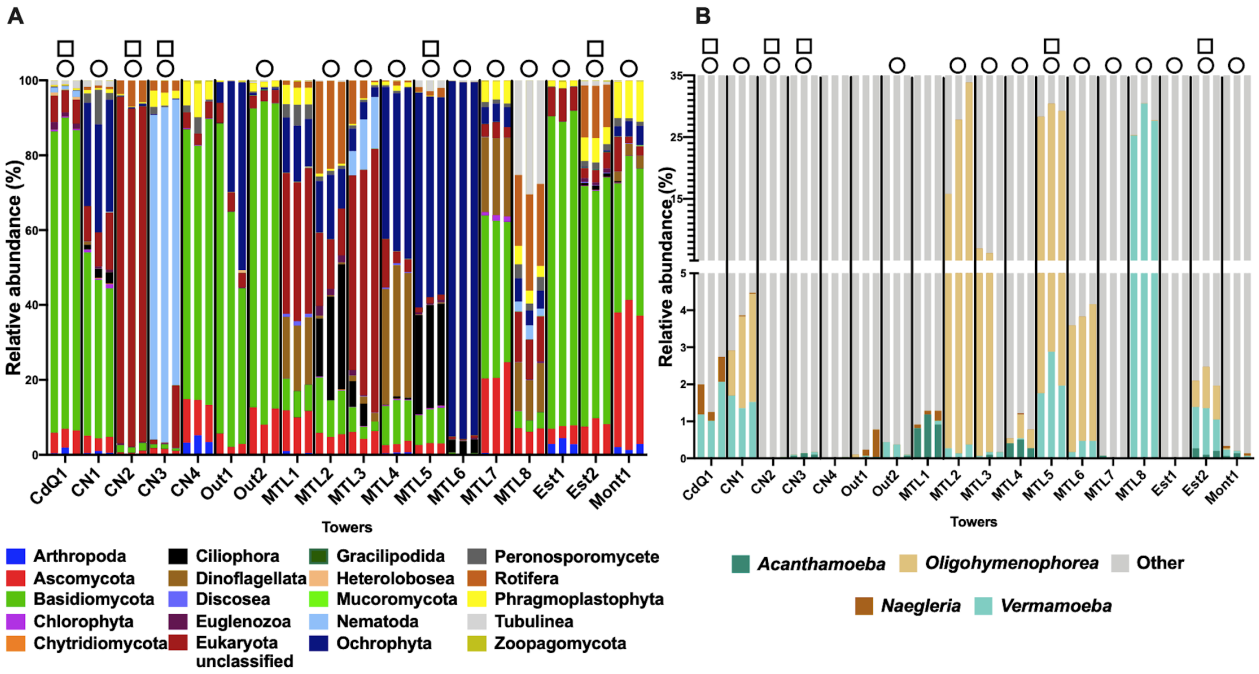

Figure 1: (A) Relative abundance of eukaryotic taxa present in cooling towers sampled in Quebec, Canada, classified at the class or phylum level. **(B)** Relative abundance of known host taxa of *L. pneumophila* and *Legionella* spp. in cooling tower samples. The group "others" represent the rest of the taxa present in the cooling towers. Circles represent towers in which *Legionella* spp. were detected (by *16S rRNA* gene amplicon sequencing), whereas squares represent towers contaminated with *L. pneumophila* (detected by qPCR).

Interestingly, several taxa harbouring known host species of *L. pneumophila* were also present in the towers, including *Ciliophora*, *Discosea*, *Heterolobosea*, *Nematoda*, and *Tubulinea* (Fig. 1A) (Boamah et al., 2017). Out of these taxa, we examined the distribution of four of the most important host taxa: the *Acanthamoeba* genus (*Discosea*), the *Vermamoeba* genus (*Tubulinea*), the *Naegleria* genus (*Heterolobosea*), and the *Oligohymenophorea* class (*Ciliophorea*) (Fig. 1B). These four taxa contain well-established host cell species, such as *Acanthamoeba castellanii*, *Vermamoeba vermiformis*, *Naegleria fowleri*, and *Tetrahymena pyriformis*, respectively (Boamah et al., 2017). The *Nematoda* class was not included as a potential host taxon, as it is still unclear whether nematodes actually promote growth or simply ensure survival of *L. pneumophila* (Brassinga et al., 2010; Rasch et al., 2016). Since we could, at most, only resolve the OTUs to the genus levels, these groups represent potential hosts of *Legionella* species, since not all species of these groups may be host cells. The relative abundance of host taxa was less than 5% in most towers (Fig. 1B). However, the host read counts reached a relative abundance of around 30% for three towers, MTL2, MTL5 and MTL8 (Fig. 1B).

3. ALPHA DIVERSITY AND BETA DIVERSITY ARE AFFECTED BY DISSOLVED ORGANIC CARBON

Alpha diversity of towers was analysed using the Shannon index and the effect of physicochemical parameters was investigated. Overall, dissolved organic carbon levels (DOC) were positively correlated with alpha diversity (Fig. 2A); however, the correlation between DOC and alpha diversity was modest (spearman's $r_s = 0.58$, $P = 0.0056$), with DOC following a non-linear regression model ($R^2=0.43$).

Beta diversity was calculated with the Bray-Curtis dissimilarity index and visualized using non-metric multidimensional scaling plot (NMDS). Analysis of similarity (ANOSIM) was used to determine statistical significance and dissimilarity between communities. The beta diversity analysis revealed that DOC levels could partially explain the clustering of the cooling tower communities when using NMDS (Fig 2B). Thus, communities that had high and low levels of DOC formed distinct clusters. Conversely, towers with medium levels of DOC shared similarity with the other two groups. When comparing just the high and low DOC towers, ANOSIM revealed high dissimilarity between these two groups, with an R-value of 0.75 (Supplemental figure S1, Appendix 1 part II). However, the p-value was around 0.0667 indicating that the two groups were not statistically different from one another.

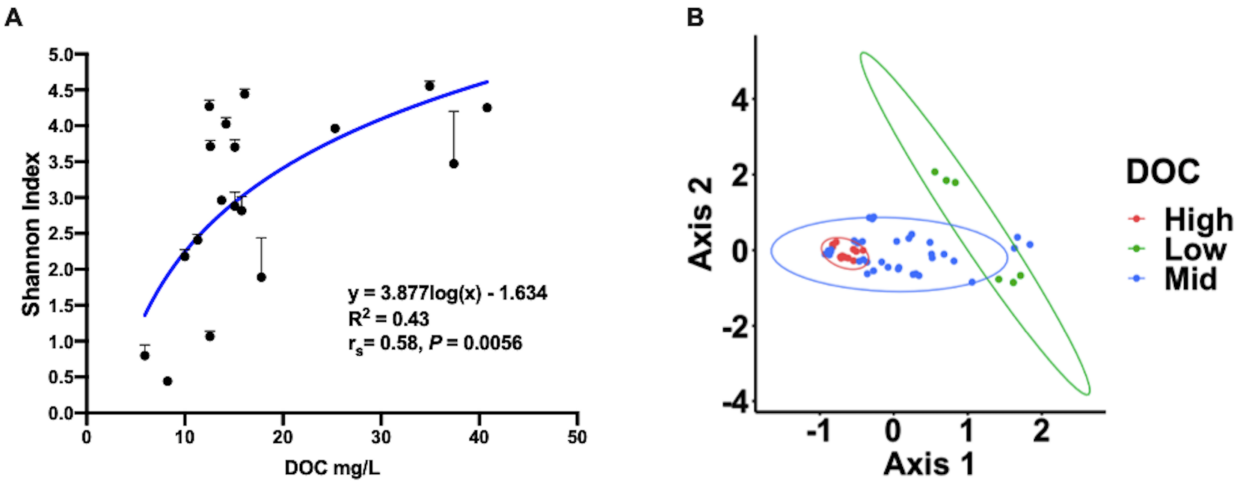

Figure 2: (A) Alpha diversity of cooling towers plotted against DOC levels of each tower. A semi-logarithmic curve fit the data best, using non-linear regression. **(B)** Non-metric multidimensional scaling plot of cooling towers eukaryotic communities categorized by DOC levels and using ANOSIM to evaluate statistical significance of dissimilarity between communities (R = 0.21, *P* = 0.118, Stress = 0.102). The categories are as followed: <10 mg/L of DOC were grouped as low;

10 to 20 mg/L were categorized as mid; 20 to 40 mg/L were categorized in the high group. The plot graphs the Bray-Curtis dissimilarity index of each replicate of each tower. Towers with high (red) and low levels (green) of DOC clustered separately (R= 0.75, P = 0.0667; see supplemental figure S1).

We hypothesized that a substantial amount of DOC in cooling towers comes from biological contaminants in the air, such as spores and pollen. These contaminants are likely captured by the water droplets and spread in the cooling tower environment. To investigate this, we grouped OTUs likely to produce spores, pollen, or seeds, as well as OTUs representing airborne insects, and plotted them as a function of DOC levels for each tower. This group was named "contaminating OTUs", as these OTUs are mostly comprised of organisms not found in water systems and likely originating from other types of ecosystems, such as green spaces. Consequently, "contaminating" OTUs were the sum of OTUs assigned to the taxa *Basidiomycota*, *Ascomycota*, *Arthropoda*, and *Phragmoplastophyta*, after rarefying the dataset. Interestingly, a modest positive correlation (Spearman r_s = 0.62, P = 0.003) was observed between DOC levels and relative abundance of contaminating OTUs (Fig. 3). Using non-linear regression, the data followed a logarithmic curve (R^2 = 0.38; Fig. 3). Thus, contaminating OTUs seems to contribute to DOC levels.

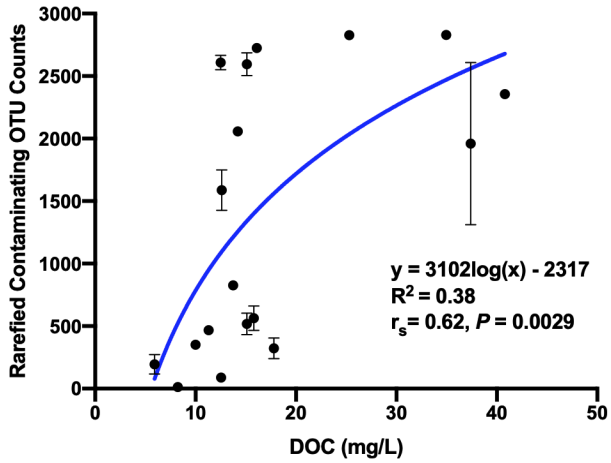

Figure 3: Rarefied contaminating OTU counts of tower as a function of the respective DOC levels for all sampled cooling towers. Non-linear regression was used to fit a semi-log curve to the data.

4. NETWORK ANALYSIS

Next, putative ecological relations between the different taxa of the microbial community of the cooling towers were identified by constructing a microbial ecological network based on co-occurrence and using Pearson's correlation. The network was constructed from our previously published bacterial profiling dataset (see (Paranjape et al., 2020) and the eukaryotic community profiles using the MENA pipeline and visualized using Cytoscape 3.7.1 (Deng et al., 2012; Shannon et al., 2003).

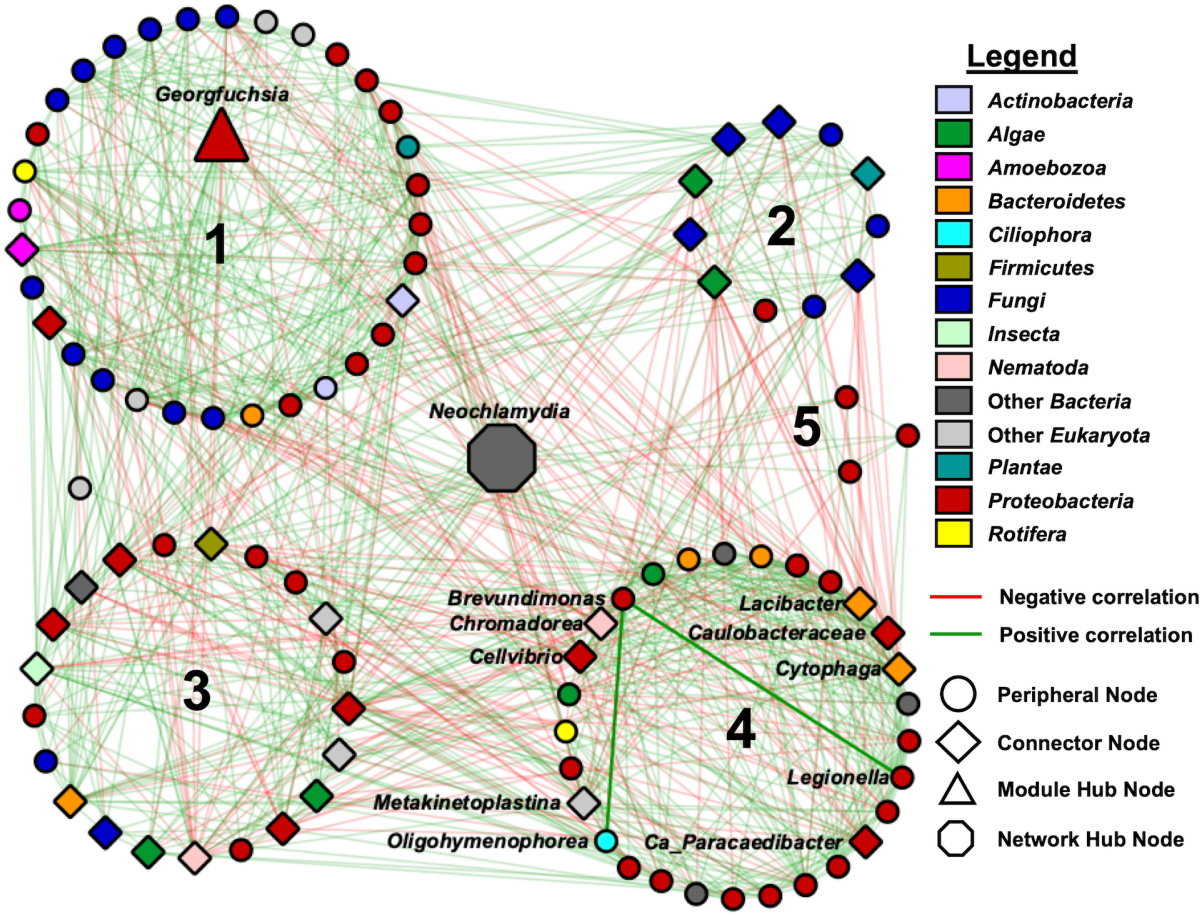

Figure 4: Microbial ecological network showing correlated taxa (Bacteria and Eukaryotes) in cooling tower samples organised into modules (1 to 5). Green edges represent positive correlations between taxa, and red edges represent negative correlations between taxa. The Octagon represents a network hub, whereas, the triangle and diamonds represent a module hub node and connector nodes, respectively. Peripheral nodes are represented by circles. A positive correlation can be observed between *Brevundimonas*, *Oligohymenophorea*, and *Legionella* in module 4

Overall, the network was constituted of 99 nodes and 847 edges (Fig. 4). The general properties of the network revealed that the network did not have scale free or small world

properties; however, it did show a low amount of modularity (M = 0.257) with the presence of 5 modules (Fig. 4) (Deng et al., 2012). The genus *Legionella* could be found in module 4 along with *Brevundimonas*, and *Oligohymenophorea*.

To understand the ecological roles of the taxa that constituted each module, the nodes were classified by their within-module (Z_i) and among-module (P_i) connectivity (Deng et al., 2012; Olesen et al., 2006). The data can be visualized in figure 4. The analysis revealed that most of the nodes were peripheral taxa, revealing a specialist ecological behaviour (Deng et al., 2012). Some of the nodes were classified as module hub and network hub nodes. These properties reveal a more generalist ecological behaviour (Deng et al., 2012). The network also contained several connector nodes with seven of them present in the same module as the one containing *Legionella* (module 4). These nodes include five bacteria (*Cellvibrio*, *Ca*. Paracaedibacter, *Cytophaga*, *Caulobacteraceae*, and *Lacibacter*), one nematode (*Chromadorea*), and one protist (*Metakinetoplastina*).

Legionella was classified as a peripheral node within module 4 of the network (Fig. 4). In order to better visualize *Legionella*'s position and role within the network, a sub-network was constructed using the first neighbour nodes of *Legionella* (Fig. 5). This sub-network revealed that *Legionella* was directly correlated with 17 different taxa. All of the *Legionella* neighbours came from the same module (module 4) and were diverse. Thus, algal groups, *Bacteroidetes*, fungal groups, *Protebacteria*, and *Gemmatimonadetes* were all identified as neighbours of *Legionella* in the network. Moreover, all of the neighbours were positively correlated with *Legionella* and with each other. We found that *Legionella* negatively correlated with other taxa, however, MENA did not identify them in the network analysis. Finally, several of the neighbour nodes are connectors,

such as *Lacibacter*, *Cytophaga*, *Caulobacteraceae*, and *Cellvibrio*. Interestingly, *Cellvibrio* is node with the most connection in the network with a total of 35 connections.

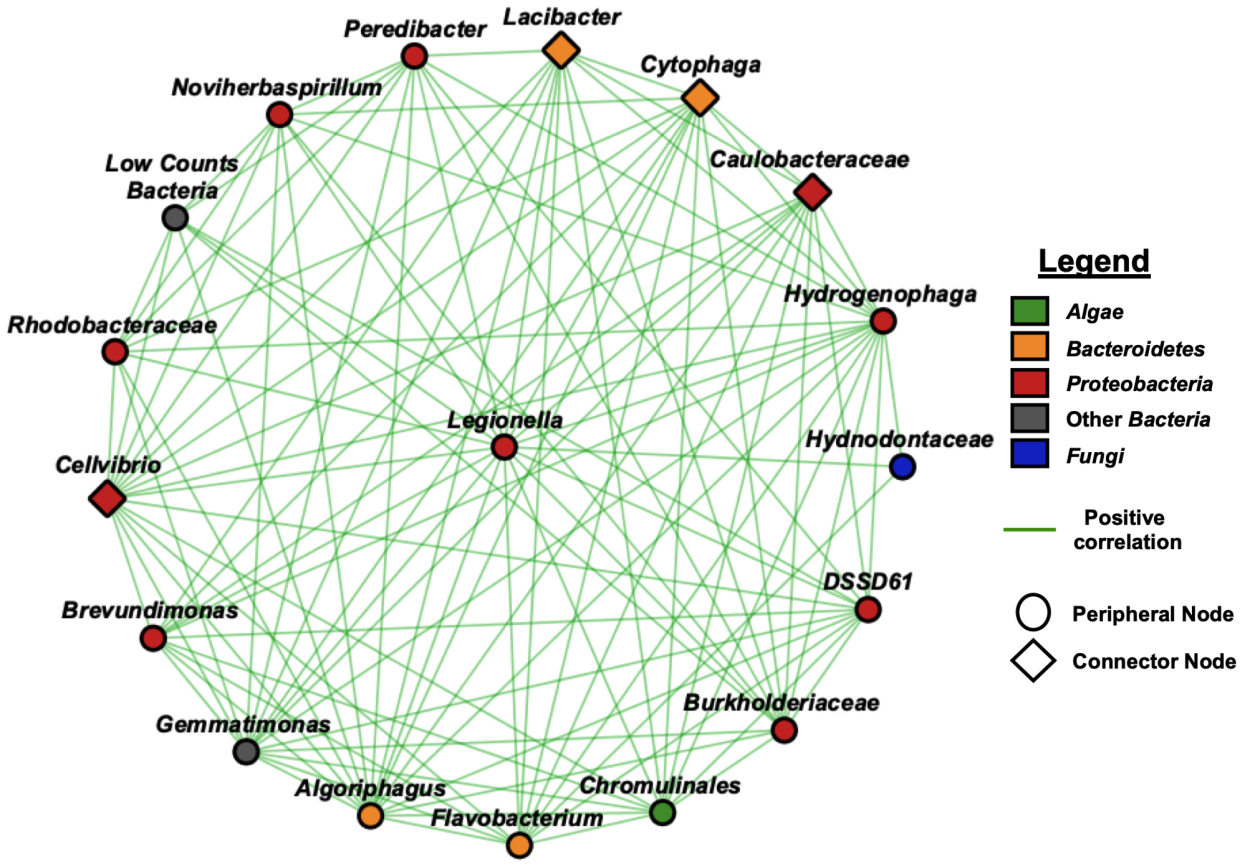

Figure 5: Sub-network showing first neighbour taxa of *Legionella*.

5. BACTERIAL PREDICTORS OF *OLIGOHYMENOPHOREA*

Eukaryotic profiling revealed that several *Legionella* host taxa were identified in the tower samples and that some of these taxa were positively correlated with *Legionella*. Indeed, *Oligohymenophorea* counts correlated positively with *Legionella* counts (Spearman's $r_s = 0.72$, $P = 0.0007$). This taxon is a class of ciliate contaning known host species of *L. pneumophila*, such

as *Tetrahymena pyriformis* (Fields et al., 1984). Since ciliates are microbial grazers and important in the ecology of *Legionella*, we performed a LEfSe analysis on our bacterial dataset to identify taxa that could predict the presence of *Oligohymenophorea* in the cooling towers (Segata et al., 2011). In order to do this, we categorized the towers based on the number of read counts of sequences classified as *Oligohymenophorea* from our eukaryotic dataset. Thus, three groups were created: high (>100 counts), low (1 to 100 counts), and absent (0 counts).

Bacterial predictors could be identified for all three *Oligohymenophorea* level categories (Fig. 6): 19 bacterial taxa were predictive of high levels, four taxa were predictive of low levels, and one taxon was predictive of an absence of *Oligohymenophorea* (Fig. 6). *Legionella* was the most predictive genus for a high level of the ciliates, whereas *Pseudomonas* was predictive of an absence of ciliates in the towers. *Brevundimonas* was also predictive of high levels. Moreover, some bacterial predictors of *Olygohymenophorea* were previously found to be predictors of the presence of *Legionella* spp. (see (Paranjape et al., 2020) or chapter 3). For instance, *Peredibacter*, *Yonghaparkia*, *Reyranella*, *Brevundimonas*, and *Sphingopyxis* were predictive of towers containing *Legionella* (Paranjape et al., 2020). Finally, several bacterial predictors were also identified as direct neighbours of *Legionella* or in the same module as *Legionella* in the network, such as *Peredibacter, Brevundimonas,* and *Ca.* Paracaedibacter (Fig. 4 and 5).

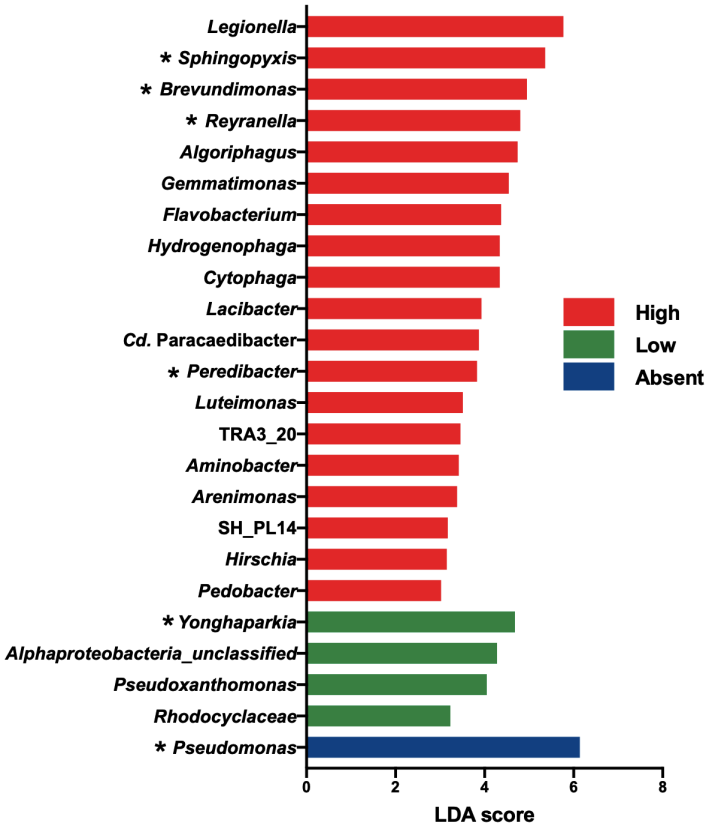

Figure 6: Bacterial taxa predicting towers containing varying levels of *Oligohymenophorea* using LEfSe. The towers were classified according to the number of sequences assigned to the *Oligohymenophorea* class: absent (0 count, blue), low (between 1 and 100, green), high (more than 100, red). Taxa previously identified as predictors of *Legionella* are indicated with "*" (Paranjape et al., 2020 or chapter 3).

6. THE BACTERIAL PREDICTOR *BREVUNDIMONAS* IS A PREY FOR *OLIGOHYMENOPHOREA* HOST CELLS

The LEfSe analysis revealed several bacterial predictors, such as the *Brevundimonas* genus, of towers with high levels of *Oligohymenophorea*. We hypothesized that the presence of these

bacterial predictors is probably due to a prey-predator relationship. *Brevundimonas* SPF441 was isolated from a cooling tower and subjected to whole genome sequencing. Analysis of the genome with MiGA revealed that our isolate is closely related to *Brevundimonas vesicularis* with a 95.49% average nucleotide identity (Rodriguez-R et al., 2018). The *16S* RDP classifier implemented by MiGA also showed that the isolate was classified within the *Brevundimonas* genus. These results indicate that the isolate is most likely a species within this genus, however identifying the species would require additional tests. Given that the network analysis revealed that the genus *Brevundimona*s was positively correlated with the genus *Legionella* and *Olygohymenophorea* class, co-culture experiments were undertaken between the isolated *Brevundimonas* SPF441 and the ciliates *T. pyriformis* or *T. thermophila*. The two species of *Tetrahymena* tested are known host species for *L. pneumophila* and belong to the *Oligohymenophoreae* class (Fields et al., 1984; Kikuhara et al., 1994).

The *Brevundimonas* SPF441 counts decreased by 5 logs after 12 h of co-culture with both *Tetrahymena* species. In contrast, no decrease in CFU numbers was seen when *Brevundimonas* SPF441 was incubated alone in the media (Fig. 7A). These drastic decreases suggest that *Brevundimonas* SPF441 is being consumed by *Tetrahymena*. However, certain *Tetrahymena* species are known to reject certain species of bacteria they consume by pelletizing them in packages and excreting them from their cells (Trigui et al., 2016). To test whether or not the *Brevundimonas* SPF441 cells were being consumed for nutrition, co-cultures where performed in Tris buffer, in which *Tetrahymena* is unable to grow. When fed with *Brevundimonas* SPF441, *T. thermophila* number increased by 9-fold and *T. pyriformis* number increased by 150-fold over four days (Fig. 7B and C). Minimal growth was observed for the ciliates in buffer alone. Our results

confirm that this bacterium is readily consumed by the ciliates and is sufficient for growth of the ciliate population.

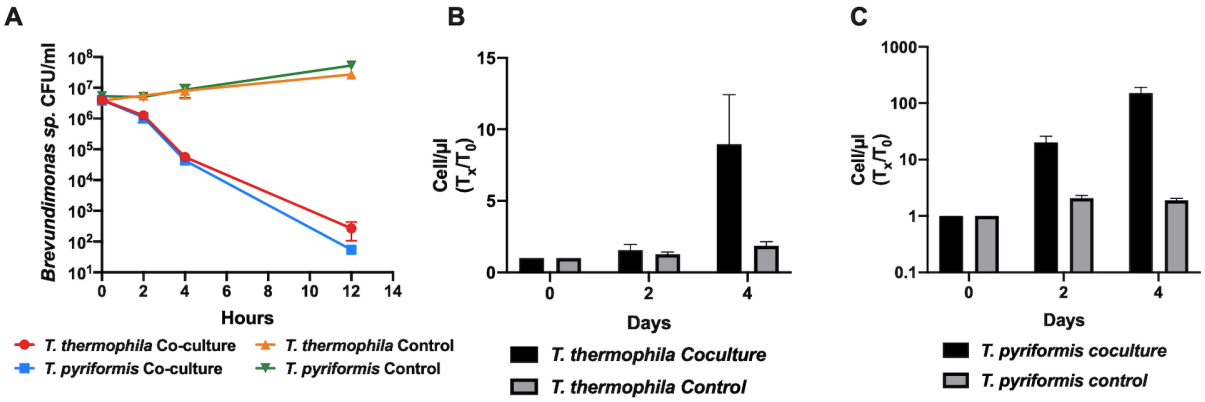

Figure 7: *Brevundimonas* SPF441 is a prey for *Tetrahymena.* Survival of *Brevundimonas* SPF441 when co-cultured with *T. thermophila* and *T. pyriformis*, in plate counting broth **(A)**. *Brevundimonas* SPF441 suspended alone in plate counting broth was used as the control. Growth rate (T_x/T_0) of *T. thermophila* **(B)** and *T. pyriformis* **(C)** fed with *Brevundimonas* SPF441, in 10 mM Tris (pH 7.5) and incubated at 30 °C and 25 °C, respectively. As a control, the two ciliates species were incubated in 10 mM Tris without feeding of *Brevundimonas* SPF441 at the same temperatures mentioned above.

7. BREVUNDIMONAS SPF441 PROMOTES GROWTH OF LEGIONELLA PNEUMOPHILA

In addition, *Brevundimonas* could also directly promote the growth of *Legionella* in water systems. To investigate this possibility, a stimulation assay was performed based on the fact that *L. pneumophila* requires supplementation of L-cysteine to grow on CYE plates. The assay showed

that *L. pneumophila* grew in a concentric circle around the colony of *Brevundimonas* SPF441 on plate lacking L-cysteine, which was visualized as a white halo (Fig. 8). This white halo around *Brevundimonas* SPF441 was not seen on plates not inoculated with *L. pneumophila* (Fig. 8B). Furthermore, the white halo was confirmed to be *L. pneumophila* by re-streaking on CYE with L-cysteine (growth) and without L-cysteine (no growth). These results indicate that *Brevundimonas sp*. isolate was able to stimulate the growth of *Lp* on CYE plates without L-cysteine.

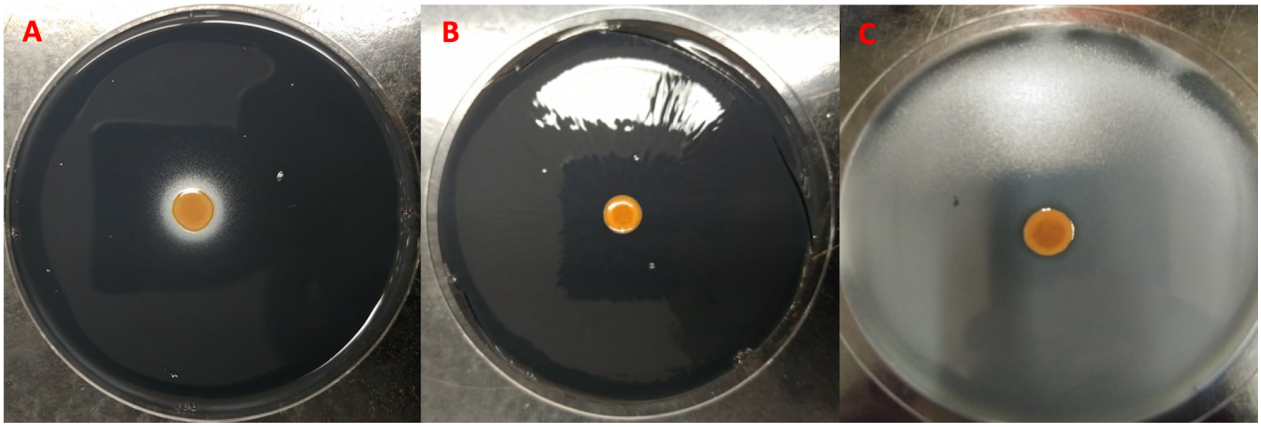

Figure 8: *Bevundimonas* SPF441 stimulates growth of *L. pneumophila*. Stimulation assay was carried on CYE agar without L-cysteine supplementation (A) or with L-cysteine supplementation (C). *L. pneumophila* was inoculated in soft agar which was poured on the surface of CYE plate (A and C). *Brevundimonas* SPF441 was spotted on each plate, and alone as control (B).

Whole genome sequencing of SPF441 was undertaken to determine genetic elements that could explain its hability to stimulate growth of *L. pneumophila*. The sequencing run generated a total of 500 485 paired reads between 35 to 301 nucleotides in length. After using Trimmomatic (see materials and methods), a total of 33 119 reads were removed, leaving 467 366 reads. Spades

assembled the reads into 66 contigs with a total sequence length of 3 201 388 bp. The N50 was 98 818 bp, with the shortest contig at 238 bp and the longest at 344 975 bp. The median depth was calculated at 7.55X. Prokka identified 3161 coding sequences (CDS), 3 rRNA elements, and 51 tRNA elements. A short description of the metabolic genes can be viewed in the supplementary document DS1 (Appendix 1 part II). Several genes related to cysteine metabolism were identified in the genome of SPF441, such as Cystathionine gamma-lyase and cysteine-S-conjugate beta-lyase.

III- DISCUSSION

Legionella outbreaks are complex phenomena that are not well understood. The presence of protozoan-host species is crucial for the bacterium's proliferation in the cooling tower environment (Loret and Greub, 2010; Murga et al., 2001; Tsao et al., 2019). Consequently, studying the ecology of the host community is key to providing insights into the mechanisms that may lead to high *Legionella* concentration in cooling towers. In the present work, we characterized the eukaryotic communities of 18 cooling towers, using an *18S rRNA* gene amplicon sequencing approach. The eukaryotic community was analyzed in relation to the bacterial and *Legionella* communities of these same towers, previously identified using a *16S rRNA* gene amplicon sequencing approach (Paranjape et al., 2020).

Though more than 4.2 million reads were sequenced from our library, around 60 % of the data was removed for analysis (Table 1). The initial denoising steps only removed 8 % of the sequences due to poor quality or presence of chimeras. Instead, it was the amplification and sequencing of bacterial reads that caused most of data loss. Indeed, around 44 % of the reads were

classified as bacterial sequences belonging to *Proteobacteria*, such as *Porphyrobacter* and unclassified *Beijerinckiaceae*. After removal of the non-eukaryotic sequences, around 1.7 million sequences remained for analysis, constituting 40 % of the data. The amplification of a high number of bacterial sequences suggests that the primers used are not specific for eukaryotic organisms. The Earth Microbiome Project recently warned that the EukBr reverse primer described in their *18S rRNA* protocol can indeed amplify bacterial sequences (Thompson et al., 2017). This notice was only known to us subsequently to the sequencing of the samples. Additionally, no bacterial sequences were amplified when we ran a blank through the same pipeline. This would suggest that the bacterial sequences were not amplified due to contamination. Certain bacteria, such as *Propionibacterium* and *Methylobacterium,* have been identified as contaminants in DNA extraction kits, making as much as 26% and 22% of the contaminating bacterial population in one study (Glassing et al., 2016). Our blank identified no bacterial sequences but did find 15 OTUs belonging to the *Embryophyta* phylum. This may be due to the use of cellulose ester filters when processing the samples for DNA extraction. As a result, the bacterial sequences identified were probably inhabitants of the cooling tower samples. Despite the data lost, we estimate that our sequencing depth was adequate to perform subsequent analysis based on Good's coverage and rarefaction curve (See table S2 in Appendix 1 part II) (Godoy-Vitorino et al., 2008; Tsao et al., 2019).

From the sequencing data, it is clear that cooling towers harbour a complex and diverse ecosystem of microorganisms. This was noted by the potential existence of a microbial loop in cooling towers. Indeed, the presence of primary producers (photoautotrophs, chemoautotrophs), microbial grazers (amoeba, ciliates, nematodes, and rotifers), and several different functional bacterial groups (heterotrophic decomposers, perchlorate reducers, nitrogen fixers,

chemolithotrophs) suggests the existence of a local microbial loop within these niches (Azam et al., 1983). In this scenario, primary producers, such as *Ochrophyta*, *Chlorophyta,* or bacterial chemoautotrophs, release dissolved organic carbon (DOC) through waste products and dead cells. Primary production through photosynthesis may be possible as cooling tower are not closed systems, and most have openings, such as air vents, that allow light to reach the basin. The DOC is then consumed by the heterotrophic bacterial and fungal populations. Subsequently, the carbon travels up the trophic levels through different groups of microbial grazers (unicellular, such as amoeba and ciliates, then multicellular, such as nematodes and rotifers). The microbial grazers re-introduce the carbon into the cycle in the form of dead cells and waste products. This dynamic indicates that cooling towers not only allow survival of microorganisms, but also, act as sustainable and active breeding grounds for microorganisms, despite the use of disinfection strategies.

This local microbial loop may impact the *Legionella* community. Previous research has shown that infected amoeba occurs more frequently in cooling towers than natural environments, and that a higher DOC level is a major predictor of infected amoeba by amoeba-associated-bacteria, such as *Legionella* species (Berk et al., 2006; Valster et al., 2009). Thus, factors controlling the release or uptake of DOC in the cooling tower environment may have important influences on the host cell population. In natural ecosystems, DOC uptake into the trophic chains is usually controlled by grazing activity, by the protozoan population, and viral lysis (Fenchel, 2008; Suttle, 1994). However, in the case of cooling towers, disinfection schedule may be more unique to these ecosystems. The use of chlorine and other biocides will cause a certain amount of cellular death, and thus, release DOC into the system (Helmi et al., 2018). Thus, different effects may be observed for continuous versus periodic applications of disinfectant. Presumably, periodic

application could release more DOC by generating peaks of cellular death, but this would require additional studies.

The importance of DOC was further demonstrated by its effect on alpha and beta diversity of the eukaryotic cooling tower communities. Alpha diversity was shown to increase with higher levels of DOC, whereas, beta diversity analysis indicated distinct clustering patterns for communities with high and low DOC levels. These findings would indicate that different populations of eukaryotes could establish themselves depending on the concentrations of DOC. However, a probable cause for effects of DOC on diversity may be due to the introduction of "contaminating" organisms. By this, we mean the presence of eukaryotic organisms not ordinarily growing in natural or engineered water systems. Cooling towers intake great volumes of air due to their function and design (Milosavljevic and Heikkilä, 2001). This can lead to the presence of airborne fungal spores, fungal tissues, insects, plant tissues and seeds in cooling tower water. Several of these organisms were detected in our dataset. Therefore, we defined contaminating OTUs as OTUs belonging to the fungal groups *Ascomycota* and *Basidiomycota*, as well as, insects (*Arthropoda*) and plants (*Phragmoplastophyta*). Although yeast and mould were identified, the majority of the fungal groups were associated with macroscopic fungal groups (mushrooms), usually found in forests. Furthermore, most of the *Phragmoplastophyta* OTUs were associated with land plants belonging to taxa comprising of grasses and trees. The Arthropods detected were mainly flying insects associated with *Diptera* order (flies and mosquitos) and beetles (*Coleoptera*). Consequently, when we grouped these OTUs together and plotted them as a function of DOC, a modest positive correlation was observed between these two factors. It is noteworthy to mention that this correlation was mainly driven by the *Basidiomycota* group. These results suggest that contaminating OTUs may have some effect on the concentration of DOC within the cooling tower

environment, depending on the location of the cooling tower. Indeed, cooling towers in rural areas or close to green spaces may receive much more contaminating OTUs then cooling towers in dense urban areas. Other factors surely contribute to DOC concentrations, such as disinfection strategies (as discussed above), source of makeup water, cooling tower design, as well as factors associated with wind, which would affect the presence of contaminating OTUs (Milosavljevic and Heikkilä, 2001). Our study suggests that the location and surroundings of a cooling tower may be important to consider when developing a management strategy.

Since we have determined the bacterial and the eukaryotic communities, network analysis based on co-occurrence was performed to identify putative interactions between these two communities (Fig. 4). One of the main findings of the network analysis was the identification of five modules within the network. A module represents a group of taxa that are more connected to one another than with other taxa in the network that are not part of the same module (Deng et al., 2012). One potential interpretation of the presence of these modules in the network is that they represent distinct sub-niches in the cooling tower ecosystems. In our case, this is partly supported by the presence of several taxa with similar ecological behavior in the same module. For instance, *Ca.* Paracaedibacter was found in the same module as *Legionella* (module 4). Both of these genera are intracellular parasites of various species of amoeba, such as *Acanthamoeba castellanii,* (Cirillo et al., 1999; Horn et al., 1999) due to the similarity in their life cycles and would indicate a shared environment requiring similar characteristics, in this case the presence of amoeba. Another example is the presence of several algal groups within module two, suggesting a niche that allows a higher proportion of photosynthesis, most likely due to towers that allow the penetration of more light.

Moving forward with the assumption that modules represent different sub-niches, the identification of several connector nodes and hub nodes within the modules may indicate that several taxa may be able to inhabit various niches or that certain niches may overlap. Connector nodes and hub nodes are believed to represent generalist ecological behaviour (Deng et al., 2012; Olesen et al., 2006). In our case, the connections between two taxa are based on Pearson's correlation between the different taxa. These correlations could be due to ecological interactions, such as competition or mutualism, or could be due to specific characteristics of the environment, such as physicochemical parameters. In both cases, this implies the presence of connector nodes in several sub-niches. This may indicate that these taxa are important keystone species, as they could potentially affect the members of several sub-niches.

This network analysis identified correlations between *Legionella* and 17 other taxa (Fig. 5). These correlations were all positive and may indicate potentially novel and uncharacterized mutualistic interactions. They further give support to the idea that specific groups of organisms are crucial for *Legionella* ecology. This was mainly supported by the *Brevundimonas*-*Oligohymenophorea* interaction. Our results showed that bacterial species belonging to the *Brevundimonas* genus could be used as predictors for identifying towers with high levels of *Oligohymenophorea* (Fig. 6) and *Legionella* (Paranjape et al., 2020). We hypothesised that this prediction was due to *Brevundimonas* being a food source for ciliates. The predator-prey interaction between *Tetrahymena* and *Brevundimonas* was confirmed *in vitro* (Fig. 6). In this scenario, *Brevundimonas* would be used as a food source for the growth of the ciliate community, which would grow in numbers. This would then allow *Legionella* species to grow in the cooling tower environment, as they would use these ciliates as host cells. These results suggest that ciliates may be more important than previously thought for *Legionella* ecology, as most research mainly

focuses on the free-living amoeba population (Buse et al., 2013; Kuiper et al., 2006; Valster et al., 2009; Valster et al., 2011). The importance of the ciliate community was recently suggested (Tsao et al., 2019). Growth of *L. pneumophila* in *A. castellanii* has been linked to increased virulence in human host cells (Cirillo et al., 1994). It is tempting to speculate that the ciliate population may increase the virulence of *L. pneumophila.*

Finally, our results showed that *Brevundimonas* SPF441 could stimulate the growth of *L. pneumophila* on CYE agar without L-cysteine supplementation (Fig. 7). This stimulation may be due to production of essential amino acids, and more specifically production of cysteine, by the *Brevundimonas* isolate. Indeed, whole genome sequencing identified several genes involved in the synthesis of cysteine or cysteine derivatives in *Brevundimonas* SPF441. Cystathionine gamma-lyase and cysteine-S-conjugate beta-lyase are both enzymes involved in production of thiocysteine and L-cysteine from L-cystine (Cooper and Pinto, 2006). Cysteine from the yeast extract in CYE agar is quickly oxidizes to L-cystine and cannot be used by *L. pneumophila*, hence the necessity to supplement the CYE medium with L-cysteine (Ewann and Hoffman, 2006). Consequently, *Brevundimonas* SPF441 may supplement *L. pneumophila* with an exogenous source of L-cysteine by converting the L-cystine to L-cysteine and thiocysteine. In addition, *L. pneumophila* is auxotrophic for several amino acids, such as arginine, cysteine, isoleucine, leucine, methionine, threonine, and valine (Eisenreich and Heuner, 2016). *Brevundimonas* SPF441 possesses all the genes necessary for the production of these amino acids. Thus, our results suggest that *Brevundimonas* species may be important for *Legionella* ecology, through direct and indirect interactions resulting in the promotion of *Legionella* survival and growth in water systems. Further research should look into the *Brevundimonas* population of cooling towers and its effect on the *Legionella* population.

IV- CONCLUSION

In conclusion, our research indicates that cooling tower ecosystems are much more complex than previously thought. This was mainly supported by the potential presence of a microbial loop that suggested that cooling towers are thriving ecosystems for microbes. Dissolved organic carbon was an important factor to shape the diversity of the cooling tower eukaryotic community. These two components may be important factors for *Legionella* ecology. More importantly, network analysis revealed the presence of various correlations between the bacterial and eukaryotic taxa. This was further observed with *Legionella*, which was directly correlated to 17 different taxa. These correlations may be due to ecological interactions, such as mutualism or competition, between the various organisms. This was further supported between the interaction of the bacterial genus *Brevundimonas* and ciliates. Indeed, our results indicated that *Brevundimonas* could stimulate the growth of *Legionella* by being a food source for *Legionella* host species, as well as, directly stimulate the growth through nutritional supplementation of L-cysteine. These findings suggest that the host community is not the single most important factor for *Legionella* outbreaks, but that instead, *Legionella* ecology is dependent on various groups of microorganisms. As a result, this may indicate that the microbiome of cooling towers is a crucial component in *Legionella* ecology. It is warranted to examine how the other correlated organisms may promote the growth of *Legionella* in cooling towers.

V- MATERIALS AND METHODS

1. SAMPLING OF COOLING TOWERS AND PARAMETER MEASUREMENTS

A total of 18 cooling towers were sampled from six different regions in Quebec, Canada, between the 10th and 21st of July 2017. Details were presented in a previous work (Paranjape et al., 2020). Briefly, water was sampled in one-litre sterile bottles in three times, from the water basin of cooling towers (Supplementary Table S3). The Biomass was collected by filtration (0.45µm pores) and DNA was extracted using the DNeasy Power water kit from Qiagen (Cat. No. 14900-100-NF) (see (Paranjape et al., 2020) and chapter 3). The *16S rRNA* gene targeted amplicon sequencing using the Illumina MiSeq platform (NCBI Sequence Read Archive accession number PRJNA507738) and the quantification of *L. pneumophila* using qPCR was reported previously (Paranjape et al., 2020).

2. EUKARYOTIC COMMUNITY PROFILING OF COOLING TOWERS

18S rRNA amplicon sequencing was performed using the Illumina MiSeq platform (Illumina, inc) as described in the Earth Microbiome Project's (EMP) 18S Illumina Amplicon Protocol (Amaral-Zettler et al., 2009; Stoeck et al., 2010; Thompson et al., 2017). This protocol targets the V9 region of the *18S rRNA* gene by using primer 1391F (5'-GTA CAC ACC GCC CGT C-3') and EukBR (5'- TGA TCC TTC TGC AGG TTC ACC TAC-3') (Amaral-Zettler et al., 2009; Stoeck et al., 2010). The Illumina two-step indexing protocol was used, where the Illumina overhang adapters were added to the primers described above. The V9 hypervariable region of the *18S rRNA* gene was amplified by PCR, using the Paq5000 PCR Hotstart master mix (Agilent Technologies, California, USA) with 10 µM of each primer and 2 µl of DNA. The cycling program

consisted of an initial denaturation step at 94°C for 3 minutes, followed by 30 cycles of 94°C for 45 seconds, 57°C for 60 seconds, and 72°C for 90 seconds, and a final elongation step of 10 minutes at 72°C. The size of the PCR products (260 bp +/- 50bp) was confirmed on a 2% agarose gel. The PCR products were purified using the Ampure XP bead kit (Beckman Coulter, Indianapolis, IN, USA) following the manufacturer's instructions. The purified PCR products were then indexed using the Nextera XT indexing kit, according to the manufacturer's instructions (Illumina, inc). The indexed PCR products were then purified using the Ampure XP bead kit and visualized on a 2% agarose gel. The purified DNA was quantified using the Quant-iT PicoGreen dsDNA assay kit (Thermofisher, MA, USA). The DNA samples were normalized to a concentration of 4 nM. The samples were pooled, diluted and denatured with NaOH to a final concentration of 20 pM in 1mM NaOH and HT1 buffer (Illumina, inc). This solution was further diluted down to 4 pM in pre-chilled HT1 buffer. Following the same dilution protocol, 4 pM PhiX control (Illumina, inc) was produced. The solutions were combined in a microcentrifuge tube to produce a 15% PhiX spike in, with 90µl of the PhiX solution and 510µl DNA library. This solution was heat denatured for two minutes at 96°C and then chilled on ice for five minutes. The sample (600 µl) was loaded in a Miseq platform using the 600 cycles MiSeq Reagent Kit v3.

Sequencing data was processed using the Mothur pipeline (Schloss et al., 2009). Briefly, the paired reads were assembled into contigs. Any contig with ambiguous bases or lengths exceeding 310 bp were culled. The sequences were aligned to the eukaryotic Silva Reference Database release 132. We customized the database so that it contained only the V9 region of *18S rRNA* genes. This provides better alignments and ensures that the reads overlap with the appropriate region of the database. Then, the ends and gaps from the sequence alignment were trimmed so that all sequences had the same alignment coordinates. The sequences were further

denoised using a pre-cluster algorithm within Mothur. The resulting unique sequences were purged of chimeras using the VSEARCH algorithm implemented by Mothur. Additionally, any remaining undesirable sequences, such as sequences from Bacteria, Archaea, chloroplasts, and mitochondria were removed by, first, classifying the sequences with Bayesian classifier algorithm within Mothur, and, then removing the undesirable sequences. The sequences were then assigned *de novo* into OTUs using the cluster.split command with a cutoff of 0.03. An OTU table and a respective taxonomy file for each OTU was created. Since we had sampled each tower three times, the OTU table was created by averaging the OTU counts of each replicate for each cooling tower sample. The clustering created a total of 44 183 OTUs. The data were analysed with MicrobiomeAnalyst (Dhariwal et al., 2017), which performs data filtration and several ecological analyses, such as community profiling, clustering and biomarker analyses. The low count filter was set so that OTUs are retained only if at least 20% of their values contains at least 2 counts. This removed a total of 12 382 low count OTUs. Additionally, the default low variance filter (remove OTU with low variance at 10% using inter-quantile range) was used to remove any OTUs that were constant throughout the samples. This removed a total of 69 OTUs. Next, each sample was rarefied to the minimum library size (3 440 counts per sample) and total sum scaling was used to scale the data. Table 1 and the supplementary table S1 (appendix 1 part II) give the read count for each replicate before processing and after processing the sequences. Furthermore, the raw reads of the *18S rRNA* amplicon sequencing have been uploaded to NCBI's sequence read archive under the accession number PRJNA563440.

Next, microbiome analyst was used to create the taxonomic abundance bar graphs, the coverage analysis, the alpha and beta diversity analysis, and the LEfSe analysis (Dhariwal et al.,

2017). Good's coverage was calculated on the unrarefied and rarefied eukaryotic OTU table (Supplemental Table S2, appendix 1 part II).

The Shannon index was calculated using the microbiome analyst, however, GraphPad prism version 8.3.1 for macOS was used to visualise the data and to test statistical significance using the Kruskal Wallis test followed by Dunn's multiple comparison test using a p value cut-off of 0.05. In the case of the regression analysis (Fig. 2A and 3), the data was plotted using GraphPad prism and non-linear regression using a semi-log line to model the data. Spearman's rank correlation test was also used to find possible correlations in the data sets. We used a p-value cut-off of 0.05 to assess statistical significance of the correlation.

The microbiome analyst was also used to perform beta diversity analysis using NMDS as ordination method and the Bray-Curtis index as dissimilarity metric. The beta diversity analysis was done at the OTU level. Statistical significance was calculated using ANOSIM. This was provided by the microbiome analyst, however, visualisation was done with the R package Tidyverse (Team, 2019; Wickham and Wickham, 2017).

The LEfSe analysis was performed on the bacterial profiles of the same cooling tower samples we had sequenced in a previous experiment (Paranjape et al., 2020). The raw reads for the bacterial data set have been uploaded on NCBI Sequence Read Archive under the accession number PRJNA507738. Briefly, LEfSe was performed on the bacterial genus table described previously (Paranjape et al., 2020) and the towers were categorized by the relative abundance of *Oligohymenophorea* (using the rarefied eukaryotic OTU data): absence (less then 0 read counts per sample), low level (between 1 and 100 read counts per sample) and high level (more than 100 read counts per sample). Then, LEfSe was used to find significant taxa in each group. LEfSE was

conducted through the microbiome analyst but GraphPad prism was used to create the bar plots and visualise the data. We used a p value cut-off of 0.05 for the Kruskal Wallis test and the Wilcoxon test and a LDA score (log scale) of 3 to identify significant bacterial taxa for the different conditions studied.

A negative control was run separately to determine contamination levels. This blank consisted of running an unused sterile filter through the same pipeline as aforementioned. Thus, the clean filter was processed for DNA extraction, *18S rRNA* PCR amplification, and sequencing on the MiSeq Illumina platform (V3 reagent kit, 600 cycles). The same mothur pipeline was used to process the raw reads.

3. Network Construction and Analysis

A network, based on co-occurrence, was constructed between the eukaryotic and bacterial taxa of the cooling towers using the Molecular Ecological Network Analysis pipeline (MENAp) (Deng et al., 2012). Briefly, eukaryotic OTUs were regrouped into their respective families. Bacterial OTUs from the previous study were grouped according to their respective genera (Paranjape et al., 2020). Taxa with an average count of lower than 5 counts per sample, were merged together into a group called low_count. This was done for the bacterial dataset (low_count_Bacteria) and the eukaryotic dataset (low_count_Eukaryota). Both bacterial and eukaryotic OTU tables were merged and then processed with the MENA pipeline via the following website: http://ieg2.ou.edu/MENA (Deng et al., 2012). The network was constructed using the default settings of the pipeline with the exception of the following parameters: the "Majority" setting was set to 1, the "Logarithm" function was not used, and Pearson's correlation was selected

to calculate correlations between different OTUs. MENA uses random matrix theory to identify a reliable Pearson's correlation coefficient as a cut-off based on the χ^2 test with Poisson distribution (Deng et al., 2012; Weiss et al., 2016). In our case, MENA identified a 0.60 Pearson coefficient as cut-off when using the strictest threshold of $\chi^2 > 0.05$. Cytoscape 3.7.1 was used to visualize the network (Shannon et al., 2003).

4. ISOLATION OF *BREVUNDIMONAS* SP. FROM COOLING TOWER

Bacterial colonies were isolated from a cooling tower on R2A agar and re-streaked three times to ensure pure cultures. Glycerol stocks (15% glycerol in R2A medium) cultures were made for each strain for downstream applications. The identities of morphologically different colonies were determined by sequencing the *16S rRNA* gene. Briefly, DNA was extracted from pure cultures using the Wizard genomic DNA purification Kit (Promega). The *16S rRNA* gene was amplified by PCR, using bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR product was cloned into the pGEM-T Easy vector system (Promega). Clones were selected by blue white screening. Plasmids containing the *16S rRNA* insert were extracted using a Miniprep plasmid extraction kit (Qiagen). The insert was sequenced by Sanger sequencing at the Plateforme Génomique de l'Université Laval, Canada. The sequence was then analysed using NCBI BLAST. One of the isolates of interest showed 99.51% identity with *Brevundimonas sp.* strain HES1 (Accesion MN081030.1). We named the strain *Brevundimonas* SPF441

5. WHOLE GENOME SEQUENCING OF *BREVUNDIMONAS* SPF441 ISOLATE

Genomic DNA was extracted from the *Brevundimonas* SPF441 isolate using the Wizard genomic DNA purification kit (Promega). The genomic DNA quality was verified on a 0.8% agarose gel and the concentration was determined using the Quant-iT PicoGreen dsDNA assay kit (Thermofisher). The DNA library for whole genome sequencing was prepared using the Nextera XT DNA library prep kit (Illumina), according to the manufacturer's instructions. The library was analyzed on an Agilent Technology 2100 Bioanalyzer (Agilent) to evaluate proper DNA fragment size. The library was normalized to 2 nM and then pooled together. The pooled library was denatured with 0.2 N NaOH and diluted to 12 pM loading concentration with HT1 buffer as per the manufacturer's instructions (Illumina). The library was spiked with PhiX control (20 pM) at 1%. The library was then loaded on the MiSeq sequencing platform (Illumina) with the MiSeq Reagent kit V3 (600 cycles).

A total of 500 485 paired reads were generated. The read quality was evaluated using FastQC (Andrews, 2010). The forward and reverse sequences were processed using Trimmomatic (v0.39) with the following commands: LEADING: 10 TRAILING: 10 SLIDINGWINDOW: 5: 20 MINLEN: 36 (Bolger et al., 2014). This removed low quality reads, leaving 467366 reads (93.38% of initial data). The forward and reverse reads were assembled using SPades (v3.13) (Nurk et al., 2013). The reads were first corrected using the "only-error-correction" option, then the corrected reads were assembled using the "only-assembler" option. When assembling the reads, the k-mer length was set to 21, 33, 55, 77, 99, and 127. The assembled genome was uploaded to MiGA (Microbial Genome Atlas, v0.3.12) server, and the NCBI Prok module was used to identify the taxonomy and novelty of the isolate (Rodriguez-R et al., 2018). Bandage was used to infer the quality of the asembly (Wick et al., 2015). Additionally, the assembled genome was annotated

using Prokka (v1.14) (Seemann, 2014), and uploaded to the blastKOALA (v2.2) website to infer metabolic pathways present in the isolate, using the KEGG database (Kanehisa et al., 2016). The raw reads of this genome were deposited to NCBI SRA under the Bioproject number PRJNA580507. The Whole Genome Shotgun project for *Brevundimonas* SPF441 has been deposited at DDBJ/ENA/GenBank under the accession number WJWX00000000. The version described in this paper is version WJWX01000000. This deposited genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline.

6. CO-CULTURE OF *BREVUNDIMONAS* WITH *TETRAHYMENA*: EVALUATING THE FATE OF *BREVUNDIMONAS* SPF441

The fate of *Brevundimonas* SPF441 when incubated in coculture with *Tetrahymena thermophila* and *Tetrahymena pyriformis* was determined by CFU counts. Briefly, *T. thermophila* and *T. pyriformis* were grown in SPP medium (Sugar Proteose Peptone: 8 g proteose peptone, 0.8 g dextrose, 0.4 g yeast extract, and 33 nM FeCL, in 400 mL of distilled water) at 30 °C and 21 °C, respectively. Cells were passaged when the density reached 5×10^5 cells/ml. Twenty-five millilitres of the cell cultures were transferred into 50 ml conical tubes and centrifuged at 600 *g* for five minutes. The supernatant was quickly removed and 25 ml of plate counting broth (PCB: 5 g Yeast extract, 10 g Tryptone, 2 g Dextrose, 1 L water) was added to each tube. One-millilitre aliquots of each ciliate solution were transferred to six wells of two 24-well plates. Six wells on each plate were filled with 1 ml aliquots of sterile PCB to be used as controls. Each well was inoculated with 30 µl of a 0.4 $OD_{600\ nm}$ *Brevundimonas* SPF441 suspension, resulting in a final inoculum of 4×10^6 CFU/ml. The co-culture with *T. thermophila* was incubated at 30 °C while

the co-culture with *T. pyriformis* was incubated at 25 °C. CFUs were determined at 0, 2, 4, and 12 hours of incubation on nutrient agar. The plates were incubated at 30 °C for two days.

7. CO-CULTURE OF *BREVUNDIMONAS* WITH *TETRAHYMENA*: EVALUATING GROWTH OF *TETRAHYMENA* USING *BREVUNDIMONAS* SPF441 AS FOOD SOURCE

The growth of *T. pyriformis* and *T. thermophila* was determined when incubated in Tris buffer and periodically fed with the *Brevundimonas* SPF441 isolate. Briefly, both ciliates were grown in SPP media as described above to a concentration of 1.0×10^6 cells/ml. The cells were washed twice in 10 mM tris (pH 7.5). *Tetrahymena* cells were counted using a Guava easyCyte flow cytometer, using the FSC and SSC parameters. The cells were then diluted down to 1.0×10^3 cells/ml for the co-culture and 1.0×10^4 cells/ml for the control (ciliates alone), in 25 ml of 10 mM tris (pH 7.5) solution. Ciliate cultures were counted before inoculation on day 0 and incubated at 25 °C and 30 °C, for *T. pyriformis* and *T. thermophila* respectively. Every other day, starting on day 1 of incubation, 200µl of 1.000 $OD_{600\ nm}$ of *Brevundimonas* SPF441 isolate culture, washed twice in 10 mM tris solution, was inoculated into the ciliate cultures. Ciliates counts were measured on day 0, 2, and 4 using a Guava easyCyte flow Cytometer.

8. STIMULATION OF *LEGIONELLA PNEUMOPHILA* GROWTH ON CYE WITHOUT L-CYSTEINE

The stimulation assay was based on Wadowsky and Yee, (1983) with slight modification (Wadowsky and Yee, 1983). Briefly, 100 µl of 0.2 $OD_{600\ nm}$ (around a total of 10^7 CFU) of *L. pneumophila* suspension in AYE was inoculated in 5 ml of soft agar (0.5% agar). The soft agar

was overlaid on CYE without L-cysteine supplementation and on CYE with L-cysteine (control). The agar was left to solidify for 15 to 30 minutes, after which, 10 µl of *Brevundimonas* isolate, at 0.2 $OD_{(600nm)}$, was spotted in the middle of the plates. The plates were incubated at 30°C for 4 days.

VI- DECLARATIONS

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets generated and analysed during the current study are available in the NCBI Sequence Read Archive under the accession PRJNA563440 (*18S rRNA* gene raw reads), and PRJNA507738 (*16S rRNA* gene raw reads). The *Brevundimonas* SPF441 genome was deposited at DDB/ENA/GenBank under the accession WJWX00000000. The version described in this paper is WJWX01000000. The raw reads for this genome were deposited in NCBI sequence read archive under the BioProject number PRJNA580507.

Additional file

Additional file 1(.Docx file): Table S1. Read count for each replicate of every cooling tower sample at the different processing steps; Table S2. Good's coverage estimator for unfiltered and filtered eukaryotic OTU table for each cooling tower sample; Table S3. Characteristics of Cooling Tower Samples; Figure S1. Principal Coordinate Analysis (PCoA) of cooling towers showing clustering of eukaryotic community according to DOC levels using ANOSIM to evaluate dissimilarity between communities ($R = 0.817041$, $P < 0.001$). Towers with high (red) and low levels (blue) of DOC clustered separately; Document DS1. Description of metabolic features in *Brevundimonas* SPF441.

Competing Interests

The authors declare no competing interest.

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Authors' contribution

KP, EB, MP and SPF design the study. KP perform *18S rRNA* gene amplicon sequencing, whole genome sequencing and the ciliate co-culture experiments. MQ isolated *Brevundimonas*. DS and FCPC perform the *Brevundimonas* stimulation assay. KP analysed the data and wrote the first draft of the manuscript. KP, SPF, EB and MP edited the manuscript. All authors approved submission of the manuscript.

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VII- REFERENCES

Abd, H., Wretlind, B., Saeed, A., Idsund, E., Hultenby, K. and SANDSTRÖM, G. 2008. Pseudomonas aeruginosa utilises its type III secretion system to kill the free-living amoeba Acanthamoeba castellanii. Journal of eukaryotic microbiology 55(3), 235-243.

Alsam, S., Jeong, S.R., Sissons, J., Dudley, R., Kim, K.S. and Khan, N.A. 2006. Escherichia coli interactions with Acanthamoeba: a symbiosis with environmental and clinical implications. Journal of medical microbiology 55(6), 689-694.

Amaral-Zettler, L.A., McCliment, E.A., Ducklow, H.W. and Huse, S.M. 2009. A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. PloS one 4(7), e6372.

Amaro, F., Wang, W., Gilbert, J.A., Anderson, O.R. and Shuman, H.A. 2015. Diverse protist grazers select for virulence-related traits in Legionella. The ISME journal 9(7), 1607.

Andrews, S. 2010 FastQC: a quality control tool for high throughput sequence data, Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom.

Armstrong, T.W. and Haas, C.N. 2008. Legionnaires' disease: evaluation of a quantitative microbial risk assessment model. Journal of water and health 6(2), 149-166.

Azam, F., Fenchel, T., Field, J., Grey, J., Meyer-Reil, L. and Thingstad, F. 1983. The ecological role of water-column microbes. Mar. ecol. Prog. ser 10, 257-263.

Barker, J. and Brown, M. 1994. Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. Microbiology 140(6), 1253-1259.

Bédard, E., Lévesque, S., Martin, P., Pinsonneault, L., Paranjape, K., Lalancette, C., Dolcé, C.-É., Villion, M., Valiquette, L. and Faucher, S.P. 2016. Energy conservation and the promotion of Legionella pneumophila growth: the probable role of heat exchangers in a nosocomial outbreak. infection control & hospital epidemiology 37(12), 1475-1480.

Berk, S., Gunderson, J., Newsome, A., Farone, A., Hayes, B., Redding, K., Uddin, N., Williams, E., Johnson, R. and Farsian, M. 2006. Occurrence of infected amoebae in cooling towers compared with natural aquatic environments: implications for emerging pathogens. Environmental science & technology 40(23), 7440-7444.

Bhopal, R.S., Fallon, R.J., Buist, E.C., Black, R.J. and Urquhart, J.D. 1991. Proximity of the home to a cooling tower and risk of non-outbreak Legionnaires' disease. BMJ 302(6773), 378-383.

Boamah, D.K., Zhou, G., Ensminger, A.W. and O'Connor, T.J. 2017. From many hosts, one accidental pathogen: the diverse protozoan hosts of Legionella. Frontiers in cellular and infection microbiology 7, 477.

Bolger, A.M., Lohse, M. and Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30(15), 2114-2120.

Brassinga, A.K.C., Kinchen, J.M., Cupp, M.E., Day, S.R., Hoffman, P.S. and Sifri, C.D. 2010. Caenorhabditis is a metazoan host for Legionella. Cellular microbiology 12(3), 343-361.

Buse, H.Y., Lu, J., Struewing, I.T. and Ashbolt, N.J. 2013. Eukaryotic diversity in premise drinking water using 18S rDNA sequencing: implications for health risks. Environmental Science and Pollution Research 20(9), 6351-6366.

Buse, H.Y., Schoen, M.E. and Ashbolt, N.J. 2012. Legionellae in engineered systems and use of quantitative microbial risk assessment to predict exposure. Water research 46(4), 921-933.

Cirillo, J.D., Cirillo, S.L., Yan, L., Bermudez, L.E., Falkow, S. and Tompkins, L.S. 1999. Intracellular growth in Acanthamoeba castellanii affects monocyte entry mechanisms and enhances virulence of Legionella pneumophila. Infection and immunity 67(9), 4427-4434.

Cirillo, J.D., Falkow, S. and Tompkins, L.S. 1994. Growth of Legionella pneumophila in Acanthamoeba castellanii enhances invasion. Infection and Immunity 62(8), 3254-3261.

Control, C.f.D. and Prevention 2011. Legionellosis---United States, 2000-2009. MMWR. Morbidity and mortality weekly report 60(32), 1083.

Cooper, A.J. and Pinto, J. 2006. Cysteine S-conjugate β-lyases. Amino acids 30(1), 1-15.

Corre, M.-H., Delafont, V., Legrand, A., Berjeaud, J.-M. and Verdon, J. 2019. Exploiting the Richness of Environmental Waterborne Bacterial Species to Find Natural Legionella pneumophila Competitors. Frontiers in Microbiology 9(3360).

Cunha, B.A., Burillo, A. and Bouza, E. 2016. Legionnaires' disease. The Lancet 387(10016), 376-385.

Deng, Y., Jiang, Y.-H., Yang, Y., He, Z., Luo, F. and Zhou, J. 2012. Molecular ecological network analyses. BMC bioinformatics 13(1), 113.

Dhariwal, A., Chong, J., Habib, S., King, I.L., Agellon, L.B. and Xia, J. 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic acids research 45(W1), W180-W188.

Dooling, K.L., Toews, K.-A., Hicks, L.A., Garrison, L.E., Bachaus, B., Zansky, S., Carpenter, L.R., Schaffner, B., Parker, E. and Petit, S. 2015. Active bacterial core surveillance for legionellosis—United States, 2011–2013. Morbidity and Mortality Weekly Report 64(42), 1190-1193.

ECDC 2019. Legionnaires' Disease. ECDC Annual epdimilogical report for 2017.

Eisenreich, W. and Heuner, K. 2016. The life stage-specific pathometabolism of Legionella pneumophila. FEBS letters 590(21), 3868-3886.

Ewann, F. and Hoffman, P.S. 2006. Cysteine metabolism in Legionella pneumophila: characterization of an L-cystine-utilizing mutant. Appl. Environ. Microbiol. 72(6), 3993-4000.

Fenchel, T. 2008. The microbial loop–25 years later. Journal of Experimental Marine Biology and Ecology 366(1-2), 99-103.

Fields, B., Shotts, E., Feeley, J., Gorman, G. and Martin, W. 1984. Proliferation of Legionella pneumophila as an intracellular parasite of the ciliated protozoan Tetrahymena pyriformis. Appl. Environ. Microbiol. 47(3), 467-471.

Fitzhenry, R., Weiss, D., Cimini, D., Balter, S., Boyd, C., Alleyne, L., Stewart, R., McIntosh, N., Econome, A. and Lin, Y. 2017. Legionnaires' disease outbreaks and cooling towers, New York City, New York, USA. Emerging infectious diseases 23(11), 1769.

García-Fulgueiras, A., Navarro, C., Fenoll, D., García, J., González-Diego, P., Jiménez-Buñuales, T., Rodriguez, M., Lopez, R., Pacheco, F. and Ruiz, J. 2003. Legionnaires' disease outbreak in Murcia, Spain. Emerging infectious diseases 9(8), 915.

Glassing, A., Dowd, S.E., Galandiuk, S., Davis, B. and Chiodini, R.J. 2016. Inherent bacterial DNA contamination of extraction and sequencing reagents may affect interpretation of microbiota in low bacterial biomass samples. Gut pathogens 8(1), 24.

Glöckner, F.O., Yilmaz, P., Quast, C., Gerken, J., Beccati, A., Ciuprina, A., Bruns, G., Yarza, P., Peplies, J., Westram, R. and Ludwig, W. 2017. 25 years of serving the community with ribosomal RNA gene reference databases and tools. Journal of Biotechnology 261, 169-176.

Godoy-Vitorino, F., Ley, R.E., Gao, Z., Pei, Z., Ortiz-Zuazaga, H., Pericchi, L.R., Garcia-Amado, M.A., Michelangeli, F., Blaser, M.J. and Gordon, J.I. 2008. Bacterial community in the crop of the hoatzin, a neotropical folivorous flying bird. Appl. Environ. Microbiol. 74(19), 5905-5912.

Guerrieri, E., Bondi, M., Sabia, C., de Niederhäusern, S., Borella, P. and Messi, P. 2008. Effect of Bacterial Interference on Biofilm Development by Legionella pneumophila. Current Microbiology 57(6), 532-536.

Hahn, M.W. and Höfle, M.G. 2001. Grazing of protozoa and its effect on populations of aquatic bacteria. FEMS microbiology ecology 35(2), 113-121.

Helmi, K., David, F., Di Martino, P., Jaffrezic, M.-P. and Ingrand, V. 2018. Assessment of flow cytometry for microbial water quality monitoring in cooling tower water and oxidizing biocide treatment efficiency. Journal of microbiological methods 152, 201-209.

Horn, M., Fritsche, T.R., Gautom, R.K., Schleifer, K.H. and Wagner, M. 1999. Novel bacterial endosymbionts of Acanthamoeba spp. related to the Paramecium caudatum symbiont Caedibacter caryophilus. Environmental Microbiology 1(4), 357-367.

Iervolino, M., Mancini, B. and Cristino, S. 2017. Industrial Cooling Tower Disinfection Treatment to Prevent Legionella spp. International journal of environmental research and public health 14(10), 1125.

Isberg, R.R., O'connor, T.J. and Heidtman, M. 2009. The Legionella pneumophila replication vacuole: making a cosy niche inside host cells. Nature Reviews Microbiology 7(1), 13.

Kanehisa, M., Sato, Y. and Morishima, K. 2016. BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. Journal of molecular biology 428(4), 726-731.

Kikuhara, H., Ogawa, M., Miyamoto, H., Nikaido, Y. and Yoshida, S.-i. 1994. Intracellular multiplication of Legionella pneumophila in Tetrahymena thermophila. Journal of UOEH 16(4), 263-275.

Kilvington, S. and Price, J. 1990. Survival of Legionella pneumophila within cysts of Acanthamoeba polyphaga following chlorine exposure. Journal of applied bacteriology 68(5), 519-525.

Kozak-Muiznieks, N.A., Lucas, C.E., Brown, E., Pondo, T., Taylor, T.H., Frace, M., Miskowski, D. and Winchell, J.M. 2014. Prevalence of sequence types among clinical and environmental isolates of Legionella pneumophila serogroup 1 in the United States from 1982 to 2012. Journal of clinical microbiology 52(1), 201-211.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K. and Schloss, P.D. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl. Environ. Microbiol. 79(17), 5112-5120.

Kuiper, M.W., Valster, R.M., Wullings, B.A., Boonstra, H., Smidt, H. and Van Der Kooij, D. 2006. Quantitative detection of the free-living amoeba Hartmannella vermiformis in surface water by using real-time PCR. Appl. Environ. Microbiol. 72(9), 5750-5756.

Lau, H. and Ashbolt, N. 2009. The role of biofilms and protozoa in Legionella pathogenesis: implications for drinking water. Journal of applied microbiology 107(2), 368-378.

Lévesque, S., Plante, P.-L., Mendis, N., Cantin, P., Marchand, G., Charest, H., Raymond, F., Huot, C., Goupil-Sormany, I. and Desbiens, F. 2014. Genomic characterization of a large outbreak of Legionella pneumophila serogroup 1 strains in Quebec City, 2012. PLoS One 9(8), e103852.

Loret, J.-F. and Greub, G. 2010. Free-living amoebae: biological by-passes in water treatment. International journal of hygiene and environmental health 213(3), 167-175.

Milosavljevic, N. and Heikkilä, P. 2001. A comprehensive approach to cooling tower design. Applied thermal engineering 21(9), 899-915.

Mouchtouri, V.A., Goutziana, G., Kremastinou, J. and Hadjichristodoulou, C. 2010. Legionella species colonization in cooling towers: risk factors and assessment of control measures. American journal of infection control 38(1), 50-55.

Murga, R., Forster, T.S., Brown, E., Pruckler, J.M., Fields, B.S. and Donlan, R.M. 2001. Role of biofilms in the survival of Legionella pneumophila in a model potable-water system. Microbiology 147(11), 3121-3126.

National Academies of Sciences, E. and Medicine (2020) Management of Legionella in Water Systems, The National Academies Press, Washington, DC.

Nhu Nguyen, T.M., Ilef, D., Jarraud, S., Rouil, L., Campese, C., Che, D., Haeghebaert, S., Ganiayre, F., Marcel, F. and Etienne, J. 2006. A community-wide outbreak of legionnaires disease linked to industrial cooling towers—how far can contaminated aerosols spread? The Journal of infectious diseases 193(1), 102-111.

Nurk, S., Bankevich, A., Antipov, D., Gurevich, A., Korobeynikov, A., Lapidus, A., Prjibelsky, A., Pyshkin, A., Sirotkin, A. and Sirotkin, Y. 2013 Assembling genomes and mini-metagenomes from highly chimeric reads, pp. 158-170, Springer.

O'mahony, M., Stanwell-Smith, R., Tillett, H., Harper, D., Hutchison, J., Farrell, I., Hutchinson, D., Lee, J., Dennis, P. and Duggal, H. 1990. The Stafford outbreak of Legionnaires' disease. Epidemiology & Infection 104(3), 361-380.

Olesen, J.M., Bascompte, J., Dupont, Y.L. and Jordano, P. 2006. The smallest of all worlds: pollination networks. Journal of theoretical Biology 240(2), 270-276.

Orkis, L.T., Harrison, L.H., Mertz, K.J., Brooks, M.M., Bibby, K.J. and Stout, J.E. 2018. Environmental sources of community-acquired legionnaires' disease: A review. International journal of hygiene and environmental health 221(5), 764-774.

Paranjape, K., Bédard, É., Whyte, L.G., Ronholm, J., Prévost, M. and Faucher, S.P. 2020. Presence of Legionella spp. in cooling towers: the role of microbial diversity, Pseudomonas, and continuous chlorine application. Water Research 169, 115252.

Paschke, A., Schaible, U.E. and Hein, W. 2019. Legionella transmission through cooling towers: towards better control and research of a neglected pathogen. The Lancet Respiratory Medicine 7(5), 378-380.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glöckner, F.O. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41(D1), D590-D596.

Rasch, J., Krüger, S., Fontvieille, D., Ünal, C.M., Michel, R., Labrosse, A. and Steinert, M. 2016. Legionella-protozoa-nematode interactions in aquatic biofilms and influence of Mip on Caenorhabditis elegans colonization. International Journal of Medical Microbiology 306(6), 443-451.

Richardson, I. 1990. The incidence of Bdellovibrio spp. in man-made water systems: coexistence with legionellas. Journal of Applied Bacteriology 69(1), 134-140.

Rodriguez-R, L.M., Gunturu, S., Harvey, W.T., Rosselló-Mora, R., Tiedje, J.M., Cole, J.R. and Konstantinidis, K.T. 2018. The Microbial Genomes Atlas (MiGA) webserver: taxonomic and gene diversity analysis of Archaea and Bacteria at the whole genome level. Nucleic acids research 46(W1), W282-W288.

Rodríguez-Zaragoza, S. 1994. Ecology of free-living amoebae. Critical reviews in microbiology 20(3), 225-241.

Rowbotham, T.J. 1980. Preliminary report on the pathogenicity of Legionella pneumophila for freshwater and soil amoebae. Journal of clinical pathology 33(12), 1179-1183.

Samba-Louaka, A., Delafont, V., Rodier, M.-H., Cateau, E. and Héchard, Y. 2019. Free-living amoebae and squatters in the wild: ecological and molecular features. FEMS Microbiology Reviews 43(4), 415-434.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H. and Robinson, C.J. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75(23), 7537-7541.

Seemann, T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30(14), 2068-2069.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S. and Huttenhower, C. 2011. Metagenomic biomarker discovery and explanation. Genome biology 12(6), R60.

Shah, P.P., Barskey, A.E., Binder, A.M., Edens, C., Lee, S., Smith, J.C., Schrag, S., Whitney, C.G. and Cooley, L.A. 2018. Legionnaires' disease surveillance summary report, United States: 2014-1015.

Shaheen, M., Scott, C. and Ashbolt, N.J. 2019. Long-term persistence of infectious Legionella with free-living amoebae in drinking water biofilms. International journal of hygiene and environmental health 222(4), 678-686.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B. and Ideker, T. 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome research 13(11), 2498-2504.

Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M.D., BREINER, H.W. and Richards, T.A. 2010. Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. Molecular ecology 19, 21-31.

Stout, J., Yu, V. and Best, M. 1985. Ecology of Legionella pneumophila within water distribution systems. Appl. Environ. Microbiol. 49(1), 221-228.

Suttle, C.A. 1994. The significance of viruses to mortality in aquatic microbial communities. Microbial ecology 28(2), 237-243.

Team, R.C. 2019. A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2012. URL https://www. R-project. org.

Temmerman, R., Vervaeren, H., Noseda, B., Boon, N. and Verstraete, W. 2006. Necrotrophic growth of Legionella pneumophila. Appl. Environ. Microbiol. 72(6), 4323-4328.

Thompson, L.R., Sanders, J.G., McDonald, D., Amir, A., Ladau, J. and Locey, K.J. 2017. A communal catalogue reveals Earth's multiscale microbial diversity. Nature 551(7681), 457-463.

Tison, D., Pope, D., Cherry, W. and Fliermans, C. 1980. Growth of Legionella pneumophila in association with blue-green algae (cyanobacteria). Appl. Environ. Microbiol. 39(2), 456-459.

Trigui, H., Paquet, V.E., Charette, S.J. and Faucher, S.P. 2016. Packaging of Campylobacter jejuni into multilamellar bodies by the ciliate Tetrahymena pyriformis. Appl. Environ. Microbiol. 82(9), 2783-2790.

Tsao, H.-F., Scheikl, U., Herbold, C., Indra, A., Walochnik, J. and Horn, M. 2019. The cooling tower water microbiota: Seasonal dynamics and co-occurrence of bacterial and protist phylotypes. Water research 159, 464-479.

Türetgen, I. 2004. Comparison of the efficacy of free residual chlorine and monochloramine against biofilms in model and full scale cooling towers. Biofouling 20(2), 81-85.

Valster, R.M., Wullings, B.A., Bakker, G., Smidt, H. and van der Kooij, D. 2009. Free-living protozoa in two unchlorinated drinking water supplies, identified by phylogenic analysis of 18S rRNA gene sequences. Appl. Environ. Microbiol. 75(14), 4736-4746.

Valster, R.M., Wullings, B.A., van den Berg, R. and van der Kooij, D. 2011. Relationships between free-living protozoa, cultivable Legionella spp., and water quality characteristics in three drinking water supplies in the Caribbean. Appl. Environ. Microbiol. 77(20), 7321-7328.

van Heijnsbergen, E., Schalk, J.A., Euser, S.M., Brandsema, P.S., den Boer, J.W. and de Roda Husman, A.M. 2015. Confirmed and potential sources of Legionella reviewed. Environmental science & technology 49(8), 4797-4815.

Wadowsky, R.M. and Yee, R.B. 1983. Satellite growth of Legionella pneumophila with an environmental isolate of Flavobacterium breve. Appl. Environ. Microbiol. 46(6), 1447-1449.

Weiss, D., Boyd, C., Rakeman, J.L., Greene, S.K., Fitzhenry, R., McProud, T., Musser, K., Huang, L., Kornblum, J. and Nazarian, E.J. 2017. A large community outbreak of Legionnaires'

disease associated with a cooling tower in New York City, 2015. Public health reports 132(2), 241-250.

Weiss, S., Van Treuren, W., Lozupone, C., Faust, K., Friedman, J., Deng, Y., Xia, L.C., Xu, Z.Z., Ursell, L. and Alm, E.J. 2016. Correlation detection strategies in microbial data sets vary widely in sensitivity and precision. The ISME journal 10(7), 1669-1681.

Wick, R.R., Schultz, M.B., Zobel, J. and Holt, K.E. 2015. Bandage: interactive visualization of de novo genome assemblies. Bioinformatics 31(20), 3350-3352.

Wickham, H. and Wickham, M.H. 2017. Package tidyverse. Easily Install and Load the 'Tidyverse.

Winn, W.C. 1988. Legionnaires disease: historical perspective. Clinical Microbiology Reviews 1(1), 60-81.

Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies, J., Ludwig, W. and Glöckner, F.O. 2013. The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. Nucleic Acids Research 42(D1), D643-D648.

CONNECTING TEXT

The next chapter is the manuscript for my third article entitled: "Characterisation of Cooling Tower Bacterial Antagonistic Species of the Pathogenic Genus *Legionella*". This manuscript will be submitted for publication. Here we isolated several bacterial strains that were capable of inhibiting different strains of *L. pneumophila* and one strain of *L. quinlivanii* on solid growth media. These bacterial isolates were isolated from different cooling towers in Quebec. The genomes of the isolates were sequenced in order to identify potential genes that could explain the inhibition. This was done by identifying biosynthetic gene clusters in the genomes of the bacterial isolates. These clusters can produce antimicrobial compounds that could potentially inhibit *L. pneumophila*. The sequencing results revealed that the bacterial species contained a number of biosynthetic gene clusters coding for secondary metabolites. These secondary metabolites were diverse, revealing that *L. pneumophila* could potentially be inhibited through multiples ways. Furthermore, several of these clusters were not characterised, indicating that cooling towers may be an interesting environment for the discovery of novel antimicrobials. Interestingly, different *L. pneumophila* strains were resistant to various degrees to the inhibiting bacterial species. This would suggest that genetic factors in different *L. pneumophila* strains may contribute to resistance to the antimicrobial produced by the inhibiting bacterial species. Finally, the work presented here gives us better knowledge of potential interactions occurring in the cooling tower environment that can affect *L. pneumophila* ecology.

Contribution of authors: I contributed to the experimental design, performed the inhibition assay, the whole genome sequencing of the different strains, the processing and analysis of the

data, and wrote the manuscript. Sébastien P. Faucher contributed to the experimental design, writing and editing of the manuscript.

CHAPTER 5. CHARACTERISATION OF COOLING TOWER BACTERIAL ANTAGONISTIC SPECIES OF THE PATHOGENIC GENUS *LEGIONELLA*

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ABSTRACT

Legionella pneumophila is the causative agent of Legionnaires' Disease in humans. *L. pneumophila* is a bacterium found ubiquitously in engineered water systems (EWS) and is transmitted to humans by inhalation of contaminated aerosols. Growth of *L. pneumophila* in EWS is influenced by the resident microbiota. In this study we report seven bacterial species that inhibit different strains of *L. pneumophila* and one strain of *L. quinlivanii* on agar plate. These bacterial species were isolated from two different cooling towers in Montreal, Canada, and from a model cooling tower. Whole genome sequencing showed that these bacterial isolates belonged to the *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* phyla, indicating that a diverse number of species could inhibit *L. pneumophila* on plate. This diversity was also mirrored by the different antimicrobial gene clusters identified in their genomes, suggesting that *L. pneumophila* could be inhibit through various mechanisms. On the other hand, the different strains of *L. pneumophila* tested showed varying degrees of susceptibility to the antimicrobial activity, suggesting that certain genetic factors may be at play. The results also indicated that the cooling tower environment contains a number of antagonistic species that can inhibit *L. pneumophila*. As a result, these species could potentially be manipulated through a probiotic or prebiotic approach to create a non-permissive environment for *L. pneumophila* in cooling towers. Finally, the isolation of these inhibitory bacteria reinforces the notion that culture-based methods of detecting *L. pneumophila* may suffer high number of false negative.

Keywords: *Legionella*, Bacterial inhibition, Biosynthetic Gene Clusters, Whole Genome Sequencing

I- INTRODUCTION

Legionella pneumophila is the causative agent of Legionnaires' Disease, a severe pneumonia. This organism is an aquatic bacterium ubiquitously found in Engineered Water Systems (EWS), where it can colonize, survive, and grow in these environments. Examples of EWS that are known sources of *L. pneumophila* are water distribution systems, cooling towers, water reservoirs, misters, shower heads, and water faucets (van Heijnsbergen et al., 2015). These various systems produce aerosols that can be inhaled by nearby people. If *L. pneumophila* contaminates these aerosols, the bacteria can colonize the lungs and cause a pneumonia called Legionnaires' disease (LD).

L. pneumophila is now one of the more significant causes of waterborne diseases in developed countries (Benedict et al., 2017; Cassell et al., 2019). The number of cases of LD have been on the rise in recent years. For instance, the United States reported an increase of more than fivefold in incidences from 2000 to 2017, and a 1.5-fold increase from 2013 to 2017 was observed in the European Union (ECDC, 2019; National Academies of Sciences and Medicine, 2019). The number of cases of LD is believed to be underreported due to lack of a common definition of the disease, efficient diagnostic tests, and surveillance programs in many countries or states (Phin et al., 2014). Consequently, the actual number of cases is likely much higher. Mortality from LD is variable but is usually around 8% to 12% (Juda, 2009; National Academies of Sciences and Medicine, 2019; Phin et al., 2014). This variability in mortality is due to either low sample size or to the prevalence of specific risk factors associated with the disease in the affected population (Cunha et al., 2016; Phin et al., 2014). For example, previous lung disease, smoking, old age (> 50 years), immunosuppression, alcoholism, and cancer have all been linked with higher levels of mortality of LD (Cunha et al., 2016). In fact, hospital settings and assisted or senior living homes

are common grounds for *Legionella* outbreaks. Consequently, different countries and organisations have implemented laws, guidelines, or standards to reduce the risk related to LD (Parr et al., 2015; Québec, 2014; Standard, 2015). This is especially true for cooling towers and hospital water systems (Danila et al., 2018; Québec, 2014). These laws generally require monitoring of the levels of *L. pneumophila* in the system and implementation of corrective measures when the quantity of *L. pneumophila* reaches a pre-determined threshold (Québec, 2014).

As *L. pneumophila* is ubiquitously found in EWS, understanding its ecology and the parameters that lead to its proliferation in these environments is important. Several factors are known to affect *L. pneumophila*'s survival and growth.

First, *L. pneumophila* is an intracellular parasite of various protozoan species, such as amoeba and ciliates, and requires these host cells for growth (Boamah et al., 2017). The number of host species is quite extensive and spans several eukaryotic phyla (Boamah et al., 2017). These hosts are believed to be the reservoir of *L. pneumophila*, and, thus, are a major factor for the dissemination of the disease (Borella et al., 2005).

Secondly, biofilms are regarded as a stronghold for *L. pneumophila* as they promote its survival in EWS. Indeed, biofilms can provide protection from harsh chemical, nutrients for survival, and attract host species (Borella et al., 2005; Lau and Ashbolt, 2009). Consequently, biofilms allow *L. pneumophila* to persist and complicate its eradication from EWS. Interestingly, it is known that *L. pneumophila* survival depends on the species present within the biofilms. For instance, *L. pneumophila* persist well in *Klebsiella pneumoniae*, *Flavobacterium*, and *Pseudomonas fluorescens* biofilms but not in *Pseudomonas aeruginosa* biofilms (Stewart et al.,

2012). This may be due to the production of homoserine lactones 3-oxo-C_{12}-HSL, a quorum sensing autoinducers produced by *P. aeruginosa,* which was demonstrated to inhibit directly *L. pneumophila* growth (Kimura et al., 2009).

Thirdly, other bacterial species possess antimicrobial activity towards *L. pneumophila*. For instance, *Staphylococcus* and *Bacillus* species inhibit *L. pneumophila* on agar plates, through the production of hemolysins , bacteriocins, and surfactants (Loiseau et al., 2015; Marchand et al., 2011). Consequently, these findings suggest that a high number of antagonistic species exist and could be potentially used to inhibit *L. pneumophila* in water systems.

Two studies in particular have shown that a high number of anti-*Legionella* bacterial species can be isolated from these water systems (Corre et al., 2019; Guerrieri et al., 2008). Indeed, Guerrieri et *al* found that 55 out of 80 bacterial isolates from tap water showed anti-*Legionella* activity (Guerrieri et al., 2008). Most of these isolates were species or strains of *Pseudomonas* but *Stenotrophomonas maltophila*, *Aeromonas hydrophyla*, *Burkholderia cepacia*, *Alacaligenes faecalis*, *Acinetobacteria spp.*, and *Flavobactrerium spp.*, where also shown to inhibit *L. pneumophila* (Guerrieri et al., 2008). On the other hand, Corre et *al* were able to isolate 273 bacterial strains from different EWS and demonstrated that 178 of them had anti-*Legionella* activity (Corre et al., 2019). Their findings also demonstrated that a high diversity of waterborne bacteria, mainly from the *Gammaproteobacteria* and *Firmiticute* groups, can have antimicrobial activity against *L. pneumophila*. This suggests that there may be a large pool of different antimicrobials that have yet to be characterized and may have different mechanisms of inhibition. For instance, Corre *et al* showed that some of the isolated *Pseudomonas* strains could produce a volatile antimicrobial compounds with anti-*Legionella* effect, whereas other strains produced agar diffusible ones (Corre et al., 2019). As an added benefit, the inhibitory mechanisms used by the

microorganisms found in EWS, and especially cooling towers, may be more specific and efficient in inhibiting *L. pneumophila*, as all these organisms have co-evolved in the same environment.

Though these studies provide a good idea of the diversity of the inhibitive bacterial species in EWS, they are lacking in characterizing the genetic elements that govern this inhibition. We have previously analysed the bacterial community of 18 cooling towers. The results demonstrated that several bacterial taxa were negatively correlated with *Legionella* in cooling towers. However, the nature of these correlations was not characterized. In this optic, our goals were to isolate bacterial species from these cooling towers and test their antimicrobial activity towards *L. pneumophila* and *L. quinlivanii*. A second goal was to characterize the potential genetic elements that could cause the inhibition through whole genome sequencing.

II- MATERIAL AND METHODS

1. LEGIONELLA PNEUMOPHILA STRAINS USED IN THIS STUDY

Table 1 shows the characteristics of the *L. pneumophila* strains used for this study. The strains are characterized by their strain number, strain name, species, sequence base type (SBT), and the environment they were isolated from. Moreover, ATCC33152 was isolated during the first outbreak of Legionnaires' disease in Philadelphia in 1976, and ID120292 caused the 2012 outbreak in Quebec City (Lévesque et al., 2014). The other strains were either isolated from the environment (E) or from patient (P) and are a kind gift from Simon Lévesque (CIUSSS de l'Estrie). The strains were grown on CYE (yeast extract 10 g, ACES buffer 10 g, activated charcoal 2.0 g, L-cysteine 0.4 g, ferric pyrophosphate 0.25 g in 1 L of water, pH 6.90) at 30°C for 4 days.

Table 1: Information on the species, the sequence base type, and the source of the *Legionella* strains

Strain name	Strain number LSPQ number	Species	Sequence Base Type (SBT)	Source
LpPhili	*ATCC33152*	*L. pneumophila philadelphia 1 (ATCC33152)*	37	Patient
LpQcS62E	ID120292	*L. pneumophila* (2012 Quebec City Outbreak)	62	Environmental
LpS62	SPF334 LSPQ143016	*L. pneumophila*	62	NA
LpS1P	SPF330 LSPQ126851	*L. pneumophila*	1	Patient
LpS1E	SPF333 LSPQ142903	*L. pneumophila*	1	Environmental
LpS256P	SPF331 LSPQ128014	*L. pneumophila*	256	Patient
LpS256E	SPF332 LSPQ128471	*L.pneumophila*	256	Environmental
LpS213P	SPF329 LSPQ120882	*L. pneumophila*	213	Patient
Lq	SPF337	*L. quinlivanii*	NA	Patient

NA: Not Applicable

2. *ISOLATION OF INHIBITORY BACTERIAL STRAINS OF LEGIONELLA PNEUMOPHILA*

Cooling tower water samples were examined for their potential for containing bacterial species that could inhibit *L. pneumophila* on plate. Briefly, water was collected in sterile 1 L bottles from the basin of two cooling towers, in Montreal, Canada, and one model cooling towers built in our lab (Paniagua et al., 2019). The water samples were serially diluted in filter sterilized sample water, to a dilution of 10^{-6}. Charcoal Yeast Extract (CYE) agar plates were layered with 5 ml of soft agar (0.5% agar in distilled water), inoculated with 100 µl of 0.2 $OD_{600\ nm}$ of *L. pneumophila* suspension. The soft agar was let to solidify for 15 to 30 minutes in a biological safety cabinet. The dilutions were spread on the CYE agar by gently flooding 1 ml of dilution solution on to the soft agar layer. The dilution was spread by gently shaking and tilting the agar plate after which the excess liquid was aspirated with a pipette. The plates were left to dry for 30 minutes in a biological safety cabinet and then incubated at 30°C for 4 days. After incubation, inhibiting colonies could be visualized by the formation of an inhibition zone on the *L. pneumophila* lawn. These colonies were re-streaked three times on CYE to obtain pure cultures. Stock cultures of these isolates were made in 15% glycerol in AYE medium (yeast extract 10 g, ACES buffer 10 g, L-cysteine 0.4 g, ferric pyrophosphate 0.25 g in 1 L of water, pH 6.90).

3. *TESTING INHIBITION OF ISOLATES WITH DIFFERENT LEGIONELLA STRAINS*

Isolates were tested for their antimicrobial activity towards eight different strains of *L. pneumophila* and one strain of *Legionella quinlivani*. The *Legionella* strains were inoculated on CYE agar using the soft-agar approach described above. The inhibiting isolates were suspended in AYE at 0.2 $OD_{600\ nm}$ and 10 µl was spotted in the centre of the agar plates. The spots were left to dry for 15 to 30 minutes and the plates were then incubated at 30°C for 4 days. After incubation,

the inhibition zone diameters were measured as a semi-quantitative metric of antimicrobial activity.

4. WHOLE GENOME SEQUENCING OF ANTI-LEGIONELLA ISOLATES

Genomic DNA was extracted from the isolates using the Wizard genomic DNA purification kit (Promega). The genomic DNA quality was verified on a 0.8% agarose gel and the quantity was measured using the Quant-iT PicoGreen dsDNA assay kit (Thermofisher). The DNA library for whole genome sequencing was prepared using the Nextera XT DNA library prep kit (Illumina). The manufacturer's instructions were followed. The library was run on an Agilent Technology 2100 Bionalyzer (Agilent) to evaluate proper DNA fragment size. The library was manually normalized to 2 nM and then pooled together. The pooled library was denatured with 0.2 N NaOH and incubated for 5 minutes at room temperature. The solution was neutralized with 200 mM Tris-HCl (pH 7.0). The denatured library was diluted to 20 pM with HT1 buffer. The library was then diluted to a loading concentration of 12 pM. PhiX was diluted to 4 nM in HT1 buffer. The PhiX was denatured with 0.2 N NaOH and incubating at room temperature for 5 minutes. The denatured PhiX was diluted to 20 pM with HT1 buffer (Illumina). The library was spiked-in at 1% PhiX control with the denatured library. The solution (600 µl) was loaded into a MiSeq Reagent kit V3 (600 cycles) and sequenced on the MiSeq platform (Illumina).

The read quality was evaluated using FastQC (Andrews, 2010). The forward and reverse sequences were removed using Trimmomatic (v0.39) with the following commands: LEADING: 10 TRAILING: 10 SLIDINGWINDOW: 5: 20 MINLEN: 36 (Bolger et al., 2014). The forward and reverse reads were assembled *de novo* using Spades (v3.13) for each isolate (Nurk et al., 2013).

The reads were first corrected using the "only-error-correction" option, then the corrected reads were assembled using the "only-assembler" option. When assembling the reads, the k-mer length was set to 21, 33, 55, 77, 99, and 127. The assembled genome was uploaded to MiGA (Microbial Genome Atlas, v0.3.12) server, and the NCBI Prok module was used to identify the taxonomy and novelty of the isolate (Rodriguez-R et al., 2018). The assembled genomes were also uploaded to Antismash (v5.0) in order to identify, annotate, and analyse secondary metabolite biosynthesis gene clusters (Blin et al., 2019). Of note, the genome for *B. amyloliquefaciens* (SPF474) was not analysed as it had contamination issues during the sequencing run.

III- RESULTS

1. INHIBITION ASSAY

Seven bacterial colonies inhibiting *L. pneumophila* growth on plates were isolated from cooling towers and a model cooling tower. Their ability to inhibit different sequence base type (SBT) of *L. pneumophila* and *L. quinlivanii* was tested. Figure 1 shows photographic examples of the inhibition assay when testing with *L. pneumophila* strain LpS256P. Figure 2 shows the diameter of the inhibition zone created by each bacterial isolate for each strain of *Legionella* tested. For clarity the isolates are named according to the results of the whole genome sequencing (see below).

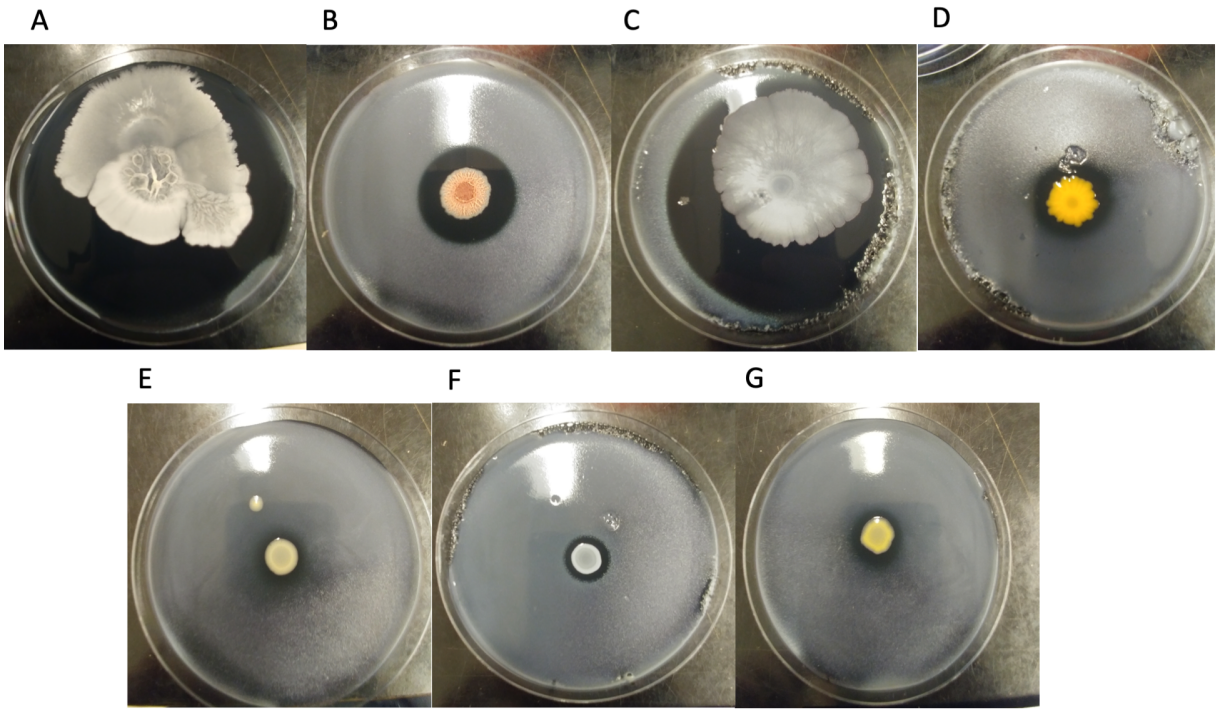

Figure 1: Isolates showing inhibition zones on lawn of *L. pneumophila* (LpS256P) on CYE agar incubated at 30°C for 4 days. Isolates are (A) *B. amyloliquefaciens* (SPF474), (B) *B. paralicheniformis* (SPF497), (C) *B. subtilis* (SPF437), (D) *Chryseobacterium sp.* (SPF475), (E) *Cupriviadus sp.* (SPF499), (F) *S. epidermidis* (SPF476), (G) *Stenotrophomonas sp.* (SPF498).

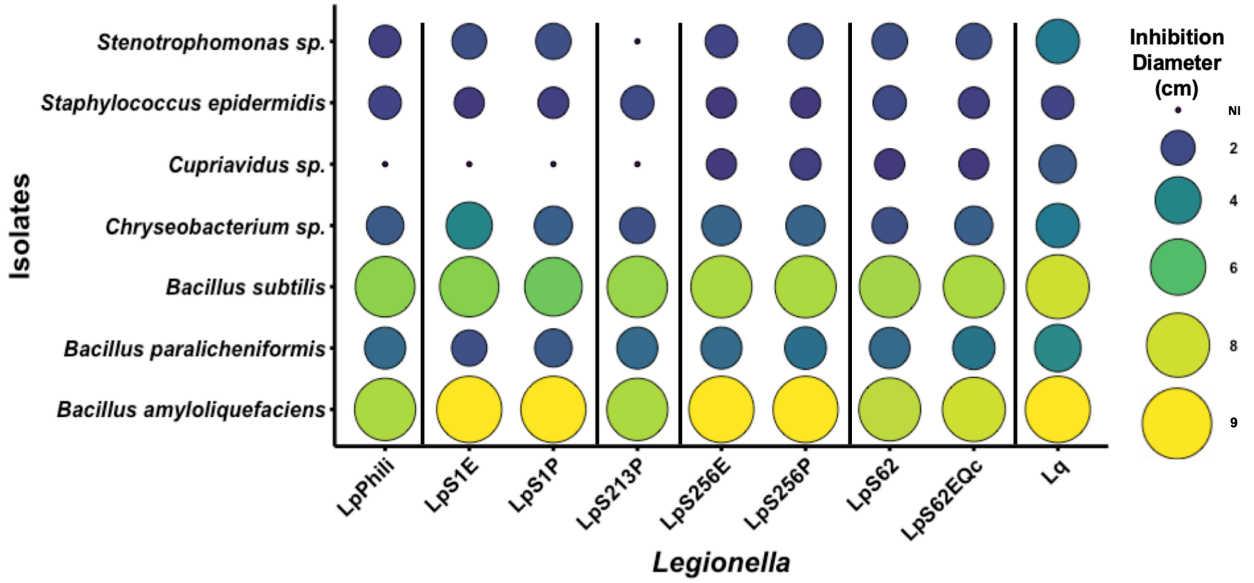

Figure 2: Inhibition zone diameters, in centimeter, produced by bacterial isolates on lawns of different strains of *Legionella* grown on CYE agar incubated at 30°C for 4 days. The inhibition zone diameter is characterized by the size of the circle and by colour scale (from purple to yellow; NI: No inhibition).

The results demonstrated that the inhibition of *L. pneumophila* varied according to the bacterial isolate tested. For instance, *B. amyloliquefaciens* and *B. subtilis* created large inhibition zones ranging from 7 cm to 9 cm (total inhibition). On the other hand, *Chryseobacterium sp.* and *B. paralicheniformis* both created intermediate inhibition zones, between 2.5 cm and 4 cm in diameter. The other isolates (*Cupriavidus sp.*, *S. epidermidis*, and *Stenotrophomonas sp.*) created small inhibition of around zones 2 cm or smaller.

Though we only tested one strain of *L. quinlivanii* (*Lq*), the results suggested that the species *pneumophila* was on average more resistant to the anti-*Legionella* activity of the bacterial isolates than the species *quinlivanii*. Indeed, *Lq* was more sensitive to most of the bacterial isolates,

showing larger inhibition zones than the other strains of *L. pneumophila*. For instance, the inhibition zone created by *Stenotrophomonas sp.* was around 4 cm for *Lq*, but only a 2 cm, or smaller, for the *L. pneumophila strain* (Fig. 2).

Finally, the results indicated that the resistance or susceptibility to the anti-*Legionella* bacterial isolate varied according to the SBT and the source from which the *L. pneumophila* strain was isolated from (Patient or Environment). Thus, the variation with SBT was most observable with the *Cupriavidus sp.* isolate, which inhibited around half of the *L. pneumophila* strain tested. As a result, SBT37 (LpPhili), SBT1 (LpS1E and P), and SBT213 (LpS213P) were resistant to *Cupriavidus sp.*, whereas, SBT256 (LpS256E and LpS256P) and SBT62 (LpS62EQc and LpS62) were susceptible, creating around 2 cm inhibition zones. In another example, *B. paralicheniformis* created an inhibition zone of between 2.2 to 2.5 cm for SBT1 (LpS1E and P), but the inhibition was between 3.1 to 3.3 cm for the other *L. pneumophila* strains.

In the case of the source of isolation, *Chryseobacterium sp.* created different sized inhibition zones for LpS1E and LpS1P. In this case, the environmental strain was more resistant, creating a 2.6 cm inhibition zone, than the patient isolated strain, which created a 4 cm zone.

2. TAXONOMIC CLASSIFICATION OF BACTERIAL ISOLATES

The genomes of the bacterial isolate, that could inhibit the different strains of *Legionella*, were sequenced and assembled, then uploaded to the Microbial Genome Atlas (MiGA) to deduce their taxonomy, with the exception of *B. amyloliquefaciens* (SPF474) (Rodriguez-R et al., 2018). Table 2 shows the results from this analysis. The taxonomic classification of four of the isolates could be identified with a high level of confidence. Indeed, SPF437, SPF476, SPF497 had average

nucleotide identities (ANI) above 99% with p-values below 0.05. The p-value indicates the probability of our query genome being wrongly classified with the reference genome from NCBI's RefSeq database (Rodriguez-R et al., 2018). Consequently, SPF437, SPF476, SPF497 were respectively classified as *Bacillus subtilis*, *Staphylococcus epidermidis*, and *Bacillus paralicheniformis*. The taxonomic classification of SPF498, SPF499 and SPF475 was less confident, as their ANI percentage varied between 86 % and 93 % at p-values sometimes above 0.05. Indeed, the MiGA results had a high probability that these strains belonged to the genera ascribed, as their p-values were below 0.05 when comparing at the genus level (SPF498 p-value = 0.0092 for *Stenotrophomonas*; SPF499 p-value = 0.012 for *Cupriavidus*; SPF475 p-value = 0.0097 for *Chryseobacterium*), but was less confident at the species level. Consequently, these strains are likely species not represented in the database.

Table 2: Taxonomic classification of the anti-*Legionella* isolates.

Strain number	Closest species (% ANI)	P-value at species level	Predicted Number of Proteins	Genome size (bp)	Source
SPF497	*Bacillus paralicheniformis* NZ CPO33389 (99.98%)	0.000	4,407	4,415,689	Cooling Tower MTL3 (Paranjape et al., 2020)
SPF498	*Stenotrophomonas spp.* MYb57 NZCP023271 (93.94%)	0.024	4,091	4,581,475	Cooling Tower MTL5 (Paranjape et al., 2020)
SPF499	*Cupriviadus pauculus* NZ CP033969 (86.84%)	0.095	6,397	6,854,167	Cooling Tower MTL3 (Paranjape et al., 2020)
SPF475	*Chryseobacterium indologenes* NZ_CP018786 (90.65% ANI)	0.053	4,971	5,302,653	Cooling Tower MTL5 (Paranjape et al., 2020)
SPF476	*Staphylococcus epidermidis* ATCC 12228 NC 004461 (99.36%)	0.001	2,422	2,530,472	Cooling Tower MTL3 (Paranjape et al., 2020)
SPF437	*Bacillus subtilis subsp inaquosorum* NZ_CP013984 (99.98% ANI)	0.000	4,123	4,195,215	Cooling Tower Model (Paniagua et al., 2019)

3. IDENTIFICATION OF PUTATIVE SECONDARY METABOLITES

In order to identify putative antimicrobial compounds produced by the different bacterial isolates, the assembled genomes were analysed using AntiSMASH server (Antibiotics and Secondary Metabolite Analysis Shell) (Blin et al., 2019). This tool allows the identification and

analysis of biosynthetic gene clusters (BGCs) within bacterial genomes. These BGCs have the potential to code for products that can have antimicrobial effects, such as antibiotics or bacteriocins (Blin et al., 2019). Table 3 represents a summary of the results obtained from AntiSMASH for the different bacterial isolates.

Overall, the genomes were found to contain several BGCs; however, the number in each isolate varied greatly from 3 to 16 BGCs. *B. paralicheniformis* and *B. subtilis* had the most BGCs and some were highly similar to BCGs producing known antimicrobial products. On the other hand, *Cupriavidus sp.* (SPF499) and *Chryseobacterium sp.* (SPF475) also possessed high number of BGCs, but they had low similarity levels to any of the antimicrobials gene products in the database. AntiSMASH only detected three BGCs for the *Stenotrophomonas* (SPF498) and *Staphylococcus* (SPF436).

Table 3: Biosynthetic gene clusters identified by AntiSMASH server in the different bacterial genomes

Strain Name	Number of BGCs	High similarity clusters	Low similarity clusters	Number of unidentified BGCs
Bacillus paralicheniformis	14	Fengycin (73%), Lichenysin (100%), Bacitracin (88%),	Bacilibactin (53%), Fengycin (23%), Butirosin (7%), Haloduracin (40%), Fengycin (20%)	6
Stenotrophomonas sp.	3	NA	Myxochelin (25%), APE Vf (35%)	1
Cupriavidus sp.	9	NA	Desferrioxamine (50%), APEVf (40%), WS9326 (12%)	6
Chryseobacterium sp.	12	NA	Desferrioxamine(50%), Flexirubin (52%), Flexirubin (22%), Caratenoid (28%)	8
Staphylococcus epidermidis	3	Staphyloferrin (100%)	NA	2
Bacillus subtilis	16	Subtilosin A (100%), Bacilysin (100%), Surfactin (82%), Bacillibactin (100%), Fengycin (80%), Sublancin (100%),	Plipastatin (53%), Zwittermycin (18%), Aurantinins (21%), Aurantinins (39%), Plipastatin (23%), Aurantinins (28%)	4

Moreover, AntiSMASH categorizes the BGCs into several types, such as bacteriocins and NRPS (Blin et al., 2019). We examined the diversity of the BGCs present throughout the different

genomes by counting the total sum of each type of BGC. The results showed that a total of 17 different types of BGCs could be identified at varying levels of confidence. These can be visualized in figure 3. Non-Ribosomal Peptides Synthetase (NRPS) were the most abundant antimicrobial clusters found in the different genomes, followed by Polyketide synthase (PKS), Terpenes, and Bacteriocins. Moreover, the other types of BGCs were found at abundance levels of less than 5 counts, and 7 BGCs (Lassopeptide, CDPS, Ladderane, phenazine, phosphonate, microviridin, and resorcinol) were only counted once.

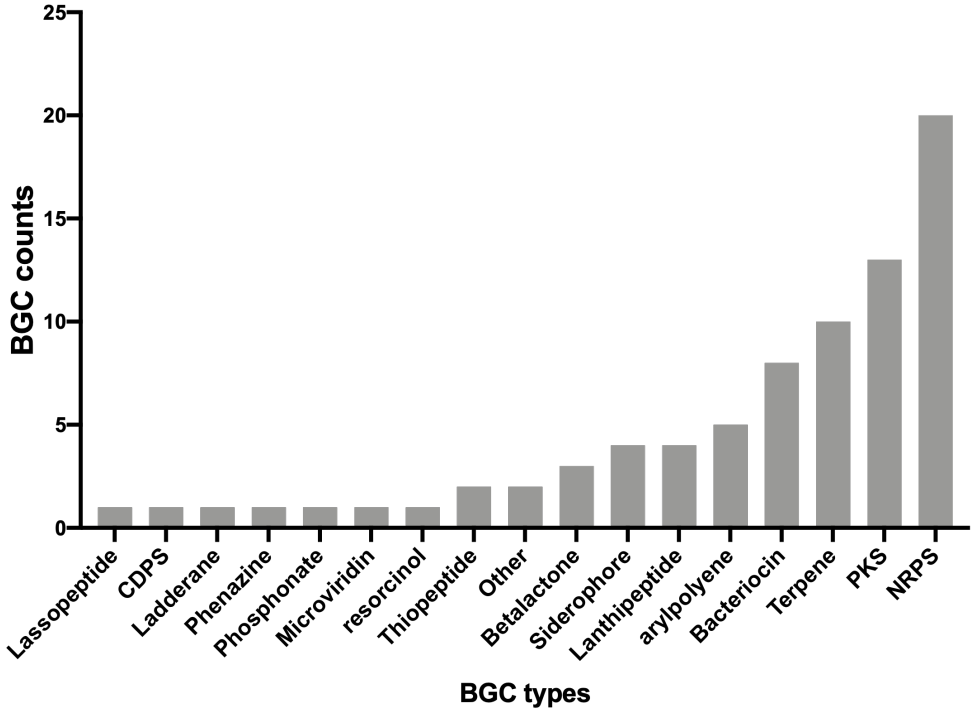

Figure 3: Diversity of BGC types identified in the genomes of the bacterial isolates using AntiSMASH.

IV- DISCUSSION

In this study, we isolated and characterized seven bacterial species from cooling tower water samples capable of inhibiting *L. pneumophila* on CYE plates. Their genomes were sequenced to get a better understanding of the potential antimicrobials that could be produced, and the genes associated with these antimicrobials. So far, research has shown that a wide variety of organism from EWS can inhibit *L. pneumophila*. However, few research articles have looked into isolating the inhibitory microorganisms and studying the genetics of their inhibition. As a result, the work done here is one of the first to use whole genome sequencing on bacterial species that inhibit *Legionella*.

First, the findings suggest that a wide variety of bacterial species can inhibit *L. pneumophila* in water systems. This diversity was shown by the fact that our isolates came from different bacterial phyla. Indeed, *Firmicutes*, *Bacteroidetes,* and *Proteobacteria* were all identified in this study, with most species belonging to the *Firmicutes*. This was in agreement with Corre et *al*, who showed that most of their anti-*Legionella* bacterial isolates belonged to these phyla (Corre et al., 2019). It is important to note that since we only tested inhibition on CYE growth medium, the actual diversity of inhibitory organisms may be underrepresented. Indeed, the number of unculturable microorganisms is far higher than the number of culturable organisms (Epstein, 2013; Rappé and Giovannoni, 2003). In our case, an obvious observation is that we most likely biased for the selection of non-fastidious mesophiles due to the incubation at 30°C on nutrient rich media. However, these growth conditions are a requirement for *L. pneumophila*. These restrictive growth conditions are a major hurdle to identify the full plethora of inhibitory organisms of *L. pneumophila*. Other studies, such as Amaro et *al* and Stewart et *al*, have used different methods to identify species that negatively interact with *L. pneumophila*, however their techniques were not

high throughput, and thus did not reveal the entire diversity and were time consuming (Amaro et al., 2015; Stewart et al., 2012). For instance, Amaro et *al* used a tedious *L. pneumophila*-protist co-cultivation method to isolate a few protozoan species that interacted with *Legionella* species, whereas, Stewart et *al* tested the persistence of *L. pneumophila* in biofilms generated from a few bacterial species. Consequently, the creation of new high throughput techniques that overcome the growth hurdle of *L. pneumophila* to show inhibition would increase the discovery of novel inhibitory organisms of the pathogen.

Second, the AntiSMASH results revealed that several bacterial isolates contained different genes coding for a wide variety of antimicrobials. Over 17 different BGC types were uncovered, suggesting *Legionella* could be inhibited through diverse mechanisms. Indeed, NRPS, PKS, terpenes, and bacteriocins were the most abundant antibacterial identified in the isolates. These compounds usually directly act on the bacterial target by inhibiting or killing it (Chakraborty et al., 2017; Mathur et al., 2017; Patil and Kumbhar, 2017; Zhang et al., 2014). On the other hand, the identification of several siderophore cluster could indicate that *L. pneumophila* may be inhibited through competition for nutrients. For instance, the staphyloferrin genes were identified in the *S. epidermidis* (SPF476) isolate. Staphyloferrin is a powerful siderophore used by *Staphylococcus* species and thus could prevent *L. pneumophila* from acquiring iron for growth (Konetschny-Rapp et al., 1990). As the growth media for *L. pneumophila* is supplemented with iron pyrophosphate, an interesting follow up experiment would be to test the inhibition with different concentration of iron supplementation. Potentially, lower levels of iron in the growth media would increase the inhibition zone created by *S. epidermidis* if siderophores are being used as competition mechanisms. Alternatively, the two others uncharacterized BGCs may be causing the inhibition or contribute in part to it. Recent findings have shown that *S. epidermidis* produces

a bacteriostatic peptide with anti-*Legionella* properties called Phenol-soluble modulin α or PSMα (Marchand et al., 2015; Marchand et al., 2011). The exact mechanisms of action of PSMα is not well understood, but it is believed that this antimicrobial peptides forms small pores in the *L. pneumophila* membrane (Marchand et al., 2011). Amino-acid substitution experiments showed that the biological function of PSMα could be altered through substitution as small as one amino-acid (Marchand et al., 2015). Interestingly, none of the BGCs from our *S. epidermidis* isolate were recognized as PSMα. This may be due to the fact that the database used by AntiSMASH does not contain this particular peptide sequence or that our isolates does not contain the genes for PSMα.

Moreover, some of the BGC sequences identified by AntiSMASH were previously shown to inhibit *L. pneumophila* on plate. For instance, Loiseau *et al* showed that surfactin produced by *B. subtilis* can create an inhibition zone on a lawn of *L. pneumophila* (Loiseau et al., 2015). However, in their case, the inhibition zone created by their strain was much smaller than our *B. subtilis* strain. Consequently, this could be due to either the use of a soft agar layer, which Loiseau did not use, allowing for better diffusion of the surfactin on the growth plate or that the strains isolated here produce more surfactin. Alternatively, the latter strain may contain other antimicrobials that could work in synergy to inhibit *L. pneumophila* growth. As shown in table 3, *B. subtilis* contained BGCs related to surfactin, bacilysin, and bacillibactin. Potentially these compounds could work in combination to inhibit *L. pneumophila*. Notably, bacilysin is an antibiotic, which works against a wide range of bacteria, and bacillibactin is a siderophore capable of chelating iron (Miethke et al., 2006; Rajavel et al., 2009). Of note, *B. paralicheniformis* was shown to contain lichenysin, a lipopeptide surfactant almost identical to surfactin (Loiseau et al., 2015; Yakimov et al., 1995). Lichenysin has been shown to have antibacterial activity with several species, such as *Acinetobacter sp*., *Bacillus sp.*, and *Pseudomonas sp.,* using agar diffusion tests,

but not in *Legionella* species (Yakimov et al., 1995). However, due to the high similarity of surfactin and lychenisin, it is of our opinion that this is most likely the compound that causes the inhibition of *L. pneumophila* on plate. Further, research is required to confirm this phenotype.

Several of the BGCs identified have very low similarity or did not relate to any known antimicrobial biosynthetic clusters from the MiBIG database used by AntiSMASH. For instance, the *Chryseobacterium sp.* (SPF475) contained several BGCs that were similar to desferrioxamine, flexirubin, and caratenoid, but at very low similarity levels (below 50%). The compounds produced by these isolates may be new variants of these antimicrobials or totally novel ones, suggesting that cooling towers are a rich source of novel and uncharacterized antimicrobial compound that could potentially be used for clinical or industrial purposes.

Our findings indicate that although *L. pneumophila* is ubiquitously found in water systems, it may encounter numerous antagonistic microorganisms in its environments. As these antagonistic species have co-evolved with *L. pneumophila* and other species, the mechanisms of their antagonisms may be more efficient and more specific than does of other bacterial species from other environments. Consequently, it could be hypothesized that if the number of antagonistic interactions outnumber the number of positive interactions, *L. pneumophila* would not be able to colonize, survive, and proliferate in that environment. As a proof of concept, Stewart et *al* demonstrated that *L. pneumophila* could not persist in monospecies biofilms of *P. aeruginosa*, potentially due to the production of homoserine lactones (Stewart et al., 2012). As a result, a potential solution to inhibit *L. pneumophila* in EWS would be to promote these antagonistic bacteria by creating conditions in water system where they would thrive. These control approaches have been suggested in the past and have been qualified as probiotic approaches (Wang et al., 2013). One possible way to do this would be to inoculate the antagonistic bacteria within the EWS.

This could be done with *B. paralicheniformis* as it is not known as a human pathogen and produces lichenysin, a surfactant which could help with biofilm removal. Another possible option would be to select for the antagonistic organisms by manipulating the physicochemical parameters that would allow the antagonistic species of *L. pneumophila* to thrive. Recent work has shown that continuous application of chlorine could be used to shape the microbial community of cooling towers to make it non-permissive to *L. pneumophila* colonization (Paranjape et al., 2020). Indeed, continuous application of chlorine was associated with the establishment of a *Pseudomonas* population in the towers that correlated negatively with the *Legionella* community (Paranjape et al., 2020). However, further research is required to better understand this process.

One possible difficulty raised by our study is that the genetics of different species of *Legionella* or different strains of *L. pneumophila* could be an issue. As we demonstrated, different SBTs were resistant, to different degrees, to various bacterial isolates. Thus, a potential drawback maybe the selection of more resistant *L. pneumophila* strains to the antagonistic species used when creating a very hostile environment. This could produce a dangerous situation by creating naturally resistant *L. pneumophila* strains better efficient at resisting the normal microbiota of water systems.

Finally, our results reveal an important problem regarding policy measures that require monitoring of the *L. pneumophila* levels in water systems. As mentioned before, culture based detection methods are still widely used for surveillance programs and are sometimes referred to as the gold standard of detection methods (Mercante and Winchell, 2015; Peci et al., 2016). These methods usually treat the sample with heat and acid treatment then plate the sample on BCYE and incubate at 37°C for 3 to 5 days (Bopp et al., 1981). After incubation, the bacterial colonies that grow and resemble *L. pneumophila* colonial morphology are enumerated. These methods are important for sequence base typing as they allow to type each colony that is suspected to be *L.*

pneumophila. Our findings seem to give further weight to the argument that culture based methods are at increased risk of false negative, especially in the case of environmental samples. The higher levels of inhibitory bacteria increase the chance of underreporting the actual number of *Legionella* due inhibition on plate, especially if total inhibition species are present. This can be problematic as managers of water systems may believe their systems are free of *Legionella*, but this may be far from the truth.

V- CONCLUSION

In conclusion, several bacterial isolates that showed anti-*Legionella* activity were isolated from different cooling towers water samples. This study confirms that cooling towers can harbour a diversity of anti-*Legionella* bacterial species from different phyla. Furthermore, the diversity of different antimicrobial cluster genes was observed by sequencing the genome of these isolates. The results revealed that several known antimicrobials were potentially causing the inhibition of *L. pneumophila* on plate; however, the number of uncharacterized antimicrobials was much greater. This would suggest that there is an untapped pool of potential antimicrobials that could be further studied either for clinical or industrial purposes. Moreover, the findings reveal that though *L. pneumophila* is ubiquitously found in water systems, it must still compete with high number of bacteria, suggesting that the microbiome of the system plays an integral part in its survival and proliferation. As a counterbalance, different strains of *L. pneumophila* can be better adapted to the antagonistic interactions of the inhibitory bacteria. Finally, our results reinforce the notion that inhibitory bacteria may be an important source of producing false negative results when detecting *L. pneumophila* through culture-based methods.

VI- REFERENCE

Amaro, F., Wang, W., Gilbert, J.A., Anderson, O.R. and Shuman, H.A. 2015. Diverse protist grazers select for virulence-related traits in Legionella. The ISME journal 9(7), 1607.

Andrews, S. 2010 FastQC: a quality control tool for high throughput sequence data, Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom.

Benedict, K.M., Reses, H., Vigar, M., Roth, D.M., Roberts, V.A., Mattioli, M., Cooley, L.A., Hilborn, E.D., Wade, T.J., Fullerton, K.E., Yoder, J.S. and Hill, V.R. 2017. Surveillance for Waterborne Disease Outbreaks Associated with Drinking Water - United States, 2013-2014. MMWR. Morbidity and mortality weekly report 66(44), 1216-1221.

Blin, K., Shaw, S., Steinke, K., Villebro, R., Ziemert, N., Lee, S.Y., Medema, M.H. and Weber, T. 2019. antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. Nucleic acids research.

Boamah, D.K., Zhou, G., Ensminger, A.W. and O'Connor, T.J. 2017. From many hosts, one accidental pathogen: the diverse protozoan hosts of Legionella. Frontiers in cellular and infection microbiology 7, 477.

Bolger, A.M., Lohse, M. and Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30(15), 2114-2120.

Bopp, C., Sumner, J., Morris, G. and Wells, J. 1981. Isolation of Legionella spp. from environmental water samples by low-pH treatment and use of a selective medium. Journal of Clinical Microbiology 13(4), 714-719.

Borella, P., Guerrieri, E., Marchesi, I., Bondi, M. and Messi, P. 2005. Water ecology of Legionella and protozoan: environmental and public health perspectives. Biotechnology annual review 11, 355-380.

Cassell, K., Gacek, P., Rabatsky-Ehr, T., Petit, S., Cartter, M. and Weinberger, D.M. 2019. Estimating the True Burden of Legionnaires' Disease. American Journal of Epidemiology 188(9), 1686-1694.

Chakraborty, K., Thilakan, B., Raola, V.K. and Joy, M. 2017. Antibacterial polyketides from Bacillus amyloliquefaciens associated with edible red seaweed Laurenciae papillosa. Food chemistry 218, 427-434.

Corre, M.-H., Delafont, V., Legrand, A., Berjeaud, J.-M. and Verdon, J. 2019. Exploiting the Richness of Environmental Waterborne Bacterial Species to Find Natural Legionella pneumophila Competitors. Frontiers in Microbiology 9(3360).

Cunha, B.A., Burillo, A. and Bouza, E. 2016. Legionnaires' disease. The Lancet 387(10016), 376-385.

Danila, R.N., Koranteng, N., Como-Sabetti, K.J., Robinson, T.J. and Laine, E.S. 2018. Hospital water management programs for Legionella prevention, Minnesota, 2017. infection control & hospital epidemiology 39(3), 336-338.

ECDC 2019. Legionnaires' Disease. ECDC Annual epdimilogical report for 2017.

Epstein, S.S. 2013. The phenomenon of microbial uncultivability. Current Opinion in Microbiology 16(5), 636-642.

Guerrieri, E., Bondi, M., Sabia, C., de Niederhäusern, S., Borella, P. and Messi, P. 2008. Effect of Bacterial Interference on Biofilm Development by Legionella pneumophila. Current Microbiology 57(6), 532-536.

Juda, T. 2009. Legionella-experience of the Provincial Specialist Hospital in Jastrzębiu Zdrój-clinical cases. Nowa Medycyna 16(1).

Kimura, S., Tateda, K., Ishii, Y., Horikawa, M., Miyairi, S., Gotoh, N., Ishiguro, M. and Yamaguchi, K. 2009. Pseudomonas aeruginosa Las quorum sensing autoinducer suppresses growth and biofilm production in Legionella species. Microbiology 155(6), 1934-1939.

Konetschny-Rapp, S., Jung, G., Meiwes, J. and Zähner, H. 1990. Staphyloferrin A: a structurally new siderophore from staphylococci. European journal of biochemistry 191(1), 65-74.

Lau, H. and Ashbolt, N. 2009. The role of biofilms and protozoa in Legionella pathogenesis: implications for drinking water. Journal of applied microbiology 107(2), 368-378.

Lévesque, S., Plante, P.-L., Mendis, N., Cantin, P., Marchand, G., Charest, H., Raymond, F., Huot, C., Goupil-Sormany, I. and Desbiens, F. 2014. Genomic characterization of a large outbreak of Legionella pneumophila serogroup 1 strains in Quebec City, 2012. PLoS One 9(8), e103852.

Loiseau, C., Schlusselhuber, M., Bigot, R., Bertaux, J., Berjeaud, J.-M. and Verdon, J. 2015. Surfactin from Bacillus subtilis displays an unexpected anti-Legionella activity. Applied microbiology and biotechnology 99(12), 5083-5093.

Marchand, A., Augenstreich, J., Loiseau, C., Verdon, J., Lecomte, S. and Berjeaud, J.-M. 2015. Effect of amino acid substitution in the staphylococcal peptides warnericin RK and PSMα on their anti-Legionella and hemolytic activities. Molecular and Cellular Biochemistry 405(1), 159-167.

Marchand, A., Verdon, J., Lacombe, C., Crapart, S., Hechard, Y. and Berjeaud, J. 2011. Anti-Legionella activity of staphylococcal hemolytic peptides. Peptides 32(5), 845-851.

Mathur, H., Field, D., Rea, M.C., Cotter, P.D., Hill, C. and Ross, R.P. 2017. Bacteriocin-antimicrobial synergy: a medical and food perspective. Frontiers in microbiology 8, 1205.

Mercante, J.W. and Winchell, J.M. 2015. Current and emerging Legionella diagnostics for laboratory and outbreak investigations. Clinical microbiology reviews 28(1), 95-133.

Miethke, M., Klotz, O., Linne, U., May, J.J., Beckering, C.L. and Marahiel, M.A. 2006. Ferri-bacillibactin uptake and hydrolysis in Bacillus subtilis. Molecular microbiology 61(6), 1413-1427.

National Academies of Sciences, E. and Medicine (2019) Management of Legionella in Water Systems, The National Academies Press, Washington, DC.

Nurk, S., Bankevich, A., Antipov, D., Gurevich, A.A., Korobeynikov, A., Lapidus, A., Prjibelski, A.D., Pyshkin, A., Sirotkin, A. and Sirotkin, Y. 2013. Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. Journal of Computational Biology 20(10), 714-737.

Paniagua, A.T., Paranjape, K., Hu, M., Bédard, É. and Faucher, S. 2019. Impact of temperature on Legionella pneumophila, its protozoan host cells, and the microbial diversity of the biofilm community of a pilot cooling tower. Science of The Total Environment, 136131.

Paranjape, K., Bédard, É., Whyte, L.G., Ronholm, J., Prévost, M. and Faucher, S.P. 2020. Presence of Legionella spp. in cooling towers: the role of microbial diversity, Pseudomonas, and continuous chlorine application. Water Research 169, 115252.

Parr, A., Whitney, E.A. and Berkelman, R.L. 2015. Legionellosis on the Rise: A Review of Guidelines for Prevention in the United States. J Public Health Manag Pract 21(5), E17-E26.

Patil, P.S. and Kumbhar, T.S. 2017. Antioxidant, antibacterial and cytotoxic potential of silver nanoparticles synthesized using terpenes rich extract of Lantana camara L. leaves. Biochem Biophys Rep 10, 76-81.

Peci, A., Winter, A.-L. and Gubbay, J.B. 2016. Evaluation and comparison of multiple test methods, including real-time PCR, for Legionella detection in clinical specimens. Frontiers in Public Health 4, 175.

Phin, N., Parry-Ford, F., Harrison, T., Stagg, H.R., Zhang, N., Kumar, K., Lortholary, O., Zumla, A. and Abubakar, I. 2014. Epidemiology and clinical management of Legionnaires' disease. The Lancet infectious diseases 14(10), 1011-1021.

Québec, G.d. 2014. Décret 454-2014 Loi sur le Bâtiment. Gazette Officielle du Québec 146(22), 1923-1927.

Rajavel, M., Mitra, A. and Gopal, B. 2009. Role of Bacillus subtilis BacB in the synthesis of bacilysin. J Biol Chem 284(46), 31882-31892.

Rappé, M.S. and Giovannoni, S.J. 2003. The uncultured microbial majority. Annual Reviews in Microbiology 57(1), 369-394.

Rodriguez-R, L.M., Gunturu, S., Harvey, W.T., Rosselló-Mora, R., Tiedje, J.M., Cole, J.R. and Konstantinidis, K.T. 2018. The Microbial Genomes Atlas (MiGA) webserver: taxonomic and gene diversity analysis of Archaea and Bacteria at the whole genome level. Nucleic acids research 46(W1), W282-W288.

Standard, A. 2015. Standard 188-2015. Legionellosis: Risk Management for Building Water Systems.

Stewart, C.R., Muthye, V. and Cianciotto, N.P. 2012. Legionella pneumophila persists within biofilms formed by Klebsiella pneumoniae, Flavobacterium sp., and Pseudomonas fluorescens under dynamic flow conditions. PloS one 7(11), e50560.

van Heijnsbergen, E., Schalk, J.A., Euser, S.M., Brandsema, P.S., den Boer, J.W. and de Roda Husman, A.M. 2015. Confirmed and potential sources of Legionella reviewed. Environmental science & technology 49(8), 4797-4815.

Wang, H., Edwards, M.A., Falkinham III, J.O. and Pruden, A. 2013. Probiotic approach to pathogen control in premise plumbing systems? A review. Environmental science & technology 47(18), 10117-10128.

Yakimov, M.M., Timmis, K.N., Wray, V. and Fredrickson, H.L. 1995. Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface Bacillus licheniformis BAS50. Appl. Environ. Microbiol. 61(5), 1706-1713.

Zhang, J., Du, L., Liu, F., Xu, F., Hu, B., Venturi, V. and Qian, G. 2014. Involvement of both PKS and NRPS in antibacterial activity in Lysobacter enzymogenes OH11. FEMS microbiology letters 355(2), 170-176.

CHAPTER 6. THESIS DISCUSSION

I- INTRODUCTION

Understanding the ecology of *Legionella pneumophila* is of great importance in order to better control the outbreaks of Legionnaires' disease (LD), a severe pneumonia. *L. pneumophila* is a bacterial species ubiquitously found in water systems, where it grows and proliferates, and is the causative agent of LD. Cooling towers are one of the most important sources of large outbreaks of LD. However, the exact mechanisms of how *L. pneumophila* survives and grows in these environments is still not well understood. Several chemical and physical parameters have been identified to correlate with *L. pneumophila* levels, however they are far from perfect correlations. As a result, it is believed that other factors are pivotal for *L. pneumophila* ecology. *L. pneumophila* is an intracellular parasite of different species of protozoa, such as amoeba and ciliates, and requires these species to proliferate in nature. These host species are usually microbial grazers that feed on the microbial populations in their ecosystems. Consequently, the growth of the host population, and indirectly the *L. pneumophila* population, is dependent on the prey population. In addition, several bacterial species have demonstrated ecological interactions (either negative or positive) with the *L. pneumophila* populations. This leads to our hypothesis that the survival and proliferation of *L. pneumophila* in cooling towers is dependent on the microbiome of these ecosystems along with certain physicochemical parameters. Our objectives were as follows:

1. Characterize the bacterial and eukaryotic community of 18 cooling towers and their relationship with *L. pneumophila* ecology.
2. Identify the factors that shape these communities and understand how these factors can affect *L. pneumophila* ecology.

3. Identify biomarkers that could potentially lead to predicting the presence or absence of *L. pneumophila* in cooling towers
4. Isolate, identify, and characterize species that can affect *L. pneumophila* either negatively or positively.

Altogether, the work done here supports the original hypothesis that certain groups of microorganisms are crucial for the colonization, survival, and proliferation of *L. pneumophila* in the cooling tower environment. Indeed, our results indicated that several microbial taxa were either predictive of the presence of *Legionella* or *L. pneumophila* in cooling towers, or positively correlated with the *Legionella* counts. For instance, *Porphyrobacter*, *Sediminibacterium*, *Yonghaparkia*, *Hyphomicrobium*, and *Brevundimonas* could predict the presence of the genus *Legionella* in the towers, whereas, *Xanthobacteraceae*, *Obscuribacteriales*, *Qipengyuania*, *Cloacibacterium*, and *Mycobacterium* could predict the presence of *L. pneumophila* in the towers, when using LEfSe. On the other hand, *Brevundimonas, Gemmatimonas, Peredibacters, Flavobacterium, Algoriphagus, Cellvibrio*, and *Cytophaga* were all positively correlated with *Legionella* counts. These positive associations between these taxa and the *Legionella* spp. population is potentially due to two reasons: 1) these taxa engage in mutualistic interactions with the *Legionella* spp. population, benefiting both populations through direct or indirect interactions, and 2) specific characteristics of the environment, such as physicochemical parameters, could be favouring the proliferation of both populations.

II- IMPORTANCE OF *BREVUNDIMONAS* ON *LEGIONELLA* ECOLOGY

Our experiments with the *Brevundimonas* sp. isolate supproted that some of the associations between the predictive or the positively correlated microbial taxa with *Legionella* or *L. pneumophila* could be due to mutualistic interactions. Indeed, *Brevundimonas* would bring nutritional benefits to *L. pneumophila* by providing a source of cysteine. In exchange, L. pneumophila would protect Brevundimonas against host cells by infecting them.

Additional experiments are needed to uncover the exact molecular mechanisms by which the stimulation occurs. Whole genome sequencing of this isolate identified genes important for converting L-cystine into L-cysteine. L-cystine is the oxidized version of L-cysteine found in non-supplemented CYE that cannot be used by *L. pneumophila* (Ewann and Hoffman, 2006). Accordingly, uncovering the exact gene sequences and regulatory elements, that are associated with these genes, in order to do knockout assays would be an interesting set of experiment to further understand this phenomenon. The annotation of the genome using PROKKA and BlastKoala revealed that the *Brevundimonas* strain contained sequences similar to cysteine-S-conjugate-beta-lyase, cysteine synthase, and cystathionine gamma-lyase, enzymes that can convert L-cystine to L-cysteine or other cysteine by-products (Cooper and Pinto, 2006). Therefore, an interesting follow up experiment would be to produce deletion mutants of *Brevundimonas sp.* to test if these genes are involved in stimulating growth of *L. pneumophila* on CYE without L-cysteine supplementation. This might be impossible to produce these mutant strains as these genes may be essential for the bacterium to grow. Thus, a second option would be to directly transform these sequences into *L. pneumophila* strains, using a plasmid vector with an inducible promoter, and then to test whether or not the resulting strains would grow on CYE without L-cysteine supplementation.

Moreover, as the stimulation assay was tested on nutrient rich growth media (CYE), an obvious follow up experiment would be to test if this stimulation occurs in water systems, such as in the bulk water phase or the biofilm phase. Koide et *al* have shown that strains of *Brevundimonas vesicularis* could promote the growth of *L. pneumophila* and *L. micdadei* in phosphate buffer with artificial sand and 0.001 % glucose (Koide et al., 2014). Since they used a liquid medium, their experiment may represent the interaction of *L. pneumophila* and *Brevundimonas sp.* in the bulk water phase in a typical EWS. However, a point of contention is that the medium used for the experiment may not represent adequately the liquid phase of water systems. Indeed, the authors used phosphate buffer and different concentration of glucose, which would have much higher levels of phosphate and glucose than what is normally found in the supply systems of EWSs. Thus, this experiment could be recreated but using an experimental liquid medium that is more representative of EWSs environments. For instance, sterilized cooling tower water could be used with an adequate carbon source.

Interestingly, when Koide et *al* tested with higher concentrations of glucose (0.1% and 0.01%), the *Legionella* counts decreased significantly, suggesting that nutrient levels may affect the relationship between *L. pneumophila* and *Brevundimonas* sp. in the bulk water (Koide et al., 2014). In their case, lower levels of nutrients were required for positive interactions between the two bacteria, whereas, higher nutrient conditions did not seem to favour *L. pneumophila* in the bulk water. However, this is opposite to our results, where *L. pneumophila* was stimulated on rich media (CYE). This could be due to differences in nutrients used for each media. Indeed, CYE is composed of yeast extract and ACES buffer, but no glucose. Thus, this would indicate that the type of nutrients and their concentrations could potentially affect the relationship between *Brevundimonas sp.* and *L. pneumophila*.

This is further substantiated by the fact that *L. pneumophila* prefers using amino acids as its carbon source rather than glucose, even though the bacterium contains all the genes necessary for glycolysis (Eisenreich and Heuner, 2016). This may be an important factor for *L. pneumophila* colonization of biofilms when *Brevundimonas sp.* species are present. Certain species of *Brevundimonas* are known to produce biofilms, such as *B. vesicularis* (Verhoef et al., 2002). Biofilms are heterogenous in the type and concentration of nutrients present (Declerck, 2010; Flemming and Wingender, 2010). Microregions are generated within biofilms due to the distribution of nutrients. Hypothetically, *Brevundimonas* sp. could stimulate the growth of *L. pneumophila* in these microregions depending on the nutrients available and their concentrations. To test this hypothesis, the survival or proliferation rates of *L. pneumophila* in mono-species biofilms with *Brevundimonas* could be investigated.

Moreover, the direct interaction between *Brevundimonas* and *L. pneumophila* suggest that this mutualistic species is probably an important factor for the initial colonization and survival of the cooling tower environment by *L. pneumophila*. Indeed, towers are harsh environments with low levels of nutrients and contain harsh chemicals and biocides. Towers with a higher population of *Brevundimonas* may increase survival, promote better health, and stimulate a small amount of growth, through nutritional support, of the initial *L. pneumophila* colonizing population than towers without a population of *Brevundimonas*. Indeed, *L. pneumophila* represses various metabolic, transcriptional, and translational machinery in order to enter a dormant state when it is exposed to water (Li et al., 2015). This is partly due to the lowered nutrient content of water, which causes the activation of a stress response in the bacterium (Li et al., 2015). Overtime, stresses will damage cellular components. *L. pneumophila* can withstand long periods in this state, however this is highly dependent on the physicochemical parameters, such as pH and temperature, of the

environment (Mendis et al., 2015). Consequently, mutualistic interactions, such as the one with *Brevundimonas*, may help *L. pneumophila* survive better in these conditions. The nutritional supplementation may help or promote the regeneration of damaged cellular components, which are acquired during long periods of stress.

Brevundimonas may also be an important factor for proliferation of *L. pneumophila* when the host cell population establishes itself in the cooling tower environment. Indeed, the initial population size and cellular physiology are important factors for growth of bacterial cultures. Thus, bacterial populations grow faster when their initial population is higher and when cells are at a better physiological state. By nutritional supplementation, towers containing *Brevundimonas* may increase the physiological activity of *L. pneumophila* cells and increase the numbers of the initial colonizing *L. pneumophila* population. This would lead to faster growth of the *L. pneumophila* population when a substantial host cell population is present in the cooling tower environment.

Finally, the dormant form of *L. pneumophila*, when exposed to water, is similar to the bacterium's MIF (Eisenreich and Heuner, 2016; Garduno et al., 2002; Li et al., 2015). MIF *L. pneumophila* is reached at the end of its intracellular lifecycle and is reported as a more infectious form than other phases of the bacterium's life cycle, such as exponential phase (Garduno et al., 2002). This increased infectivity is due to the upregulation of several virulence factors brought on by depletion of nutrients of the environment (Garduno et al., 2002). These same traits are observed when *L. pneumophila* is exposed to water and enters the dormant phase, with virulence factors such as flagellar genes being upregulated and increased resistance to antibiotics being observed (Li et al., 2015). As a result, an interesting experiment to pursue would to study if or how *Brevundimonas* could affect this dormant form. Hypothetically, the supplementation of nutrients may shift *L. pneumophila* cells to more active phase, such a pre-exponential or exponential phase.

This may reduce the upregulation of certain virulence factors causing the *L. pneumophila* cells to be less infectious. This may be an important factor for outbreaks, as the presence of *Brevundimonas* would theoretically increase survival of *L. pneumophila* but potentially lower infectivity. However, more research must be done in order to better understand this question.

III- IMPORTANCE OF INDIRECT INTERACTIONS OF *BREVUNDIMONAS* ON *LEGIONELLA* ECOLOGY

The results from our work reveal that species associated with *Legionella* could promote the growth of *L. pneumophila* through indirect interactions with the *L. pneumophila* host population. In this case, the *Brevundimonas sp.* isolate demonstrated to be a food source for *T. pyriformis* and *T. thermophila*, ciliate species of the *Olygohymenophorea* class that are hosts of *L. pneumophila*. By consuming the *Brevundimonas sp.* isolate, these two ciliates species were able to proliferate in co-culture experiments with *Brevundimonas sp*. Since *L. pneumophila* is an intracellular parasite of protozoa, the establishment and growth of these population is a necessity. Consequently, *Brevundimonas* would be a potential keystone species for the ciliate population, as it would allow their establishment and proliferation. This would benefit the *L. pneumophila* population, as more host would generate more bacterial growth. Consequently, this could be another explanation for the positive correlation between *Legionella* and *Brevundimonas*. Our findings reinforce the notion that a better understanding of the ecology of the prey community is necessary to properly understand the ecology of *L. pneumophila*. In particular, special attention should be given in identifying and characterizing the prey of the host species, as they are key factors for host population establishment in water systems.

IV- IMPORTANCE OF THE HOST AND PREY COMMUNITY

As *L. pneumophila* requires the presence of host species and host species require the presence of prey species, this suggests that proliferation of the *L. pneumophila* population requires the ecological succession of different microbial populations.

In this scenario, we can imagine that clean and newly installed cooling towers will get colonized by different populations of microbes over time. An important first step would be the establishment of a prey population in the cooling tower. This would require adequate growth conditions for these species, such as correct physicochemical parameters and few negatively interacting microbial species. Subsequently, the prey population must proliferate to high enough numbers so that the host population can feed and grow. This would allow the establishment of the host population, which could lead to proliferation of the *L. pneumophila* population. The host population must reach a certain level so that enough *L. pneumophila* can grow to dangerous levels to cause an outbreak.

Following this logic, an interesting prospective project would be to model the succession of microbial population that lead to the proliferation of *L. pneumophila* in cooling towers. Indeed, a succession model could help us predict future outbreaks as well as discovering important principles about microbial succession in other ecosystems. A potential method would be to characterize the microbial community of different cooling towers over a period of time and to discern any patterns occurring in the succession of the microbial community that would lead to an increase of *L. pneumophila*. The use of ribosomal marker amplicon sequencing may be appropriate. A pattern recognition algorithm could be used to identify any patterns that would lead to the proliferation of *L. pneumophila*. For instance, Tromas et *al* have used symbolic regression to predict when cyanobacterial blooms in lakes occur with OTU data (Tromas et al., 2017). This

method could be applied to *Legionella* outbreak data. On the other hand, a metagenomic approach could give us a more holistic view of microbial succession and thus finetune the predictive *L. pneumophila* outbreak model. This would be done by identifying the taxonomy of the microbial communities, at the species level, and the genes associated with each successive microbial community. By sequencing all of the genes in a sample, specific functions of each microbial population can be determined. Thus, metabolic functions could be of interest, as metabolic genes can give insights into the environmental conditions of the cooling tower. This could potentially give us information on environmental changes that are permissive to *L. pneumophila* proliferation. For instance, higher levels of RubisCo gene may indicate higher levels of photosynthetic organisms (Giri et al., 2004). This would imply that a high level of primary production is occurring and potentially that the systems contain higher numbers of prey species. Sunlight can penetrate the inside of a cooling tower as these systems are not closed, for example trough air vents.Furthermore, the metagenomic approach may increase the number of genetic markers that could predict *L. pneumophila* proliferation. These biomarkers could be used to increase the consistency and robustness of current *L. pneumophila* surveillance programs.

Moreover, our data suggested that ciliates may play a more important role in *Legionella* ecology. Our sequencing results revealed that the *Oligohymenophorea* class was the most prevalent ciliate taxa in the cooling towers. However, due to the lack of discriminatory power of the region used, the richness of this population was not fully observed. Most of the ciliate sequences could only be discriminated down to the *Oligohymenophorea* class and not to lower ranks. Thus, using primers with better discriminatory power for the ciliate community may be of interest to further study this population. This would give a better picture of the species present in cooling towers. Additionally, research has shown that certain species of amoeba are able to

consume *L. pneumophila* for nutrition (Amaro et al., 2015). Consequently, this may also be true for the ciliate population. Isolating and characterizing these species may be of interest as they could be used as a probiotic approach to create an inhospitable environment for *L. pneumophila*. An added advantage of using ciliates would be that they are much faster acting than amoeba, and thus would reduce *L. pneumophila* content much quicker.

Finally, owing to the fact that our data revealed several taxa were positively correlated, it is imperative to better understand how these different organisms may promote the growth of L. pneumophila in the context of outbreaks of LD. As mentioned previously, several of these positively associated bacterial and eukaryotic taxa are uncharacterized and thus require further investigation in how they can benefit *L. pneumophila* or its hosts. The benefits for *L. pneumophila* may be through direct interactions, such as supplementation of nutrients, however, the literature suggests that several of these organisms may benefit *L. pneumophila* through indirect mechanisms. For instance, *Ca.* Paracaedibacter was identified in the same module as *Legionella* according to the network analysis. This organism is an endosymbiont of *Acanthamoeba* species (Heinz et al., 2007). Consequently, *Ca*. Paracaedibacter is more likely an indicator of conditions that allow the establishment of *Acanthamoeba* species. Furthermore, this demonstrates that the correlations or predictions between *Legionella* and the other taxa may be due to common characteristics of the environment that benefit both members. On the other hand, certain positive correlations are potentially due to taxa negatively interacting with taxa that negatively interact with *L. pneumophila*. For instance, *Peredibacter* was positively correlated with *Legionella* and is a predator of *Pseudomonas* species (Davidov and Jurkevitch, 2004). Consequently, *Peredibacter* could reduce the *Pseudomonas* population. As *Pseudomonas* species can inhibit *Legionella* species, this would reduce antagonistic interactions with *Legionella* community. As a result, the

Legionella community could thrive along with the *Peredibacter* community, hence the positive correlation between these two taxa. Thus, several other organisms may benefit the *L. pneumophila* community through direct and indirect interactions. Further research should focus on characterizing these interactions, as these organisms may be potential biomarkers. In addition, this concept could be applied to the prey and host community. Indeed, research into positively interacting organisms with prey and host species may improve our knowledge into how these two populations establish themselves in the cooling tower environment.

V- NEGATIVE INTERACTING ORGANISMS WITH *LEGIONELLA*

Several taxa were negatively associated with *Legionella* and *L. pneumophila*. The *Pseudomonas* community was an example of this, as it negatively correlated with the *Legionella* community and was a predictor of the absence of *Legionella* in the cooling towers, when using LEfSe (Paranjape et al., 2020). Our findings suggested that this negative association was due to direct inhibition of the *Legionella* community and indirect mechanisms, that affected the prey and host communities, by the *Pseudomonas* community. This was supported by the fact that species of *Pseudomonas* have been demonstrated to inhibit several bacterial species, including *Legionella* species (such as *L. pneumophila*), as well as, certain host species (Corre et al., 2019; Guerrieri et al., 2008; Kimura et al., 2009; Matz et al., 2008; Stewart et al., 2012). Consequently, an approach to reduce the levels of *Legionella* species in cooling towers could be to use the inhibitive properties of the *Pseudomonas* community. This could be done through a prebiotic approach by creating the conditions necessary for the establishment and proliferation of the *Pseudomonas* species that inhibit *Legionella*, or through a probiotic approach, by directly inoculating these species in the cooling towers. However, this would require identifying the *Pseudomonas* species capable of

inhibiting *L. pneumophila*. Indeed, not all *Pseudomonas* species can inhibit *L. pneumophila* and some can even help it thrive (Stewart et al., 2012). Furthermore, several species are human pathogens and would be inappropriate to use as probiotics.

Our study revealed that several other bacterial isolates were able to inhibit *L. pneumophila* on agar plate. The isolated species were identified as *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Cupriavidus sp.*, *Stenotrophomonas sp.*, *Chryseobacterium indologenes*, *Staphylococcus epidermidis*, and *Bacillus subtilis*. Whole genome sequencing revealed that a number of different antimicrobial genes were present in the genomes of the different isolates. These antimicrobial gene clusters could be grouped into products that could potentially directly damaged the *L. pneumophila* cells, such as harmful proteins, bacteriocin, or antimicrobial fatty acids, as well as, elements that could advantage nutritional competition, such as siderophores. However, further research is required to properly identify the anti-*Legionella* compounds produced by these isolates. As some of these antimicrobials have not been characterized yet, they may prove valuable for industrial or research purposes. Additionally, as the anti-*Legionella* effect was only tested on agar, future projects should test if these isolates are capable of inhibiting *L. pneumophila* in water systems, such as in biofilms or the bulk water phase of cooling towers.

VI- COOLING TOWER MICROBIOME PERMISSIVENESS TO *LEGIONELLA*

In a more global perspective, these positively and negatively interacting organisms reveal that the permissiveness of the microbiome of cooling towers for *L. pneumophila* survival and proliferation can be described on a spectrum. Consequently, we can hypothesize that the more mutualistic species are present, the more the microbiome is permissive towards *L. pneumophila*.

However, the more antagonistic species are present, the less permissive the microbiome will be towards *L. pneumophila*. We can further divide this spectrum into three groups. Thus, the first group would contain the microbiomes that promote the growth of *L. pneumophila* and would contain a high-level of mutualistic species and low-level of antagonistic species. Extrapolating from our results, this microbiome would contain high levels of *Brevundimonas*, ciliates, and other microbial predictor of the presence of *Legionella,* such as *Porphyrobacter*, *Sediminibacterium*, *Yonghaparkia*, and *Hyphomicrobium*. The second group would contain microbiomes that are more neutral, thus mainly allowing survival of *L. pneumophila*, with very little to no inhibition or proliferation. These microbiomes would contain species that did not interact with *L. pneumophila* or *Legionella* and no or very few susceptible host cells. The third group would contain microbiomes that inhibit *L. pneumophila* and would contain high levels of antagonistic organisms and very low levels of mutualistic organisms. These microbiomes would contain high levels of *Pseudomonas* or *Sphingobium* according to our results.

Additionally, our finding revealed that the physicochemical parameters and management of the towers were important factors in moulding the microbiome of cooling towers. Thus, geographic location, water source, frequency of the application of chlorine, and DOC were major factors affecting the bacterial or eukaryotic communities. Consequently, these parameters could be manipulated to ensure control and elimination of *L. pneumophila* in the same way as hurdle technology is used for food processing (Khan et al., 2017). Hurdle technology is the approach of combining various food preservation methods in order to reduce to control and eliminate the growth of pathogens in food items (Khan et al., 2017). In this way, manipulating a combination of abiotic and biotic parameters so that the environment is inhospitable for *L. pneumophila*, may be a better approach to reducing *L. pneumophila* levels in cooling towers. For instance, the

combination of manipulating salt levels, chlorination frequency, and other parameters is potentially more efficient than just using biocides to reduce *L. pneumophila* levels. Furthermore, manipulation of certain parameters may work synergistically with other elements to reduce *L. pneumophila* levels (Khan et al., 2017). For instance, continuous chlorination of cooling towers was found to increase levels of *Pseudomonas*, which was negatively correlated with *Legionella* counts. As a result, the double effect of antimicrobial effect of chlorine and the highly competitive nature of *Pseudomonas* species may work in synergy to reduce levels of *L. pneumophila* in cooling towers. Consequently, hurdle technology may prove to be an approach to manipulate the microbiome of cooling towers.

VII- LIMITATIONS OF THE STUDY

Finally, certain drawbacks in the design of the experiments need to be addressed for future work. In our case, sampling may not have represented the entire picture, as we only sampled the water basin of the cooling towers. Cooling towers are composed of various parts, potentially with different microbiomes and different physicochemical parameters. For instance, we have demonstrated that different microbiomes populate the biofilms of the hot water and cold water pipes of a model cooling towers (Paniagua et al., 2019) (Appendix 2). One of the more interesting regions that we should have tried to investigate was the fill, which is a region in the cooling tower where the hot water exchanges heat with the ambient air. In our opinion, this is where the *Legionella* community can be most problematic as this is where aerosols are produced and evacuated. Furthermore, two basic types of fill exist, splash fill and film fill (Hensley, 2009). Splash fill are composed of slats on which the hot water is sprinkled. This causes the water to break up into small droplets and increases heat exchange. Film fill causes the water to disperse into a

thin film on which air is blown over to allow exchange of heat. These designs have different properties that may affect the microbiomes that colonize these regions. For instance, splash film contains very little channeling of water, and thus bulk water phase is not present in these types of fill (Hensley, 2009). As a result, biofilms are probably dominant in these environments and planktonic organisms may not be present. This could be problematic for the ciliate population, as they are usually planktonic. The planktonic organisms and parts of the biofilm of the fill may wash off into the collection basin or may be aerosolized (potentially heavier elements may fall into the tank whereas lighter elements will get aerosolized). Consequently, as we sampled only the water phase of the collection basins, we may have only observed a fraction of the elements that cause an outbreak of *L. pneumophila*. This is also evidenced by the fact that we did not sample biofilms in the cooling towers. Different regions of the cooling tower can contain different microbial communities. Unfortunately, the fill region is almost never accessible for sampling, as it would require disassembling and turning off the cooling towers. Thus, an easier way to study this region would be to create an experimental model, increasing accessibility and control.

Moreover, certain cooling towers were sampled through a sample port, located away from the basin. This may have biased the results, as we would have sampled the organisms present in the sampling port. However, this was mitigated by the flushing of the water in the sample ports before sampling.

Another limitation was due to the relatively small sample size (only 18 towers were sampled and this only in a two-week period in the summer of 2017). As a result, future work should recreate our experiment but with increased sampling efforts, such as increased number of samples or different time points. This would allow to gauge if our results and conclusions are reproducible or if they were due to some stochastic event during the sampling period. Possibly, reducing the

number of replicates may be a strategic way to increase the number of cooling towers we could sequence. Indeed, our results showed that the relative abundance of the microbial population was fairly consistent between replicate. Thus, sequencing three replicate per tower may not be necessary.

Sequencing cooling towers from different climatic or geographic regions may be an interesting additional step. As our samples were concentrated around Southern Quebec and the St Lawrence River, different microbiomes could be recovered from these different locations. For instance, cooling towers near marine environments may harbour more marine microorganisms, and northern regions may contain more psychrophiles.

Lastly, the archaeal and viral communities have not been characterized in cooling towers. Thus, this may be an interesting project that could give information on novel interactions between the *Legionella* community and the latter two communities. So far, these two communities are poorly explored in the context of EWS. Thus, studying these two community may prove fruitful. Metagenomics would be valuable in this case, as the entire microbiome could be sequenced (Eukaryotes, Bacteria, Archaea, and Viruses). To complement the metagenomic study, metatranscriptomics may be interesting in discriminating the active microbes from the inactive microbes in the system and how this may be important for *Legionella* ecology.

VIII- CONCLUSION

The main conclusion from this work is that the microbiome of cooling towers is a crucial element in the ecology of *L. pneumophila*. More importantly, several groups of microorganisms can have an effect on the colonization, survival, and proliferation of *L. pneumophila*. As a result,

the levels of antagonistic and mutualistic organisms could potentially indicate if a microbiome of a cooling tower is permissive or not for *L. pneumophila*. Thus, microbiomes with higher levels of *L. pneumophila* mutualistic species would be permissive, whereas higher levels of antagonistic species would create non permissive microbiomes. Furthermore, physicochemical parameters and managerial procedures impact the microbiome and its permissiveness toward *L. pneumophila*. This was demonstrated with the application of chlorine parameter. Indeed, continuous chlorine application seems to have enhanced the establishment of a *Pseudomonas* population in the cooling towers. This reduced *Legionella* levels. These findings indicate that the physicochemical parameters of the cooling tower environment could be targeted to reduce *L. pneumophila* in a probiotic manner. In this context, safe antagonistic organisms could be promoted to grow in the cooling tower environment by tweaking the physicochemical parameters for the benefit of these microorganisms. However, this would require a more thorough examination of the correlations between the microbiome and the different characteristics of the cooling tower environment. Conversely, as several microbial groups were found to negatively associate with *Legionella* and *L. pneumophila*, we could potentially directly seed them in the cooling tower environment as probiotic measure. This work also demonstrated that *L. pneumophila* ecology relies on a network of different microorganisms. Most of the interactions have not been yet characterized and thus represent a pool of novel ecological interactions. Furthermore, as we only characterized the eukaryotic and bacterial community, future research could be concentrated on characterizing the Archaeal and viral communities of cooling towers. These communities could potentially play important factors in *L. pneumophila* ecology, as viruses are known to shape bacterial community and Archaea can play important roles in certain nutrient cycles.

IX- REFERENCE

Amaro, F., Wang, W., Gilbert, J.A., Anderson, O.R. and Shuman, H.A. 2015. Diverse protist grazers select for virulence-related traits in Legionella. The ISME journal 9(7), 1607.

Cooper, A.J. and Pinto, J. 2006. Cysteine S-conjugate β-lyases. Amino acids 30(1), 1-15.

Corre, M.-H., Delafont, V., Legrand, A., Berjeaud, J.-M. and Verdon, J. 2019. Exploiting the Richness of Environmental Waterborne Bacterial Species to Find Natural Legionella pneumophila Competitors. Frontiers in Microbiology 9(3360).

Davidov, Y. and Jurkevitch, E. 2004. Diversity and evolution of Bdellovibrio-and-like organisms (BALOs), reclassification of Bacteriovorax starrii as Peredibacter starrii gen. nov., comb. nov., and description of the Bacteriovorax–Peredibacter clade as Bacteriovoracaceae fam. nov. International journal of systematic and evolutionary microbiology 54(5), 1439-1452.

Declerck, P. 2010. Biofilms: the environmental playground of Legionella pneumophila. Environmental microbiology 12(3), 557-566.

Eisenreich, W. and Heuner, K. 2016. The life stage-specific pathometabolism of Legionella pneumophila. FEBS letters 590(21), 3868-3886.

Ewann, F. and Hoffman, P.S. 2006. Cysteine metabolism in Legionella pneumophila: characterization of an L-cystine-utilizing mutant. Appl. Environ. Microbiol. 72(6), 3993-4000.

Flemming, H.-C. and Wingender, J. 2010. The biofilm matrix. Nature reviews microbiology 8(9), 623.

Garduno, R.A., Garduno, E., Hiltz, M. and Hoffman, P.S. 2002. Intracellular growth of Legionella pneumophila gives rise to a differentiated form dissimilar to stationary-phase forms. Infection and immunity 70(11), 6273-6283.

Giri, B.J., Bano, N. and Hollibaugh, J.T. 2004. Distribution of RuBisCO genotypes along a redox gradient in Mono Lake, California. Appl. Environ. Microbiol. 70(6), 3443-3448.

Guerrieri, E., Bondi, M., Sabia, C., de Niederhäusern, S., Borella, P. and Messi, P. 2008. Effect of Bacterial Interference on Biofilm Development by Legionella pneumophila. Current Microbiology 57(6), 532-536.

Heinz, E., Kolarov, I., Kästner, C., Toenshoff, E.R., Wagner, M. and Horn, M. 2007. An Acanthamoeba sp. containing two phylogenetically different bacterial endosymbionts. Environmental microbiology 9(6), 1604-1609.

Hensley, J.C. 2009. Cooling tower fundamentals, SPX cooling technologies. Overland, KS.

Khan, I., Tango, C.N., Miskeen, S., Lee, B.H. and Oh, D.-H. 2017. Hurdle technology: A novel approach for enhanced food quality and safety–A review. Food Control 73, 1426-1444.

Kimura, S., Tateda, K., Ishii, Y., Horikawa, M., Miyairi, S., Gotoh, N., Ishiguro, M. and Yamaguchi, K. 2009. Pseudomonas aeruginosa Las quorum sensing autoinducer suppresses growth and biofilm production in Legionella species. Microbiology 155(6), 1934-1939.

Koide, M., Higa, F., Tateyama, M., Cash, H.L., Hokama, A. and Fujita, J. 2014. Role of Brevundimonas vesicularis in supporting the growth of Legionella in nutrient-poor environments. The new microbiologica 37(1), 33-39.

Li, L., Mendis, N., Trigui, H. and Faucher, S.P. 2015. Transcriptomic changes of Legionella pneumophila in water. BMC genomics 16(1), 637.

Matz, C., Moreno, A.M., Alhede, M., Manefield, M., Hauser, A.R., Givskov, M. and Kjelleberg, S. 2008. Pseudomonas aeruginosa uses type III secretion system to kill biofilm-associated amoebae. The ISME journal 2(8), 843.

Mendis, N., McBride, P. and Faucher, S.P. 2015. Short-term and long-term survival and virulence of Legionella pneumophila in the defined freshwater medium Fraquil. PloS one 10(9), e0139277.

Paniagua, A.T., Paranjape, K., Hu, M., Bédard, É. and Faucher, S. 2019. Impact of temperature on Legionella pneumophila, its protozoan host cells, and the microbial diversity of the biofilm community of a pilot cooling tower. Science of The Total Environment, 136131.

Paranjape, K., Bédard, É., Whyte, L.G., Ronholm, J., Prévost, M. and Faucher, S.P. 2020. Presence of Legionella spp. in cooling towers: the role of microbial diversity, Pseudomonas, and continuous chlorine application. Water Research 169, 115252.

Stewart, C.R., Muthye, V. and Cianciotto, N.P. 2012. Legionella pneumophila persists within biofilms formed by Klebsiella pneumoniae, Flavobacterium sp., and Pseudomonas fluorescens under dynamic flow conditions. PloS one 7(11), e50560.

Tromas, N., Fortin, N., Bedrani, L., Terrat, Y., Cardoso, P., Bird, D., Greer, C.W. and Shapiro, B.J. 2017. Characterising and predicting cyanobacterial blooms in an 8-year amplicon sequencing time course. The ISME journal 11(8), 1746.

Verhoef, R., de Waard, P., Schols, H.A., Rättö, M., Siika-aho, M. and Voragen, A.G. 2002. Structural elucidation of the EPS of slime producing Brevundimonas vesicularis sp. isolated from a paper machine. Carbohydrate research 337(20), 1821-1831.

APPENDIX 1

I- SUPPLEMENTAL FIGURES FOR CHAPTER 3

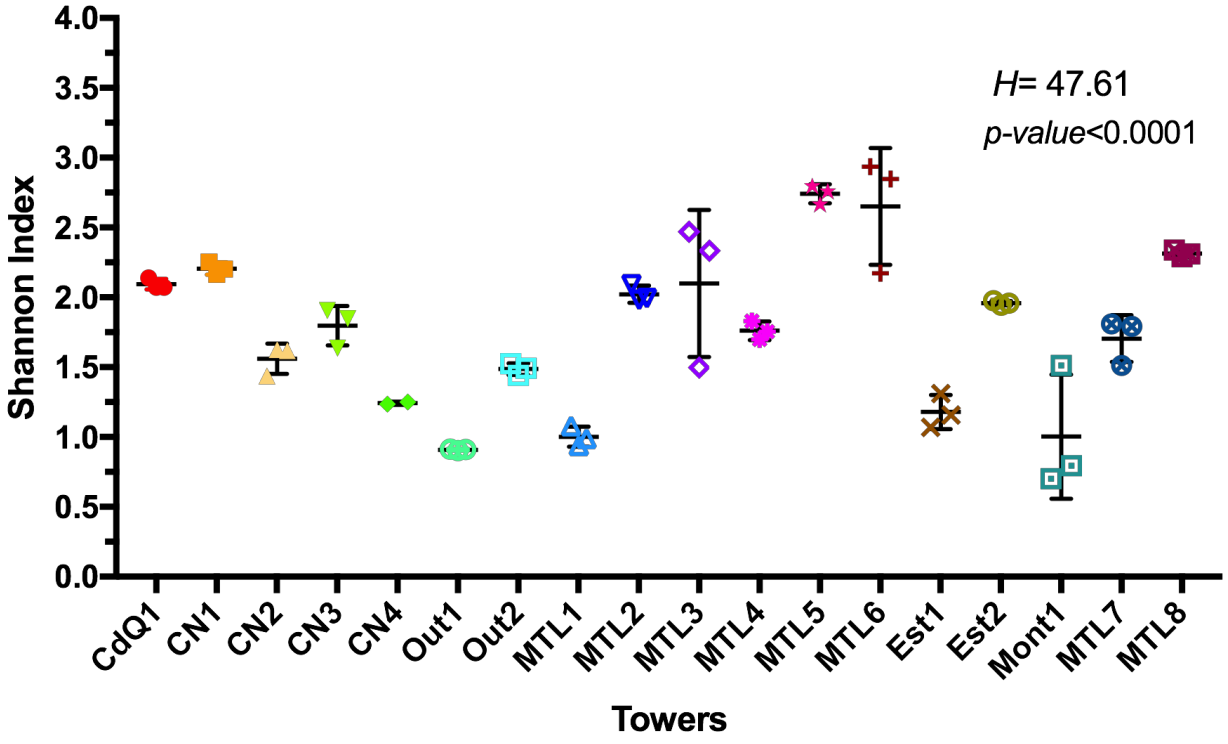

Figure S1: Alpha diversity levels of sampled cooling towers using the Shannon index. The Kruskal-Wallis test was used to assess statistical significance.

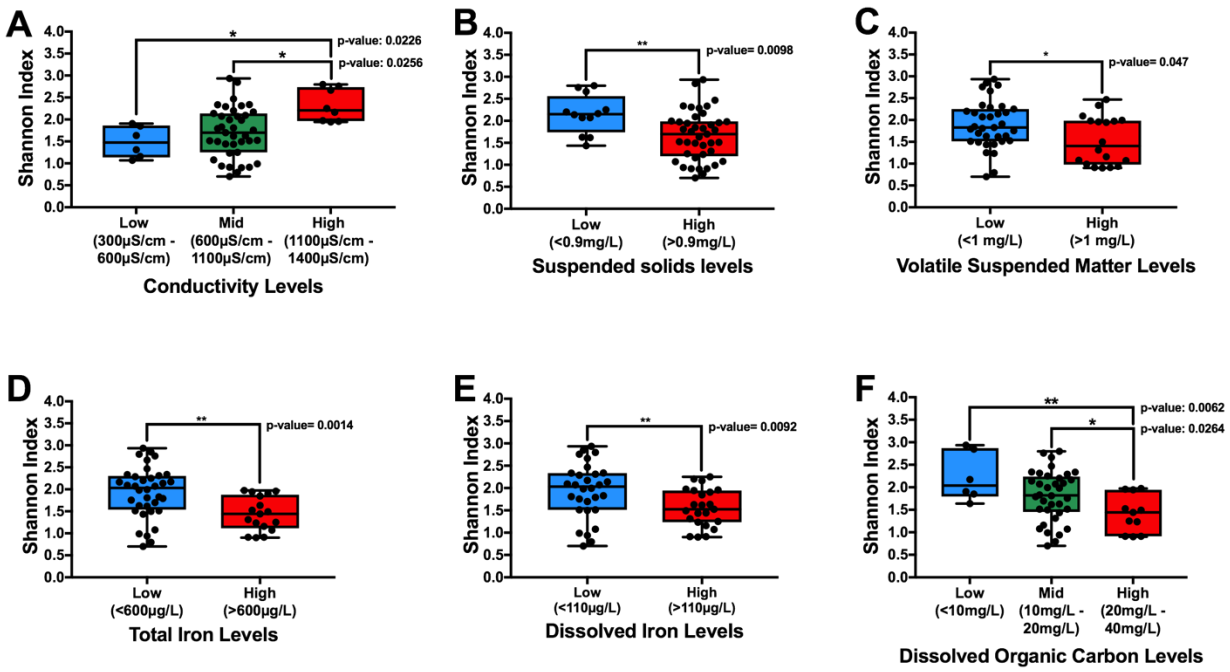

Figure S2: Effect of levels of conductivity (A), suspended solids (B), volatile suspended matter (C), total iron (D), dissolved iron (E), and dissolved organic carbon (F) on alpha diversity (Shannon index) of cooling towers. Higher levels of suspended solids, volatile suspended matter, total iron, dissolved iron, and dissolved organic carbon decreased alpha diversity. Higher levels of conductivity increased alpha diversity of towers.

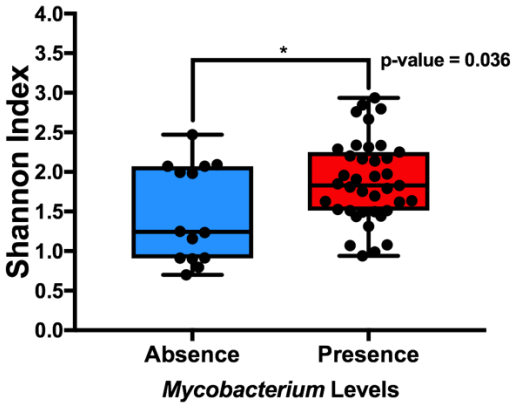

Figure S3: Alpha diversity and the presence of *Mycobacterium* in sampled cooling towers. Towers with the presence of *Mycobacterium* had higher levels of alpha diversity than towers without Mycobacterium.

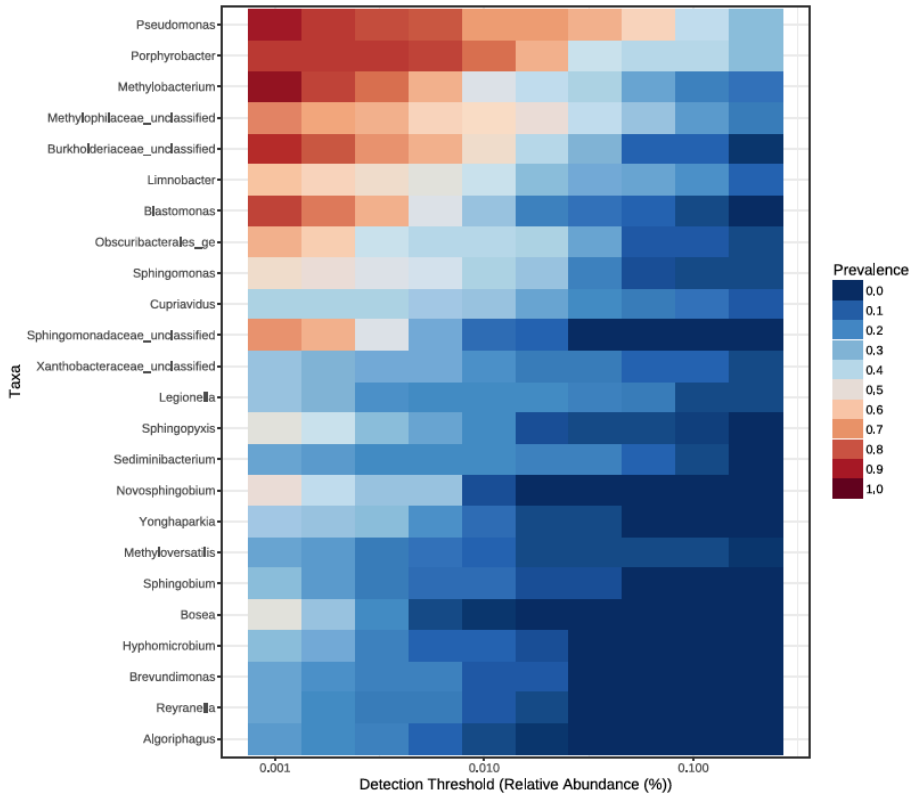

Figure S4: Most prevalent genera present in sampled cooling towers representing the core bacterial community.

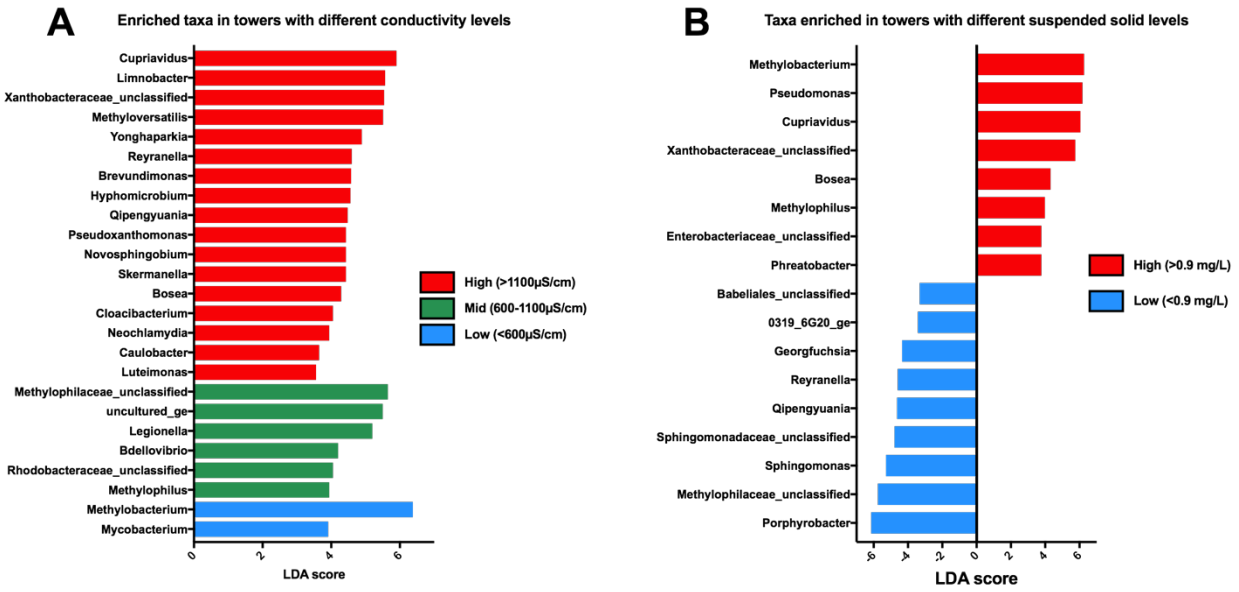

Figure S5: Enriched taxa in towers with different levels of conductivity (A), and with different levels of suspended solids (B) using LEfSe. *Legionella* was enriched in towers with mid levels of conductivity, and *Pseudomonas* was enriched in towers with high levels of suspended solids.

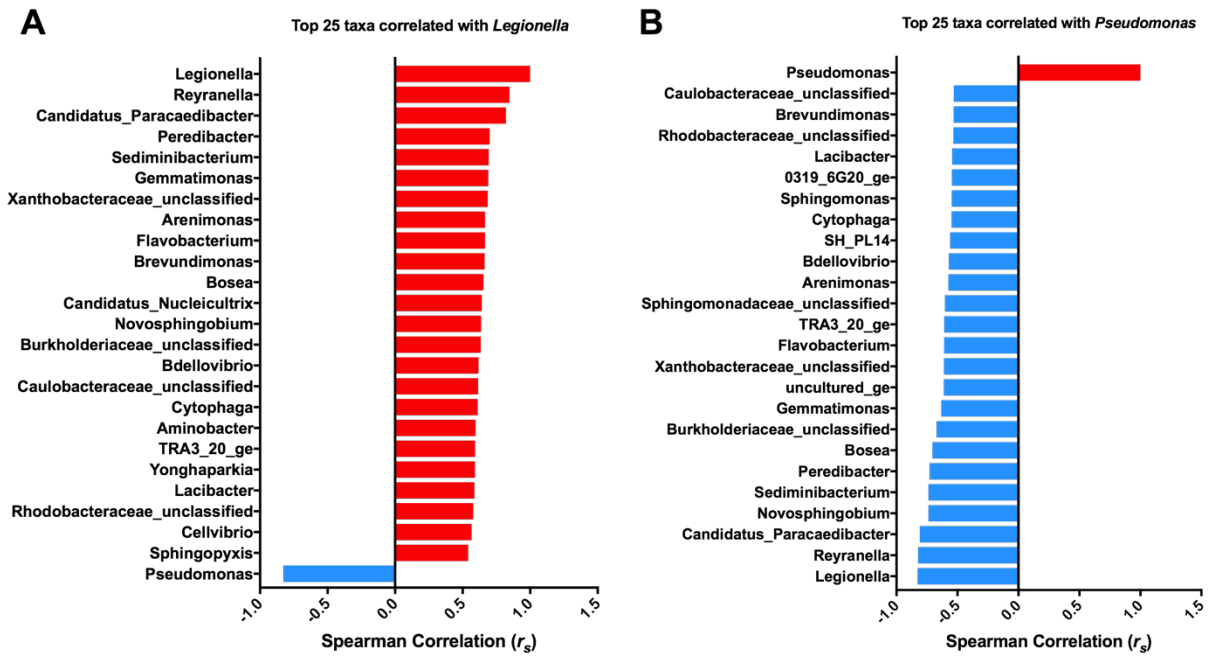

Figure S6: Spearman correlation of top 25 taxa correlated with *Legionella* (A), and with *Pseudomonas* (B). *Legionella* was positively correlated with many taxa; *Pseudomonas* was negatively correlated with most taxa.

II- SUPPLEMENTAL FIGURES FOR CHAPTER 4

Table S1: Read count for each replicate before and after processing with mothur

Sample_code	Replicate_name	Raw_MiSeq_Counts	Mothur_Processed_Counts
CdQ1	K1	63456	19199
CdQ1	K2	50722	22346
CdQ1	K3	48048	18790
CN1	K4	72928	37468
CN1	K5	75319	38667
CN1	K6	91802	45820
CN2	K7	75168	12598
CN2	K8	32897	5662
CN2	K9	59825	9242
CN3	K10	54482	22243
CN3	K11	55766	22991
CN3	K12	83860	27998
CN4	K13	69819	29196
CN4	K14	57272	22320
CN4	K15	88797	34262
Out1	K16	59462	5786
Out1	K17	54536	17241
Out1	K18	89458	18219
Out2	K19	89207	3532
Out2	K20	78715	3959
Out2	K21	77539	4789
MTL1	K22	56547	16655
MTL1	K23	59115	18143
MTL1	K24	70161	16520
MTL2	K25	71880	39064
MTL2	K26	70341	44526
MTL2	K27	52595	22852
MTL3	K28	74566	29768
MTL3	K29	50005	27419
MTL3	K30	64320	22644
MTL4	K31	82739	38448
MTL4	K32	53462	20983
MTL4	K33	81401	37623

MTL5	K34	71913	39709
MTL5	K35	62509	28797
MTL5	K36	42745	19542
MTL6	K37	164961	134440
MTL6	K38	78263	69383
MTL6	K39	135408	115850
Est1	K43	75382	41268
Est1	K44	91752	59256
Est1	K45	94505	45164
Est2	K46	120235	83033
Est2	K47	126295	87797
Est2	K48	105882	76811
Mont1	K49	151506	11431
Mont1	K50	168579	9350
Mont1	K51	135235	5409
MTL7	K54	79056	13023
MTL7	K55	83847	25517
MTL7	K56	69941	21320
MTL8	K57	56615	31371
MTL8	K58	65532	34261
MTL8	K59	84207	43034
Total	Total	4280578	1752739

Table S2: Good' coverage estimator for each sample before and after filtration step

	Before filtration			After filtration		
Sample name	no.singleton	no.seqs	good's coverage estimator	no.singleton	no.seqs	goods coverage estimator
CdQ1	450	18684	97.5915222	100	3440	97.0930233
CN1	298	36547	99.1846116	76	3440	97.7906977
CN2	52	8351	99.3773201	15	3440	99.5639535
CN3	133	23589	99.4361779	22	3440	99.3604651
CN4	647	26652	97.5724148	87	3440	97.4709302
Out1	295	12873	97.7083819	98	3440	97.1511628
Out2	220	3573	93.8427092	77	3440	97.7616279
MTL1	449	15889	97.1741456	66	3440	98.0813953
MTL2	464	34103	98.6394159	88	3440	97.4418605

MTL3	389	25154	98.4535263	51	3440	98.5174419
MTL4	394	30713	98.7171556	94	3440	97.2674419
MTL5	293	28093	98.9570356	56	3440	98.372093
MTL6	190	105739	99.8203123	14	3440	99.5930233
Est1	1040	45815	97.7300011	112	3440	96.744186
Est2	1102	78669	98.5991941	119	3440	96.5406977
Mont1	324	7994	95.9469602	72	3440	97.9069767
MTL7	626	18488	96.6140199	117	3440	96.5988372
MTL8	396	32461	98.7800746	61	3440	98.2267442

Table S3: Characteristics of Cooling Tower Samples

Tower name	Total Iron (µl/L)	Dissolved Organic Carbon (DOC, mg/L)	Disinfection schedule	Administrative regions	Source of water
CdQ1	11	12.5	Weekly	Centre du Québec	Nicolet River
CN1	387	12.6	Daily	Capitale Nationale	St-Lawrence river
CN2	427	12.6	Continuous	Capitale Nationale	St-Charles lake, St-Lawrence river
CN3	805	5.92	Daily	Capitale Nationale	St-Charles lake, St-Lawrence river
CN4	603	35.0	Weekly	Capitale Nationale	St-Charles lake, St-Lawrence river
Out1	970	37.4	Continuous	Outaouais	Ottawa river
Out2	715	25.3	Continuous	Outaouais	Ottawa river

MTL1	109	13.8	Continuous	Montréal	St-Lawrence river
MTL2	55	15.8	NA	Montréal	St-Lawrence river
MTL3	209	17.8	Continuous	Montréal	St-Lawrence river
MTL4	72	11.3	Continuous	Montréal	St-Lawrence river
MTL5	82	10.0	Weekly	Montréal	St-Lawrence river
MTL6	155	8.24	Daily	Montréal	St-Lawrence river
Est1	738	16.1	Weekly	Estrie	St-François river
Est2	1384	40.8	Weekly	Estrie	St-François river
Mont1	134	15.1	Continuous	Montérégie	St-Lawrence river
MTL7	182	14.2	Weekly	Montréal	St-Lawrence river
MTL8	14	15.1	Weekly	Montréal	St-Lawrence river

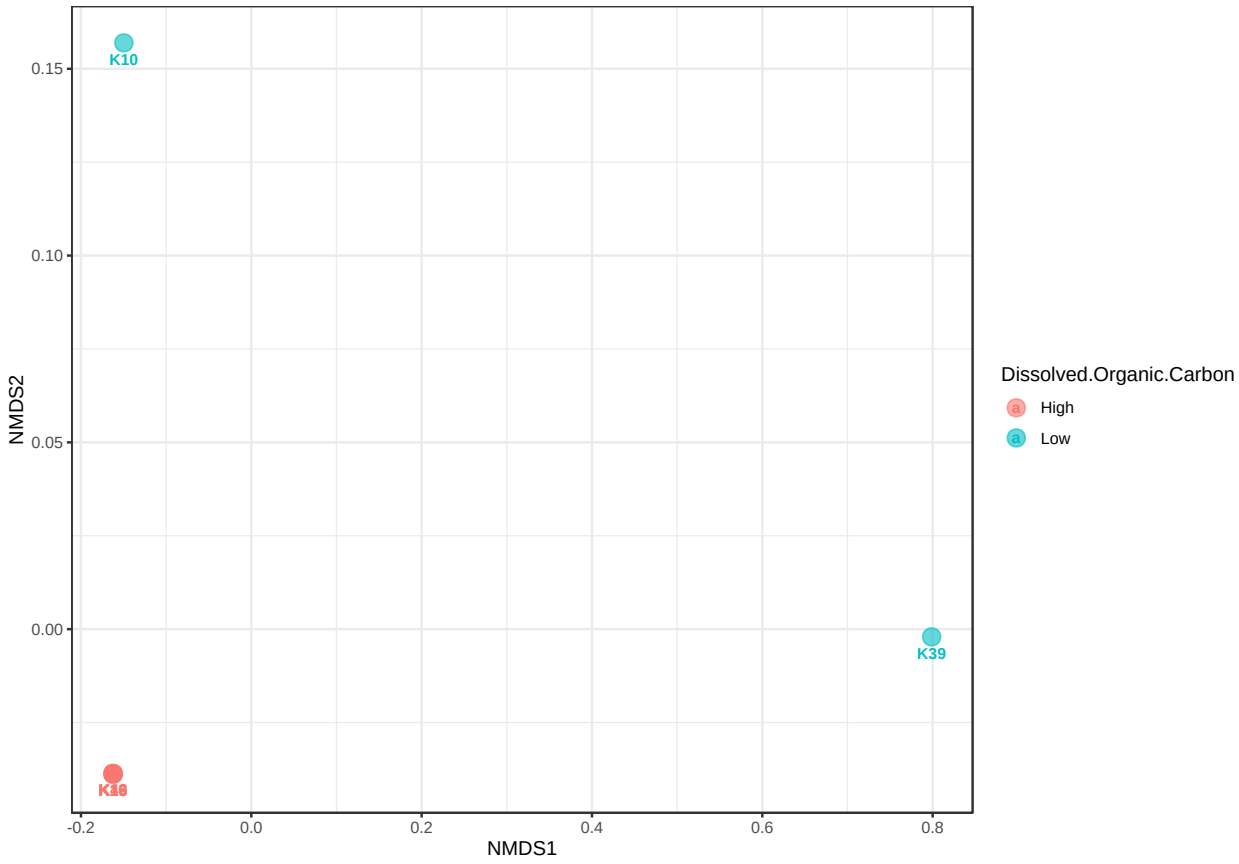

Figure S1: NMDS of cooling towers showing clustering of eukaryotic community according to DOC levels using ANOSIM to evaluate dissimilarity between communities (R = 0.75, P = 0.066; stress = 6.54e-05). Towers with high (red) and low levels (blue) of DOC clustered separately. A total of 4 samples are represented for the high category; however, due to very similar ordination results, these samples are superimposed on each other.

Document DS1: Description of metabolic features of *Brevundimonas* SPF441

Further analysis of the genome, using BlastKOALA, revealed that the *Brevundimonas* SPF441 isolate had 38% of its genes related to metabolism. This was shown by the number of different carbon metabolism cycles present in its genome. The carbohydrate metabolic pathways

glycolysis, gluconeogenesis, the TCA cycle, the Pentose phosphate pathway, PRPP biosynthesis, the Entner-Doudoroff pathway, nucleotide sugar biosynthesis, Glyoxylate cycle, and propanoyl-CoA metabolism were all identified in this isolate. The genomic analysis also identified the presence of malate dehydrogenase, pyruvate orthophosphate dikinase, and phosphoenolpryuvate carboxylase (PEPC). These genes are usually associated with carbon fixation in plants, but can also be found in bacteria, such as *E. coli* (Smith, 1970). PEPC is an enzyme that fixes bicarbonate with phosphoenolpyruvate to form oxaloacetate (Smith, 1970) . Thus, this may be important in several metabolic pathways. In addition, *Brevundimonas* SPF441 possesses the complete set of genes for biosynthesis of several amino acids, such as threonine, cysteine, methionine, valine, isoleucine, leucine, lysine, arginine, proline, histidine, and tryptophan. Genes for assimilatory sulphate reduction were also identified, which is an important pathway for incorporating sulphate molecules into different biological molecules, such as certain amino acids like cysteine (Rückert, 2016).

Reference:

Smith, Thomas E. "Escherichia coli phosphoenolpyruvate carboxylase: competitive regulation by acetyl-coenzyme A and aspartate." *Archives of biochemistry and biophysics* 137.2 (1970): 512-522.

Rückert, Christian. "Sulfate reduction in microorganisms—recent advances and biotechnological applications." *Current opinion in microbiology* 33 (2016): 140-146.

APPENDIX 2

I authored three articles as second author during my doctorate:

1) Paniagua, Adriana Torres, et al. "Impact of temperature on Legionella pneumophila, its protozoan host cells, and the microbial diversity of the biofilm community of a pilot cooling tower." *Science of The Total Environment* 712 (2020): 136131.

2) Bédard, Emilie, et al. "Legionella pneumophila levels and sequence-type distribution in hospital hot water samples from faucets to connecting pipes." *Water research* 156 (2019): 277-286.

3) Bédard, Emilie, et al. "Energy conservation and the promotion of Legionella pneumophila growth: the probable role of heat exchangers in a nosocomial outbreak." *infection control & hospital epidemiology* 37.12 (2016): 1475-1480.

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Impact of temperature on *Legionella pneumophila*, its protozoan host cells, and the microbial diversity of the biofilm community of a pilot cooling tower

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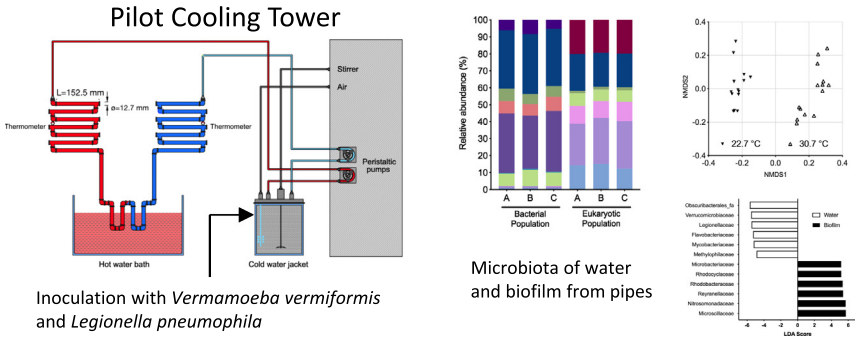
HIGHLIGHTS

- A pilot cooling tower was constructed to study colonization by *Legionella*.
- *Legionella* was more abundant in the water phase than in the biofilms.
- Ciliates and amoeba were more abundant in the cold and warm biofilm, respectively.
- The microbial populations of the cold and warm biofilms were different.

GRAPHICAL ABSTRACT



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ABSTRACT

Legionella pneumophila is a waterborne bacterium known for causing Legionnaires' Disease, a severe pneumonia. Cooling towers are a major source of outbreaks, since they provide ideal conditions for *L. pneumophila* growth and produce aerosols. In such systems, *L. pneumophila* typically grow inside protozoan hosts. Several abiotic factors such as water temperature, pipe material and disinfection regime affect the colonization of cooling towers by *L. pneumophila*. The local physical and biological factors promoting the growth of *L. pneumophila* in water systems and its spatial distribution are not well understood. Therefore, we built a lab-scale cooling tower to study the dynamics of *L. pneumophila* colonization in relationship to the resident microbiota and spatial distribution. The pilot was filled with water from an operating cooling tower harboring low levels of *L. pneumophila*. It was seeded with *Vermamoeba vermiformis*, a natural host of *L. pneumophila*, and then inoculated with *L. pneumophila*. After 92 days of operation, the pilot was disassembled, the water was collected, and biofilm was extracted from the pipes. The microbiome was studied using *16S rRNA* and *18S rRNA* genes amplicon sequencing. The communities of the water and of the biofilm were highly dissimilar. The relative abundance of *Legionella* in water samples reached up to 11% whereas abundance in the biofilm was extremely low (≤0.5%). In contrast, the host cells were mainly present in the biofilm. This suggests that *L. pneumophila* grows in host cells associated with biofilm and is then released back into the water following host cell lysis. In addition, water temperature shaped the bacterial and eukaryotic community of the biofilm, indicating that different parts of the systems may have different effects on *Legionella* growth.
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1. Introduction

Legionella pneumophila is a Gram negative, intracellular, waterborne pathogen known for causing Legionnaires disease (LD), a severe pneumonia, contracted by the inhalation of contaminated aerosols (Buse et al., 2012; Fields, 1996; Fliermans, 1996; McDade et al., 1979). *L. pneumophila* is the main cause of waterborne disease in the United States with an incident rate of 1.89 cases per 100,000 inhabitants in 2015 (Centers for Disease Control and Prevention, 2018). The estimated annual cost of hospitalization due to LD in the United States exceeds $716 million USD per year (Giambrone, 2013; Whiley et al., 2014). The incidence of outbreaks of LD is on the rise; the CDC reported that between 2000 and 2014, there was an increase of 286% in cases of LD and Pontiac fever in the United States (Centers for Disease Control and Prevention, 2015). A similar trend was reported in Europe (Beauté, 2013; Beauté, 2017).

L. pneumophila is a natural inhabitant of many aquatic ecosystems such as lakes, hot springs and rivers (Borella et al., 2005; Carvalho et al., 2008; Fliermans et al., 1981; Lin et al., 2007; Ortiz-Roque and Hazen, 1987; Sheehan et al., 2005). There, it can be found as an intracellular parasite of free living amoeba and ciliates (Fields et al., 2002; Rowbotham, 1980). Importantly, *Legionella* is ubiquitous in engineered water systems (Alary and Roy, 1992). *Legionella* has been detected in pools, water fountains, dental units, humidifiers, domestic potable water distribution systems, cooling towers, hospital and hotel hot water systems (Atlas et al., 1995; Hampton et al., 2016; Kyritsi et al., 2018; Leoni et al., 2018; Leoni et al., 2001; Llewellyn et al., 2017; Moran-Gilad et al., 2012; Paranjape et al., 2020; Pereira et al., 2017; Smith et al., 2015; Stout et al., 1992).

The first recognized outbreak of LD that sickened 182 people in 1976 in Philadelphia was associated to a contaminated cooling tower (Kurtz et al., 1982; McDade et al., 1979). Since then, cooling towers have been reported as the source of several outbreaks of LD (Addiss et al., 1989; Bell et al., 1996; Breiman et al., 1990; Brown et al., 1999; Fitzhenry et al., 2017; Greig et al., 2004; Isozumi et al., 2005; Mitchell et al., 1990; Shelton et al., 1994; Wang et al., 2014). Currently, cooling towers are a major source of outbreaks and cause up to 28% of sporadic cases of LD (Fitzhenry et al., 2017). This is due to the large amounts of aerosols produced by these towers, which are dispersed over long distances of up to 12 km (Beauté, 2017; Bhopal et al., 1991; Cunha et al., 2016; Fisman et al., 2005; Klaucke et al., 1984; Nguyen et al., 2006; White et al., 2013; Walser et al., 2014).

Understanding the conditions affecting growth of *L. pneumophila* in water is critical to elucidate the risk factors linked to outbreaks and improve monitoring and management of water systems. *Legionella* spp. can be detected at low levels in the majority of cooling towers; however, promoting factors are required for *Legionella* to reach sanitary risk levels (Llewellyn et al., 2017). Several physical and chemical factors contributing to *Legionella* colonization have been identified. A temperature between 25 °C and 50 °C is optimal for *L. pneumophila* growth and proliferation (Bedard et al., 2015; Katz et al., 2009; Wadowsky et al., 1985; Yamamoto et al., 1992). A long-term study conducted by Pereira et al. (2017), in which the microbiome of the water of a cooling tower was analyzed, confirmed that temperature is highly correlated with the presence of *Legionella*. Moreover, the material of the pipes greatly influence the abundance of *Legionella* in water systems and some materials, such as PVC, promote the presence of *L. pneumophila* (Buse et al., 2014; Moritz et al., 2010; Proctor et al., 2017; Rogers et al., 1994b; van der Kooij et al., 2005). The use of disinfectant also impacts the presence of *L. pneumophila*. In many countries, cooling towers are under surveillance and management plans are carried out to prevent the proliferation of *Legionella* (Kim et al., 2002; McCoy et al., 2012; Springston and Yocavitch, 2017; Whiley, 2016; WHO, 2007).

Biotic factors also affect the presence of *L. pneumophila* in cooling towers. High heterotrophic plate counts (HPC) in poorly managed water distribution systems seem to increase the odds of colonization of *L. pneumophila* (Messi et al., 2011; Serrano-Suarez et al., 2013). In contrast, some cooling towers that have high HPC do not harbor *L. pneumophila*, suggesting that they may host a microbial population resistant to *Legionella* colonization (Duda et al., 2015). The presence of some organisms such as *Cyanobacteria* (Tison et al., 1980) and *Flavobacterium* (Wadowsky and Yee, 1983) contribute to the growth of *L. pneumophila*. Interestingly, other bacteria such as *Pseudomonas* and *Staphylococcus warneri* seem to have an antagonistic effect on the proliferation of *Legionella* (Guerrieri et al., 2008; Hechard et al., 2006; Paranjape et al., 2020). Therefore, the growth and proliferation of *L. pneumophila* in water systems seem to be impacted by the resident microbes. The identity and relative abundance of these microbes is influenced by several parameters. The microbial population residing in cooling towers is shaped by local climate and water sources (Llewellyn et al., 2017; Paranjape et al., 2020). Additionally, the microbiota is affected by the disinfectant residuals and application schedule (Hwang et al., 2012; Paranjape et al., 2020). An important limitation of these studies is that they focus on the microbiota of the water. Biofilm plays a crucial role in *Legionella* proliferation and survival (Cooper and Hanlon, 2010; Flemming et al., 2002; Rogers and Keevil, 1992; Simões et al., 2010). In addition, the composition of the microbial communities in water systems is different in the biofilm and in the water phase (Di Gregorio et al., 2017; Wang et al., 2014). Therefore, analysing the microbial interaction between *L. pneumophila* and the resident microbiota in the water and in the biofilm is warranted to fully understand its life cycle and propose better strategies to control its growth.

Pilot-scale water systems have been developed to study disinfection methods (Farhat et al., 2012; Liu et al., 2011; Zhang et al., 2016), *L. pneumophila* growth and integration in biofilm (Taylor et al., 2013; Turetgen and Cotuk, 2007), corrosion, scaling, and biofouling (Chien et al., 2012). Of note, *L. pneumophila* can be detected in the biofilm in such pilot systems. Nevertheless, few studies have been conducted on pilot cooling towers and, to our knowledge, none accurately depict the complexity of real cooling towers.

Cooling towers are heat exchange devices in which hot water that comes from an external process such as refrigeration, is cooled due to heat exchange between water and air. Hot water is sprayed from the top of the cooling tower by a distribution system through a filling material that breaks the water into small droplets to increase the heat exchange between the air and the water. While water is sprayed, atmospheric air flows from the bottom to the top of the tower. A heat exchange will take place between the air and the water. The water will be cooled and collected at the bottom of the tower and returned to the process that needs cooling. Therefore, a cooling tower system consists of two sections characterized by different temperatures. In addition, the massive input of air in the system increases oxygen availability in the water. It is conceivable that the oxygen concentration is high initially in the basin, but decreases thereafter due to microbial consumption, reaching minimal concentration at the end of the warm pipe section. As a result, the microbial composition in the biofilm formed on the surface of the different parts of a cooling tower is likely different.

To better understand the growth of *L. pneumophila* in cooling towers and its interaction with the resident microbiome, it is therefore crucial to study the biofilm. It is difficult to perform such study on real cooling towers since the pipes are not easily accessible and sampling the biofilm of the pipes requires dismantling the system. As an alternative, we built a lab-scale cooling tower pilot to study the dynamics of *L. pneumophila* colonization in relationship to resident microbiota and spatial distribution. This pilot consists of cold and warm water pipe sections connected to an aerated cooling vessel, simulating a typical open evaporative cooling tower. It was filled with water from an operating cooling tower. The objective of the study was to characterize the microbial community in the pilot cooling tower residing in the biofilm and in the water, and to relate it with the dynamics of *L. pneumophila* colonization and local temperature.

2. Materials and methods

2.1. Cooling tower pilot

A lab-scale cooling tower pilot was designed to mimic critical components of a real cooling tower (Fig. 1). The pilot was installed in a biological safety cabinet to ensure the safety of the laboratory personnel. The system consisted of two symmetrical arrangements of PVC pipes coupled, on one side, to an aerated cooling bioreactor set at 15 °C (Sartorius Stedim Biostat Q Plus, Germany). On the other side, a loop heated by a warm water bath set at 34.4 °C was connected. Each arrangement consisted of eight PVC threaded pipes (McMaster-Carr, USA) of a length of 6 in. and a diameter of 0.5 in., connected to each other by a threaded T connector and an elbow at the end of each pipe section. A treaded thermocouple type K probe (McMaster-Carr, USA) was fitted in the T connector after the fourth pipe in each arrangement of pipes. The temperature was recorded with a 4-channel portable thermometer/datalogger (OMEGA, USA). A total water volume of 1.05 L was circulated through the pipes by a peristaltic pump using BTP PharMed tubes (Cole Parmer, USA) at a flowrate of 1 L/h. The temperatures of the water in the pipes were constant during the whole experiment: 22.7 °C for the cool section and 30.7 °C for the warm section. Ambient air was injected in the system at a flowrate of 3 L/min using an aquarium air pump equipped with a 0.2 μm air filter (Millipore, USA). Prior to the start of the experiment, the whole system was disinfected by circulating a 3-ppm sodium hypochlorite solution that was changed every 2 days. Chlorine residual was measured before changing the chlorine solution using the *N,N*-diethyl-*p*-phenylenediamine Colorimetric method 4500-Cl (American Public Health Association (APHA) et al., 2017) and a DR/2010 spectrophotometer (HACH Company, Loveland, CO, USA). In total, 2 weeks were required to reach stable chlorine residual in the system. Following system disinfection, the pilot was rinsed with unchlorinated sterile distilled water for 24 h. At this point, the HPC count was 1.4×10^3 CFU/L. The pilot was then filled with water from an actual cooling tower harboring undetectable levels of *L. pneumophila* at the time it was collected. An aliquot of water from this cooling tower was kept in a 10 L polypropylene carboy (Nalgene, USA) at room temperature for 3 months as control water, to distinguish the impact of stagnation from the impact of the pilot system on the water microbiome. After 64 days, the pilot was seeded with *Vermamoeba vermiformis* to a final concentration of 6×10^6 cells/L. At day 72, the pilot was seeded with *L. pneumophila* to a final concentration of 3.5×10^5 cells/L. The pilot was dismantled after 92 days, 3 weeks after the inoculation with *L. pneumophila*.

2.2. Inoculation with V. vermiformis

V. vermiformis (ATCC 50237) was freshly purchased from the American Type Culture Collection and grown in modified PYNFH medium at 30 °C in 75 cm^2 cell culture flask (Fields et al., 1990). Cells were passaged at a ratio of 1 in 5 when confluence was reached. For inoculation in the pilot cooling tower, the cells were harvested by centrifugation at 800*g* and washed three times in Page's Amoeba Saline. The cells were counted with a hematocytometer and a volume corresponding to 6×10^6 cells was added to the pilot through a sampling port in the bioreactor vessel on day 64.

2.3. Inoculation with L. pneumophila

L. pneumophila isolated during the Quebec City outbreak in 2012 (*lp120292*) was inoculated in the pilot (Levesque et al., 2014). The strain was maintained at −80 °C in 10% glycerol and grown on BCYE (ACES-Buffered charcoal yeast extract) agar supplemented with 0.25 mg/L L-cysteine and 0.4 mg/L ferric pyrophosphate for 3 days at 37 °C. Several colonies were suspended in filtered sterilized water from the cooling tower to a concentration of 3.5×10^5 cells/mL. One milliliter was added to the pilot through a sampling port of the bioreactor vessel on day 72. This dose was chosen because it is high enough to enable the detection of a decline in *L. pneumophila* population, yet low enough to notice growth by *16S rRNA* gene amplicon sequencing. Furthermore, this value is just below the regulated level in the province of Québec (10^6 CFU/L) Gouvernement du Québec, 2014.

2.4. Periodic water sampling

Water sampling was carried out from the bioreactor sampling port. One milliliter samples were taken twice a week for heterotrophic plate count (HPC) on R2A agar. The plates were incubated at 30 °C for 48 h. During the first 43 days, a 20 mL sample was collected weekly for DNA extraction. Starting from day 43, the volume was increased to 60 mL. Additional samples of 60 mL were taken after inoculation with *V. vermiformis* and *L. pneumophila*. The volume loss was compensated by adding filter sterilized water from the cooling tower that was kept at 4 °C. Due to a considerable decrease in HPC, the volume of sampling

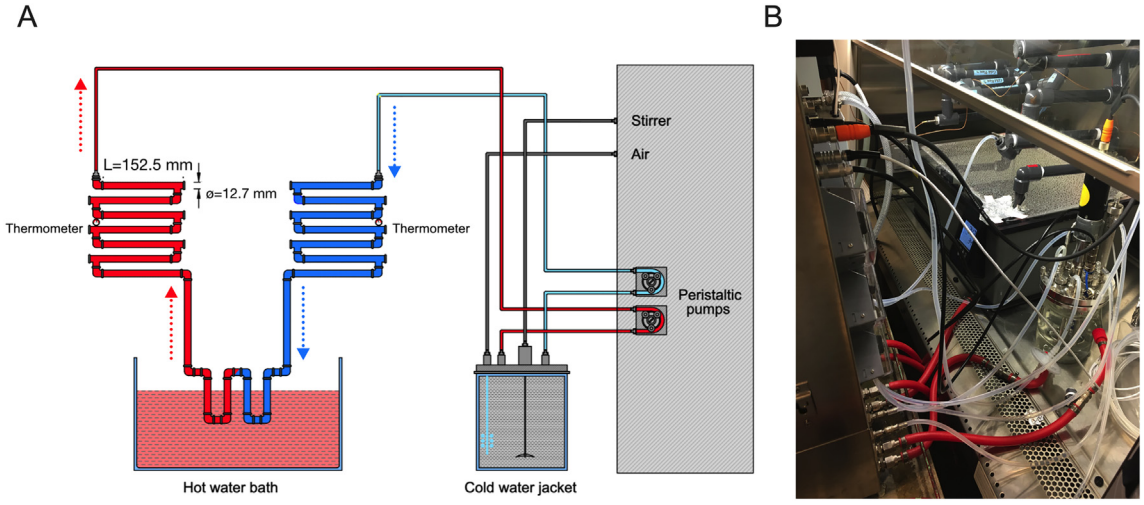

Fig. 1. Schematic representation (A) and picture (B) of the pilot tower used in this study. The pilot is composed of a water-jacketed bioreactor vessel connected to a series of cold water pipes (blue), a loop heated by a warm water bath and a series of warm water pipes (red). Water is pumped to the network of pipe and returned to the bioreactor using 2 peristaltic pumps. The bioreactor was maintained at 15 °C while the water bath was set at 34.4 °C. The direction of water is indicated with doted arrows.

was reduced back to 20 mL on day 64 until the end of the experiment. All water samples collected for DNA extraction were filtered through a 0.45 μm pore size filter (Millipore, USA), and the filters were kept at −20 °C until DNA extraction.

2.5. Pilot disassembly and biofilm sampling

After 92 days of operation, the pilot was disassembled. The water from the bioreactor was first collected. Then, the water was drained from the pipes. The pipes were disassembled, and the attached biofilm was collected as previously described (Proctor et al., 2016; Proctor et al., 2018). Briefly, ten 6-inch pipes (five pipes from the cold part and five from the warm part of the system) were unthreaded. Pipes were capped and filled with 10 mL of 3 mm sterile glass beads. The remaining volume was filled with filter-sterilized water collected from the pilot. The pipes were sonicated for 5 min in a sonication bath (Cole Parmer, Canada). Supernatant was collected, and the process was repeated 5 times. An aliquot of the resulting slurry was kept for CFU counts on R2A agar while the rest was filtered through 0.45 μm nitrocellulose filters and kept at −20 °C until DNA extraction.

2.6. 16S rRNA gene amplicon library preparation

DNA was extracted from filters using DNeasy PowerWater Kit from Qiagen (Qiagen, USA), following the manufacturer's protocol. Each replicate was treated separately. *16S rRNA* gene amplicon sequencing was performed using the dual-index paired-ends approach described by Kozich et al. (2013). Selected samples were analyzed in triplicate. Due to the sampling methodology on the day of dismantlement, the sample of September 13 (day 92) contained detached flocs. Briefly, the extracted DNA was amplified with the 515F and 806R primers targeting the V4 region of the bacterial 16S rRNA gene (Kozich et al., 2013). The PCR amplification was carried out using the Paq5000 Hotstart PCR Master Mix following the manufacturer's protocol (Agilent, USA). Cycling was performed on an Applied Biosystems Thermal Cycler with cycles consisting of an initial denaturation step at 95 °C for 2 min, 25 cycles of 95 °C for 2 s, 55 °C for 15 s and 72 °C for 5 min followed by a final elongation at 72 °C for 10 min. PCR products were purified with AMPure XP beads (Beckman Coulter, USA) according to the manufacturer's instruction. The purified DNA was quantified with Picogreen using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, USA). Normalized samples (1.5 ng/μl) were pooled together and mixed with 10% PhiX sequencing control (Illumina, USA). The DNA was diluted to a concentration of 4 pM and denatured with 0.2 N NaOH. The library was sequenced on the MiSeq platform with the MiSeq Reagent V2 250 cycles kit, according to the manufacturer's instructions.

2.7. 18S rRNA genes amplicon library preparation

18S rRNA gene amplicon sequencing was performed using a two-step PCR strategy. Selected samples were analyzed in triplicate. The V9 region of the *18S rRNA* was amplified in a first PCR with primers described in the Earth Microbiome Project protocol (http://www.earthmicrobiome.org/emp-standard-protocols/18s/) (Amaral-Zettler et al., 2009; Stoeck et al., 2010). The cycle of the amplicon PCR consisted of an initial denaturation step at 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, followed by a final elongation at 72 °C for 5 min. PCR products were purified using AMPure XP beads (Beckman Coulter, USA) according to the manufacturer's instructions. An indexing PCR was next carried out using the Nextera XT Index Kit (Illumina, USA). The index PCR cycle consisted of an initial denaturation step at 95 °C for 3 min, 8 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, followed by a final elongation at 72 °C for 5 min. Both PCR amplifications were carried out using the Paq5000 Hotstart PCR Master Mix (Agilent, USA). The library was sequenced on the MiSeq platform with the MiSeq Reagent V2 250 cycles kit, as described above for *16S rRNA* gene amplicon sequencing.

2.8. Data processing

The raw sequence reads were deposited in Sequence Read Archive under the BioProject accession number PRJNA588467. The sequenced reads were processed using the Mothur pipeline (Schloss et al., 2009). Paired-end reads were first assembled into contigs. Contigs that presented ambiguous bases or that were longer than 275 bp for the *16S rRNA* gene sequencing and 373 bp for the *18S rRNA* gene sequencing were removed. The SILVA 132 database was used to align the sequences. Ends and gaps were trimmed in order to have the same alignment coordinates for all the sequences. Chimeras were removed using the VSEARCH algorithm. Two of the replicates for the *16S rRNA* gene analysis had significantly lower read counts than the rest; hence, they were removed from the analysis (Warm pipe 7 c, Cold pipe 7 b). The rest of the samples were rarified to the next sample with the lower number of reads (3038 read counts). For the 18S *rRNA* gene analysis, the samples included in the analysis were rarified to the lowest read count sample (6325 read counts). For *16S rRNA* gene sequencing, non-bacterial sequences such as Eukaryotes, chloroplasts, Archaea and Mitochondria were removed. For *18S rRNA* gene sequencing, only Eukaryotic sequences were considered. For *18S rRNA* gene sequencing, only Eukaryotic sequences were considered. Operational Taxonomic Units were defined at an identity cut-off of 97%, by assigning the OTUs de novo. The OTU data was analyzed with the MicrobiomeAnalyst web-based tool (Dhariwal et al., 2017). Default parameters were used to filter OTU with low counts (OTUs with <2 counts in at least 20% of the samples were removed). Beta diversity was calculated with the Bray-Curtis dissimilarity index to analyze differences between samples. Non-metric multidimensional scaling (NMDS) and principle coordinate analysis (PCOA) were used to visualize the data. PERMANOVA analysis was performed to analyze the statistical significance between groups.

3. Results and discussion

3.1. Physical and microbial characteristics of the pilot

The pilot was designed to mimic as accurately as possible the operation of a real cooling tower. The temperatures in the cold and hot pipe were remarkably stable at 22.7 °C and 30.7 °C respectively, reproducing the temperature range typically seen in a cooling tower (ASHRAE, 2008). The pH was also stable around 8.1 during the whole experiment. The pilot was seeded with water collected from an operating cooling tower and filter-sterilized water from that tower was used as make up water.

HPCs in the reactor water were between 10^5 and 10^6 CFU/mL during the first 40 days of the experiment, showing a relative stability (Fig. 2A). A decrease in the HPC was noticed between day 43 and 64. During this period, the volume of water collected from the bioreactor for DNA extraction was increased from 20 mL per week to 60 mL, which increased the addition of makeup water. This apparently caused over dilution of the microbial population in water. A rise of the CFU in the water was observed when the volume taken was decreased back to 20 mL around the time of inoculation with *V. vermiformis* on day 64. Inoculation of *L. pneumophila* did not seem to affect CFU counts. On the last day (92), there were 1.40×10^5 CFU/mL in the water, for an estimated total cultivable biomass of 1.47×10^8 CFU in the system, assuming a volume of water of 1.05 L. Of note, some flocs were visible in the water collected from the bioreactor on the last day.

Biofilms were extracted from the pipes using a sonication and glass beads method (Proctor et al., 2016; Proctor et al., 2018). There was no significant difference (Mann–Whitney test) between the HPC counts from the biofilm samples taken from the cold pipes and the ones

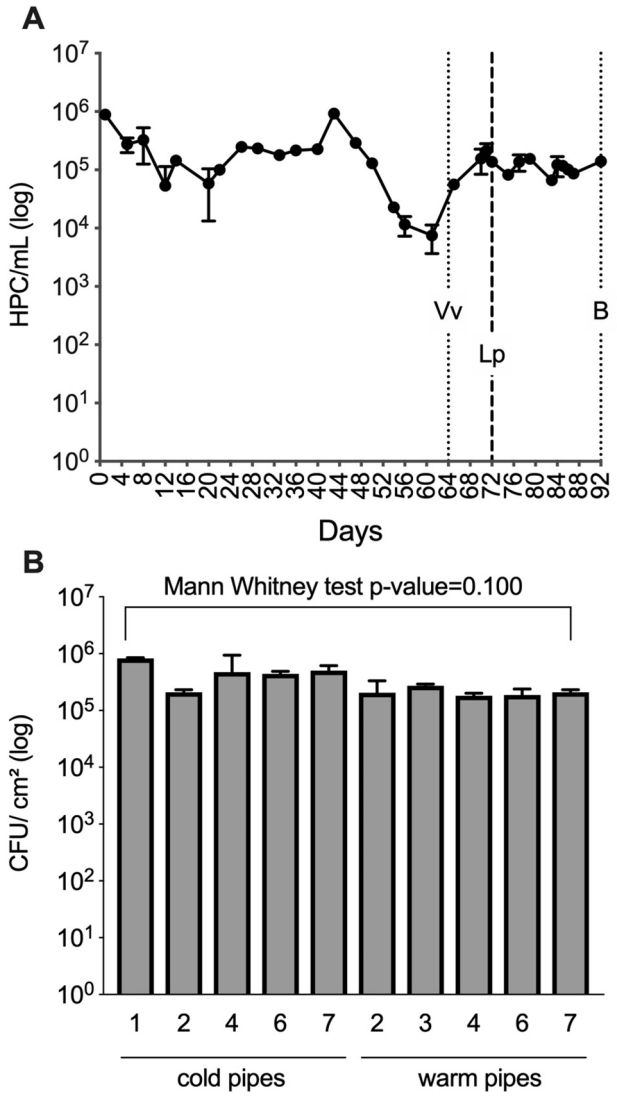

Fig. 2. The system was monitored by heterotrophic plate count during operation and at the time of dismantlement. Water samples of 1 mL were taken twice a week for 92 days and HPC counts were performed on R2A agar (A). Data represents the mean of triplicate samples with standard deviation. The time of inoculation with *V. vermiformis* (Vv) and with *L. pneumophila* (Lp) is indicated. On day 92, the system was dismantled and biofilm (B) samples from five pipe segments from the cold section and from the warm section were harvested by sonication with glass beads and HPC was determined on R2A agar (B). Data represents the average of CFU per cm^2 with standard deviation. Statistical significance between biofilm grown at the different temperatures was determined using a Mann–Whitney test.

taken from warm pipes (Fig. 2B). The average cultivable biomass in the biofilm was 3.51×10^5 CFU/cm^2. Using an estimated surface of 2257.5 cm^2 for the pipe system, the total cultivable biomass present in the biofilm is estimated to 7.92×10^8 CFU. This is at the upper range of what was previously reported for biofilm sampled inside drinking water distribution systems (Wingender and Flemming, 2011). There were only 5 times more cultivable microorganisms in the biofilm than in the water of our pilot system at the time biofilm was sampled (day 92). This is not consistent with the literature reporting that about 95% of bacterial cells in water systems are fixed on surfaces (Flemming et al., 2002). This can be due to the fact that the temperature range in the system and the lack of disinfection methods was ideal for the organisms to be in the planktonic state. Alternatively, the relatively high surface-to-volume ratio of our system (2.15 cm^{-1}) is known to promote cell release from the biofilm into the water (Bedard et al., 2018).

3.2. Characterization of the eukaryotic and bacterial communities in the pilot cooling tower

Bacterial community profiling was carried out on the samples collected on day 1, 57, 79, 83, 86 and 92. The bacterial community of the water changed drastically between day 1 and day 57 but seemed relatively stable afterward. The inoculation with *V. vermiformis* and *L. pneumophila* had only a minor effect on the general composition of the water microbiota (Fig. 3A). This indicates that the initial inoculated microbiota was modified by the system during the first 2 months and eventually reached a relative equilibrium by day 57. *Obscuribacterales* and *Verrucomicobiceae* were the most predominant bacterial families in the water samples, with relative abundance going from 7.7% to 27.9% and 8.8 to 22.2% respectively, excluding the samples taken at inoculation. Several water samples analyzed with 18S rRNA gene amplicon sequencing produced very low number of reads and were therefore excluded from the analysis. *Poterioochromonas* was the most predominant eukaryotic genus in the water samples taken on day 57 but was reduced to 15% on day 92, apparently being replaced by other organisms. Interestingly, *Poterioochromonas* is a flagellated protist that preys on other microbes, including bacteria (Saleem et al., 2013). Possibly, its favorite prey type disappeared from the system during day 87 and day 92, perhaps because of over predation, which resulted in a decline in the population of *Poterioochromonas*. Alternatively, it might be a previously unknown host of *L. pneumophila*, since the decline of *Poterioochromonas* coincide with the growth of *L. pneumophila*. Additional experiments will be required to confirm this hypothesis. *Vermamoeba* was already present at day 57, before inoculation with *V. vermiformis*, and was also present at day 92. Other OTUs harboring potential hosts for *L. pneumophila* were also detected such as *Oligohymenophorea* and *Naegleria*, but only at the later time point (Fig. 3B). In the biofilm sampled on day 92 (Fig. 3C), *Nitrosomonadaceae* was the most predominant bacterial family in the warm pipes (17.1% to 22.5%). In contrast, the levels of *Burkholderiaceae* (9.3% to 22.2%), *Microscillaceae* (7.9% to 16.8%) and *Rhodocyclaceae* (8.0% to 17.1%) seemed higher in the biofilm formed in the cold pipes. *Oligohymenophorea* was the most abundant eukaryotic genus in the biofilm samples (Fig. 3D), having a higher abundance in the biofilm formed in the cold pipes (31.3% to 73.7%) compared to the hot pipes (3.1% to 18.5%). *Vermamoeba* and *Naegleria* were also detected in the biofilm samples in pipes at both temperatures.

It is difficult to compare the microbiome of this pilot cooling tower with other studies since water source and regional climate shape the microbiome of cooling towers (Llewellyn et al., 2017; Paranjape et al., 2020). Nevertheless, some similarities are observed between our pilot cooling tower and other studies. For instance, our results showed that *Burkholdericeae* was abundantly found in the water of the pilot cooling tower. This is in agreement with other studies reporting the microbial communities of similar environments (Paranjape et al., 2020; Tsao et al., 2019). *Verrumicrobiaceae* was also an abundant family in the system, which is consistent with its presence in natural water reservoirs (Boucher et al., 2006; Zwart et al., 2002). Taxa previously identified as organisms capable of forming biofilm such as *Pirellulaceae*, *Rhodobacteraceae and Caulobacteraceae*, were identified in the biofilm samples of the pilot (Elifantz et al., 2013; Entcheva-Dimitrov and Spormann, 2004; Miao et al., 2019).

Beta diversity analysis was used to evaluate the effect of the pilot on the microbiota by comparing the bacterial communities of the pilot tower water at day 92, of the initial water used for inoculation, and of the initial water incubated at room temperature on the bench for 92 days (Fig. 4). The microbiome of the pilot water was significantly different than the initial water used to seed the system and shows a significant difference from the microbiome of the stagnant water (PERMANOVA F-value: 269.21; R^2: 0.98901; p-value < 0.001). This result indicates that specific characteristics and operating parameters of our pilot tower, such as temperature, dissolved oxygen, and water

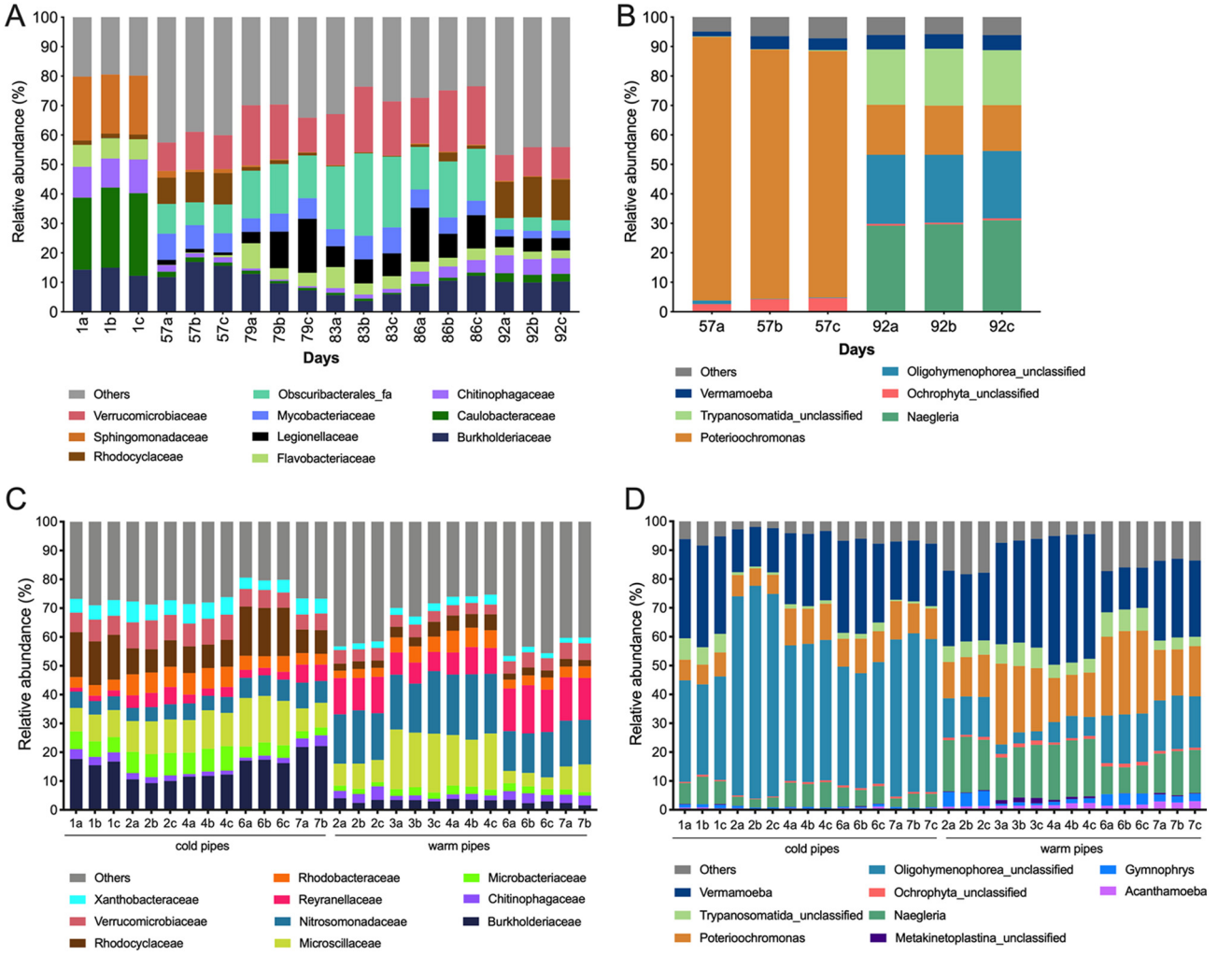

Fig. 3. The microbial community composition of water samples (A and B) and biofilm samples (C and D) was determined. The bacterial community was analyzed by *16S rRNA* gene amplicon sequencing (A and C) while the eukaryotic community was determined using *18S rRNA* gene amplicon sequencing (B and D). The data are presented as the relative abundance of OTUs classified at the most appropriate taxonomic level.

flow, shaped the resident microbiota. These parameters were identified as the main factors influencing the resident microbiota of a model water distribution system (Douterelo et al., 2017).

3.3. Presence of Legionella in the system

The presence of *Legionella* in the system was evaluated using the results of the *16S rRNA* gene amplicon sequencing (Fig. 5). The relative abundance of *Legionella* in the water at the beginning of the experiment (day 1) was almost null (0.02%). An increase in the relative abundance of *Legionella* was observed after 57 days reaching 3.0%. Right after the inoculation with *L. pneumophila* on day 72, the relative abundance of *Legionella* in water was 11% reaching 13% at day 86, but then dropping to 4% on day 92 (Fig. 5B). The relative abundance of *Legionella* in the biofilm samples was extremely low, but detectable (Fig. 5B). One of the objectives of this study was to observe the spatial distribution of *Legionella* within cooling towers. While we were expecting to see significant differences in the relative abundance of *Legionella* in biofilm at different temperatures, this was not observed. It is tempting to conclude that most of *Legionella* was in the water phase in the system. The presence of *Legionella* in biofilms within water distribution systems has been reported in several studies (Abdel-Nour et al., 2013; Abu Kweek and Amer, 2018; Armon et al., 1997; Buse et al., 2014; Buse et al., 2012; Declerck, 2010; Lau and Ashbolt, 2009; Moritz et al., 2010; van der Kooij et al., 2005). Several factors influence the formation of biofilm by *Legionella* and its ability to integrate biofilms (Buse et al., 2017; Piao et al., 2006; Rhoads et al., 2017; Rogers et al., 1994a). Indeed, *Legionella* incorporates in pre-established biofilms as a secondary colonizer. Instead of attaching to surfaces and growing biofilm, the bacterium will form an association with other microbes that previously developed biofilm (Buse et al., 2017). Thus, integration of *Legionella* into biofilms is affected by water temperature, surface material, water quality, microbial composition of the biofilm and biofilm age (Buse et al., 2017). Potential host of *Legionella*, such as *Vermamoeba*, *Acanthamoeba*, *Naegleria* and ciliates (*Oligohymenophorea*) were detected in the water samples (Fig. 3B) as well as in the biofilm. Intracellular growth of *L. pneumophila* in biofilm is dependent on the concentration of host cells (Shaheen et al., 2019). The presence of host cells in the biofilm (Fig. 3B) as well as the temperature being between 22.7 and 30.7 °C in the pipes suggest that *Legionella* had ideal growth conditions (Ashbolt, 2015; Fields et al., 2002; Moffat and Tompkins, 1992; Rowbotham, 1980). Furthermore, it was previously shown that *Legionella* is able to integrate biofilm formed on PVC, the material used for the pipe in our studies (Armon et al., 1997; Rogers et al., 1994a). Therefore, we were expecting to find a larger proportion of *Legionella* in the biofilm than in the water. However, the conditions found in the water of our pilot, including the presence of specific host species, might be ideal for *L. pneumophila* growth, favoring its presence in water. This is supported by the presence of host cells in the water phase. The lack of time points for the analysis of the composition of the biofilm prevents us from

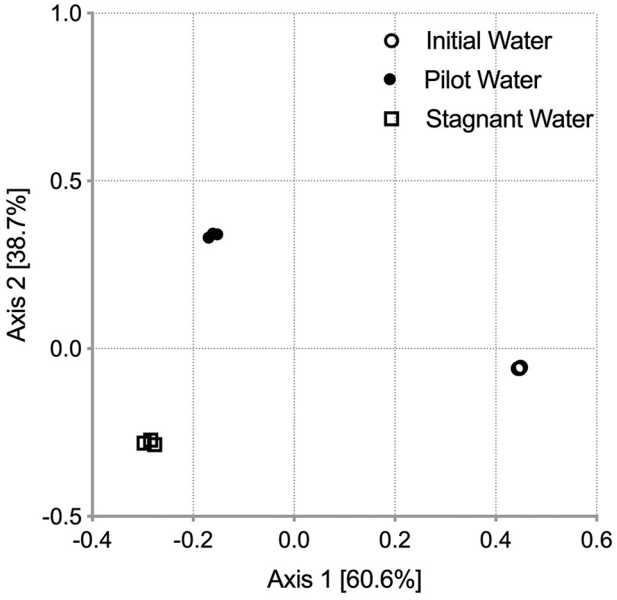

Fig. 4. Beta-diversity was used to analyze the effect of the pilot cooling tower on the bacterial community. A principal coordinate analysis (PCoA) plot of bacterial profiles of the water samples from the cooling tower at day 92 (pilot water), from the initial water (day 1) and control stagnant water was used. Statistical significance was determined using PERMANOVA.

making any assumptions about the dynamics of the microbiota in the biofilm. It is possible that *Legionella* concentration in the biofilm was higher at an earlier time point. The spatial distribution of *L. pneumophila* in cooling towers will need to be studied further.

3.4. Difference in the water and biofilm communities

At first sight, the composition of the microbial communities of the water and of the biofilm seems different. To characterize the communities further, the Shannon Index was calculated to measure alpha diversity (Fig. 6A and B) while beta diversity was used to assess dissimilarities between the communities (Fig. 6C and D). For the water samples, the analysis was performed only with the samples from day 79 to 92, to avoid changes induced by inoculations of *V. vermiformis* and *L. pneumophila*. There was a slight but significant difference between the alpha diversity in the biofilm samples and in the water samples for the bacterial communities but not for eukaryotic communities. Beta diversity analysis revealed that the water and biofilm bacterial communities were dissimilar, clustering in distinct groups (Fig. 6C, PERMANOVA F-value: 36.174; R^2: 0.488; $P < 0.001$; Stress = 0.0765). This was also observed for the eukaryotic communities (Fig. 6D, PERMANOVA F-value: 11.076; R^2: 0.246; $P < 0.001$; Stress = 0.0945); however, the samples from day 92 clustered with the biofilm samples (open circle samples in Fig. 6D). This could be due to contamination of the water samples with biofilm fragments as flocs were observed in the bioreactor during dismantlement. Similarly, the bacterial communities at day 92 seem to share characteristics between the water and biofilm group (Fig. 6 C), although the similarity was less pronounced then for the eukaryotic community.

Next, the machine-learning algorithm LEfSe was used to identify bacterial and eukaryotic taxa associated with either the water samples or the biofilm samples (Segata et al., 2011). Only water samples taken after day 79 were considered for the LEfSe analysis of bacterial communities. The algorithm was able to identify significant taxa associated with water and biofilm (Fig. 7). Of note, *Legionellaceae* were enriched in the water while its hosts, including *Vermamoeba*, *Acanthamoeba*, and *Oligohymenophorea*, were enriched in the biofilm. The enrichment of amoebas in the biofilm of our pilot system is consistent with what

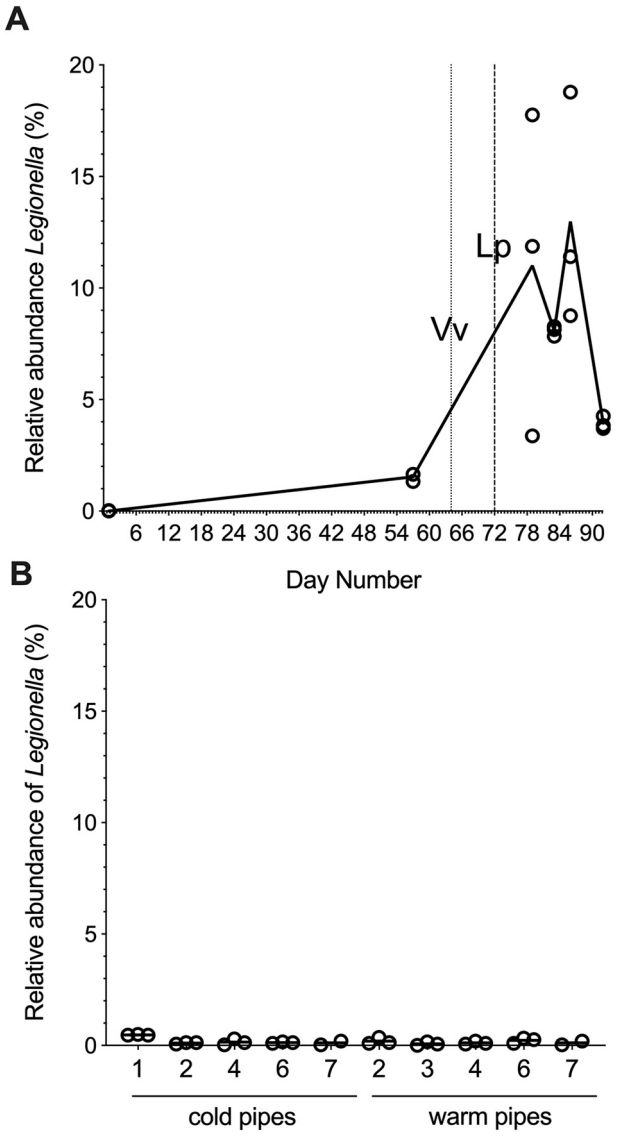

Fig. 5. The relative abundance of *Legionella* in the pilot water (A) and biofilm (B) was determined from the *16S rRNA* gene amplicon sequencing data. The percentage of abundance of the reads of the *Legionella* OTU was calculated according to the rarefied number of reads for each sample after rarefaction. Individual replicates are shown. The line in (A) connects the means of each time points.

was previously reported for the biofilm in water distribution systems (Taravaud et al., 2018; Thomas et al., 2004; Thomas et al., 2008). Therefore, it seems that *Legionella* is mostly in the water while its hosts are mostly in the biofilm, which seems counterintuitive. A possible explanation is that *Legionella* actively grows in the biofilm, where the hosts are located, but it is released into the water after intracellular replication, as previously shown (Greub and Raoult, 2004; Lau and Ashbolt, 2009). The bacterium can also be expelled in cysts from ciliates and amoeba such as a *Tetrahymena* and *Acanthamoeba*, respectively (Berk et al., 2008; Bouyer et al., 2007; Hojo et al., 2012).

3.5. Influence of the temperature on bacterial and eukaryotic communities in the biofilm

Beta diversity was calculated to analyze the difference between biofilm samples (Fig. 8). Grouping the samples according to the temperature produced significantly different clusters for the bacterial (Fig. 8A, PERMANOVA F-value: 37.838; R^2: 0.59272, $P < 0.001$, Stress = 0.10321) and for the eukaryotic communities (Fig. 8B, PERMANOVA F-

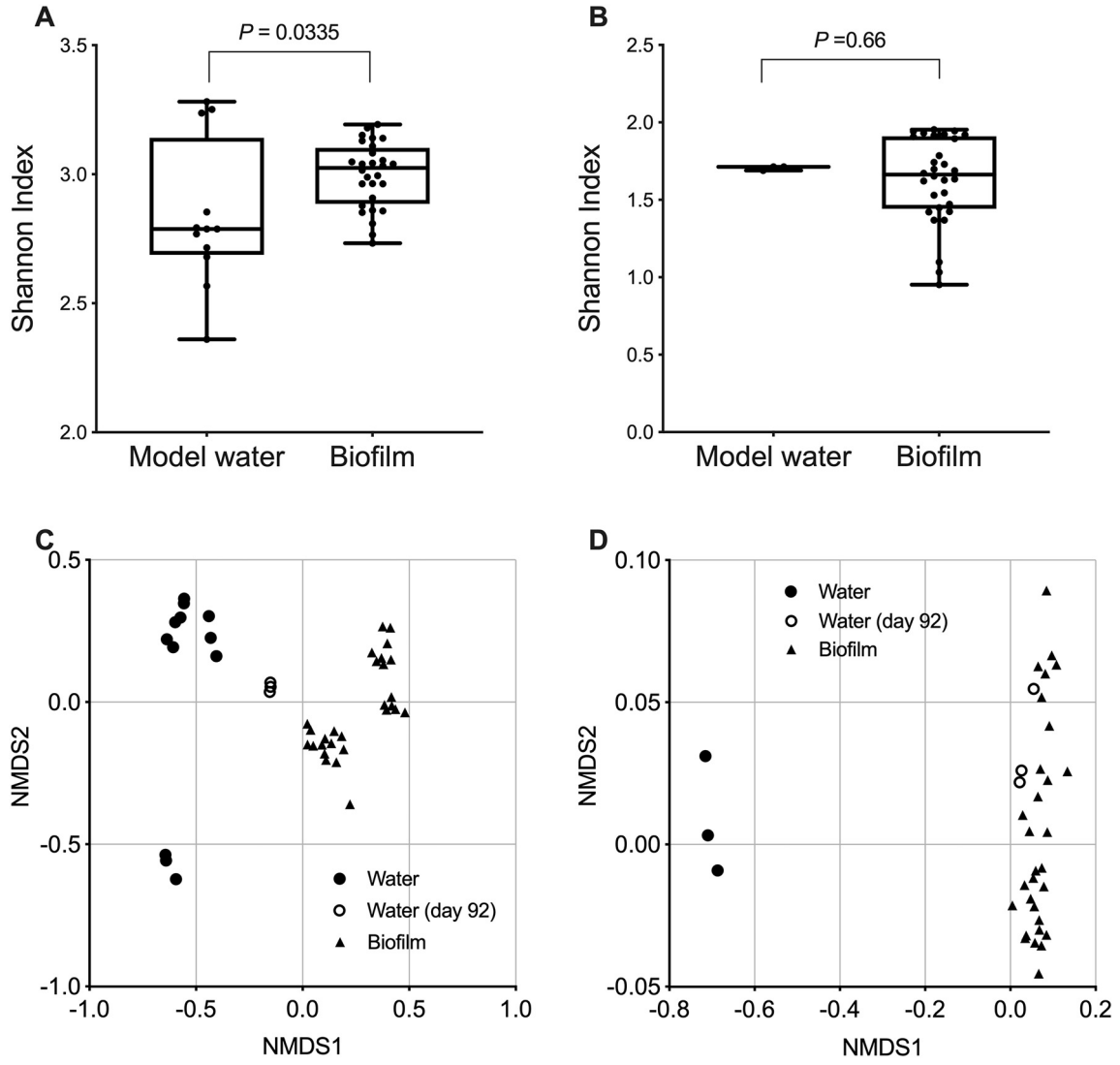

Fig. 6. Alpha diversity of samples from the pilot for the bacterial (A) and eukaryotic community (B), categorized by the type of samples: water and biofilm. A Mann-Whitney test was performed to determine the statistical significance. Beta diversity was calculated for the bacterial (C) for the eukaryotic (D) community categorized by the type of samples. PERMANOVA was used to assess statistical significance. Only day 79 to day 92 were analyzed for the bacterial community of the water to avoid noise introduced by addition of *V. vermamoeba* and *L. pneumophila*.

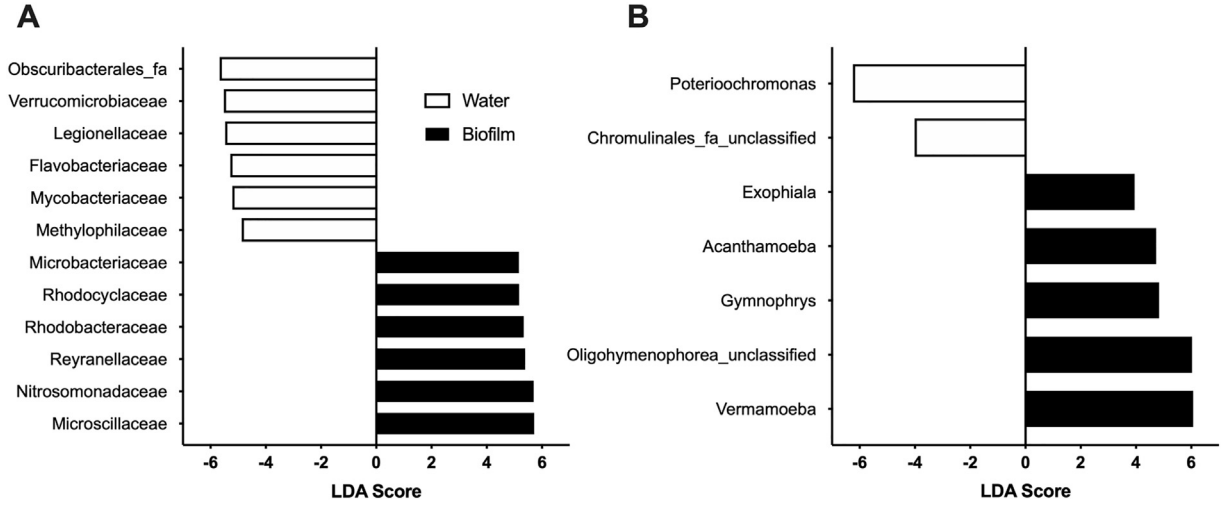

Fig. 7. The machine learning algorithm LEfSe was used to identify significant bacterial (A) and eukaryotic (B) taxa associated with either water (open bars) or biofilm (black bars). Only significant taxa ($P < 0.02$) are shown.

value: 37.717, R^2: 0.57393, $P < 0.001$; Stress = 0.15982). This is not surprising since temperature is known to affect biofilm formation and composition, as well as the presence of *L. pneumophila* (Buse et al., 2017). The strength of our study is that our unique pilot design allows us to decipher the effect of temperature in a single system were the different surfaces are inoculated with the same microbiota. The specific biofilm communities present at the different temperatures likely established gradually from the original inoculum eventually reaching a specific composition. It is not clear if the composition of the biofilms was stable at the time of disassembly. A time course study will need to be performed to understand the dynamic of biofilm establishment at different temperatures in the same system. To our knowledge, there is a scarcity of study assessing this particular point. Next, LEfSe was used to identify bacterial and eukaryotic taxa enriched in biofilm at 22.7 °C and at 30.7 °C. Bacterial families such as *Burkholderiaceae*, *Rhodocylaceae* and *Microbacteriaceae* were predictive of biofilm at 22.7 °C while *Nitrosomonadaceae* and *Reynellaceae* were predictive of biofilm at 30 °C. Interestingly, the ciliate class *Olygohymenophorea* was predictive of cold biofilm while amoeba such as *Naegleria* and *Acanthamoeba* were predictive of warm biofilm. It is possible that the species of *Oligohymenophorea* present in the system have an optimal growth temperature closer to 20 °C. Indeed, a recent study of the microeukaryote communities in the St-Charles river in Quebec, Canada, revealed that ciliates are more abundant during the winter period (Cruaud et al., 2019). It is tempting to speculate that ciliates might be more important for intracellular growth of *L. pneumophila* at low temperature and amoebas at higher temperature in water systems. The role of ciliates in the life cycle of *Legionella* in water systems running at low temperature should be investigated further.

4. Conclusion

This study illustrates the importance of studying the microbial composition of the water as well as the biofilm to fully understand *Legionella* ecology in water systems. From our study, three main observations emerge.

- In our pilot, the temperature had a great impact in the composition of the resident microbiota of the biofilm, indicating that the cold and warm pipe section of actual cooling towers are likely to harbor different microbial population.
- The host cells were mainly present in the biofilm, while *Legionella* was present in a lower proportion in the biofilm at the time of sampling. This support the notion that *Legionella* grows in the biofilm but is released back in the water afterward, following a host-prey cycle within hosts population.
- Ciliates and amoebas seem to inhabit different parts of the system, the former preferring the colder part. Therefore, additional research is needed to appreciate the role of ciliates in *Legionella* growth at lower temperature. Finally, our study supports the usefulness of pilot systems in studying the ecology of *Legionella* and other waterborne pathogens.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

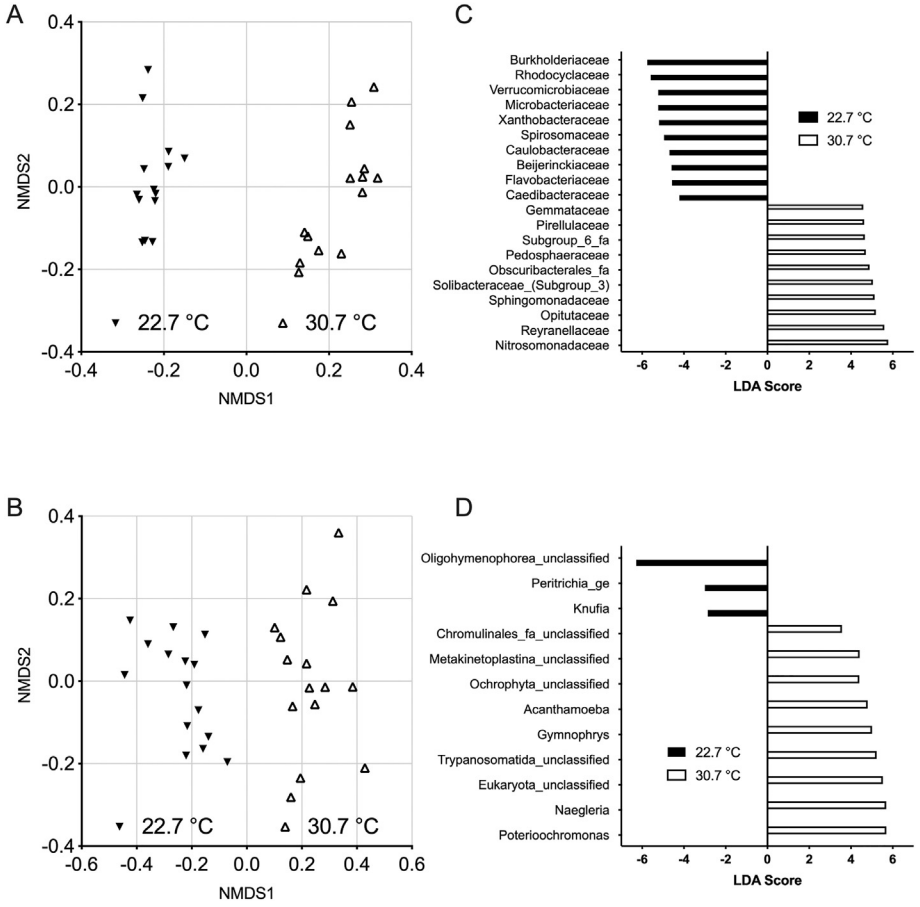

Fig. 8. Temperature affects the microbial composition of the biofilm. Beta-diversity of the bacterial (A) and eukaryotic (B) communities was calculated using the Bray-Curtiss index and a non-metric dimensional scaling (NMDS) plot. The samples were grouped by temperature. PERMANOVA was used to assess statistical significance. A LEfSe analysis was performed to identify bacterial (C) and eukaryotic (D) taxa associated with each temperature. Only statistically significant taxa ($P < 0.02$) are shown.

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References

Abdel-Nour, M., Duncan, C., Low, D.E., Guyard, C., 2013. Biofilms: the stronghold of Legionella pneumophila. Int. J. Mol. Sci. 14, 21660–21675.

Abu Kweek, A., Amer, A.O., 2018. Factors mediating environmental biofilm formation by Legionella pneumophila. Front. Cell. Infect. Microbiol. 27.

Addiss, D.G., Davis, J.P., Laventure, M., Wand, P.J., Hutchinson, A., McKinney, R.M., 1989. Community-adquired Legionnaires' disease associated with a cooling tower: evidence for longer-distance transport of Legionella Pneumophila. Am. J. Epidemiol. 130, 557–568.

Alary, M., Roy, J., 1992. Factors contributing to the contamination of hospital water distribution systems by legionellae. J. Infect. Dis. 165, 565–569.

Amaral-Zettler, L.A., McCliment, E.A., Ducklow, H.W., Huse, S.M., 2009. A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. PLoS One 4. https://doi.org/10.1371/journal.pone.0006372.

American Public Health Association (APHA), American Water Works Association (AWWA), Water Environment Federation (WEF), 2017. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington DC https://doi.org/10.2105/SMWW.2882.078.

Armon, R., Starosvetzky, J., Arbel, T., Green, M., 1997. Survival of Legionella pneumophila and Salmonella typhimurium in biofilm systems. Water Sci. Technol. 35, 293–300.

Ashbolt, N.J., 2015. Environmental (saprozoic) pathogens of engineered water systems: understanding their ecology for risk assessment and management. Pathogens 4, 390–405. https://doi.org/10.3390/pathogens4020390.

ASHRAE, 2008. Cooling towers. 2008 ASHRAE Handbook: Heating, Ventilating, and Air-conditioning Systems and Equipment. ASHRAE, Atlanta, GA.

Atlas, R.M., Williams, J.F., Huntington, M.K., 1995. Legionella contamination of dental-unit waters. Appl. Environ. Microbiol. 61, 1208–1213.

Beauté, J., 2013. Legionnaires' disease in Europe, 2009–2010. Euro Surveill 18, 10.

Beauté, J., 2017. Legionnaires' disease in Europe, 2011 to 2015. Euro Surveill 22. https://doi.org/10.2807/1560-7917.ES.2017.22.27.30566.

Bedard, E., Fey, S., Charron, D., Lalancette, C., Cantin, P., Dolce, P., Laferriere, C., Deziel, E., Prevost, M., 2015. Temperature diagnostic to identify high risk areas and optimize Legionella pneumophila surveillance in hot water distribution systems. Water Res. 71, 244–256. https://doi.org/10.1016/j.watres.2015.01.006.

Bedard, E., Laferriere, C., Deziel, E., Prevost, M., 2018. Impact of stagnation and sampling volume on water microbial quality monitoring in large buildings. PLoS One 13. https://doi.org/10.1371/journal.pone.0199429.

Bell, J.C., Jorm, L.R., Williamson, M., Shaw, N.H., Kazandjian, D.L., Chiew, R., Capon, A.G., 1996. Legionellosis linked with a hotel car park–how many were infected? Epidemiol. Infect. 116, 185–192.

Berk, S.G., Faulkner, G., Garduno, E., Joy, M.C., Ortiz-Jimenez, M.A., Garduno, R.A., 2008. Packaging of live Legionella pneumophila into pellets expelled by Tetrahymena spp. does not require bacterial replication and depends on a Dot/Icm-mediated survival mechanism. Appl. Environ. Microbiol. 74, 2187–2199. https://doi.org/10.1128/AEM.01214-07.

Bhopal, R.S., Fallon, R.J., Buist, E.C., Black, R.J., Urquhart, J.D., 1991. Proximity of the home to a cooling tower and risk of non-outbreak Legionnaires' disease. BMJ 302, 378–383.

Borella, P., Montagna, M.T., Stampi, S., Stancanelli, G., Romano-Spica, V., Triassi, M., Marchesi, I., Bargellini, A., Tato, D., Napoli, C., Zanetti, F., Leoni, E., Moro, M., Scaltriti, S., Ribera D'Alcala, G., Santarpia, R., Boccia, S., 2005. Legionella contamination in hot water of Italian hotels. Appl. Environ. Microbiol. 71, 5805–5813. https://doi.org/10.1128/AEM.71.10.5805-5813.2005.

Boucher, D., Jardillier, L., Debroas, D., 2006. Succession of bacterial community composition over two consecutive years in two aquatic systems: a natural lake and a lake-reservoir. FEMS Microbiol. Ecol. 55, 79–97. https://doi.org/10.1111/j.1574-6941.2005.00011.x.

Bouyer, S., Imbert, C., Rodier, M.H., Hechard, Y., 2007. Long-term survival of Legionella pneumophila associated with Acanthamoeba castellanii vesicles. Environ. Microbiol. 9, 1341–1344. https://doi.org/10.1111/j.1462-2920.2006.01229.x.

Breiman, R.F., Cozen, W., Fields, B.S., Mastro, T.D., Carr, S.J., Spika, J.S., Mascola, L., 1990. Role of air sampling in investigation of an outbreak of legionnaires' disease associated with exposure to aerosols from an evaporative condenser. J. Infect. Dis. 161, 1257–1261.

Brown, C.M., Nuorti, P.J., Breiman, R.F., Hathcock, A.L., Fields, B.S., Lipman, H.B., Llewellyn, A.C., Hofmann, J., C., M., 1999. A community outbreak of Legionnaires' disease linked to hospital cooling towers: an epidemiological method to calculate dose of exposure. Int. J. Epidemiol. 28, 353–359.

Buse, H.Y., Schoen, M.E., Ashbolt, N.J., 2012. Legionellae in engineered systems and use of quantitative microbial risk assessment to predict exposure. Water Res. 46, 921–933. https://doi.org/10.1016/j.watres.2011.12.022.

Buse, H.Y., Lu, J., Struewing, I.T., Ashbolt, N.J., 2014. Preferential colonization and release of Legionella pneumophila from mature drinking water biofilms grown on copper versus unplasticized polyvinylchloride coupons. Int. J. Hyg. Environ. Health 217, 219–225. https://doi.org/10.1016/j.ijheh.2013.04.005.

Buse, H.Y., Ji, P., Gomez-Alvarez, V., Pruden, A., Edwards, M.A., Ashbolt, N.J., 2017. Effect of temperature and colonization of Legionella pneumophila and Vermamoeba vermiformis on bacterial community composition of copper drinking water biofilms. Microb. Biotechnol. 10, 773–788. https://doi.org/10.1111/1751-7915.12457.

Carvalho, F.R., Nastasi, F.R., Gamba, R.C., Foronda, A.S., Pellizari, V.H., 2008. Occurrence and diversity of Legionellaceae in polar lakes of the Antarctic peninsula. Curr. Microbiol. 57, 294–300. https://doi.org/10.1007/s00284-008-9192-y.

Centers for Disease Control and Prevention, 2015. Notice to readers: Final 2014 reports on nationally notifiable inectious diseases. MMWR Morbidity and Mortality Weekly Report. 64, pp. 1019–1033.

Centers for Disease Control and Prevention, 2018. Legionnaires' Disease Surveillance Summary Report, United States, 2014–2015. Centers for Disease Control and Prevention, United States.

Chien, S.H., Hsieh, M.K., Li, H., Monnell, J., Dzombak, D., Vidic, R., 2012. Pilot-scale cooling tower to evaluate corrosion, scaling, and biofouling control strategies for cooling system makeup water. Rev Sci Instrum 83, 024101. https://doi.org/10.1063/1.3680563.

Cooper, I.R., Hanlon, G.W., 2010. Resistance of Legionella pneumophila serotype 1 biofilms to chlorine-based disinfection. J Hosp Infect 74, 152–159. https://doi.org/10.1016/j.jhin.2009.07.005.

Cruaud, P., Vigneron, A., Fradette, M.-S., Dorea, C.C., Culley, A.I., Rodriguez, M.J., Charette, S.J., 2019. Annual Protist community dynamics in a freshwater ecosystem undergoing contrasted climatic conditions: the Saint-Charles River (Canada). Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.02359.

Cunha, B.A., Burillo, A., Bouza, E., 2016. Legionnaires' disease. Lancet 387, 376–385. https://doi.org/10.1016/s0140-6736(15)60078-2.

Declerck, P., 2010. Biofilms: the environmental playground of Legionella pneumophila. Environ. Microbiol. 12, 557–566.

Dhariwal, A., Chong, J., Habib, S., King, I.L., Agellon, L.B., Xia, J., 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res. 45, 180–188. https://doi.org/10.1093/nar/gkx295.

Di Gregorio, L., Tandoi, V., Congestri, R., Rossetti, S., Di Pippo, F., 2017. Unravelling the core microbiome of biofilms in cooling tower systems. Biofouling 33, 793–806. https://doi.org/10.1080/08927014.2017.1367386.

Douterelo, I., Jackson, M., Solomon, C., Boxall, J., 2017. Spatial and temporal analogies in microbial communities in natural drinking water biofilms. Sci. Total Environ. 581–582, 277–288. https://doi.org/10.1016/j.scitotenv.2016.12.118.

Duda, S., Baron, J.L., Wagener, M.M., Vidic, R.D., Stout, J.E., 2015. Lack of correlation between Legionella colonization and microbial population quantification using heterotrophic plate count and adenosine triphosphate bioluminescence measurement. Environ. Monit. Assess. 187, 393. https://doi.org/10.1007/s10661-015-4612-5.

Elifantz, H., Horn, G., Ayon, M., Cohen, Y., Minz, D., 2013. Rhodobacteraceae are the key members of the microbial community of the initial biofilm formed in Eastern Mediterranean coastal seawater. FEMS Microbiol. Ecol. 85.

Entcheva-Dimitrov, P., Spormann, A.M., 2004. Dynamics and control of biofilms of the oligotrophic bacterium Caulobacter crescentus. J. Bacteriol. 186, 8254–8266.

Farhat, M., Moletta-Denat, M., Frère, J., Onillon, S., Trouilhé, M.-C., Robine, E., 2012. Effects of disinfection on Legionella spp., Eukarya, and biofilms in a hot water system. Appl. Environ. Microbiol. 78, 6850–6858. https://doi.org/10.1128/aem.00831-12.

Fields, B.S., 1996. The molecular ecology of legionellae. Trends Microbiol. 4, 286–290.

Fields, B.S., Nerad, T.A., Sawyer, T.K., King, C.H., BJ, M., Martin, W.T., Morrill, W.E., Sanden, G.N., 1990. Characterization of an axenic strain of Hartmannella vermiformis obtained from an investigation of nosocomial Legionellosis. The Journal of Protozoology 37, 581–583. https://doi.org/10.1111/j.1550-7408.1990.tb01269.x.

Fields, B.S., Benson, R.F., Besser, R.E., 2002. Legionella and Legionnaires' disease: 25 years of investigation. Clin. Microbiol. Rev. 15, 506–526. https://doi.org/10.1128/cmr.15.3.506-526.2002.

Fisman, D.N., Lim, S., Wellenius, G.A., Johnson, C., Britz, P., Gaskins, M., Maher, J., Mittleman, M.A., Spain, C.V., Haas, C.N., Newbern, C., 2005. It's not the heat, it's the humidity: wet weather increases legionellosis risk in the greater Philadelphia metropolitan area. J. Infect. Dis. 192, 2066–2073.

Fitzhenry, R., Weiss, D., Cimini, D., Balter, S., Boyd, C., Alleyne, L., Stewart, R., McIntosh, N., Econome, A., Lin, Y., Rubinstein, I., Passaretti, T., Kidney, A., Lapierre, P., Kass, D., Varma, J.K., 2017. Legionnaires' disease outbreaks and cooling towers, New York City, New York, USA. Emerg. Infect. Dis. 23. https://doi.org/10.3201/eid2311.161584.

Flemming, H.C., Percival, S.I., Walker, J.T., 2002. Contamination potential of biofilms in water distribution systems. Water Sci. Technol. 47.

Fliermans, C.B., 1996. Ecology of Legionella: from data to knowledge with a little wisdom. Microb. Ecol. 32, 203–228.

Fliermans, C.B., Cherry, B.W., Orrison, L.H., Smith, S.J., Tison, D.L., Pope, D.H., 1981. Ecological distribution of Legionella pneumophila. Appl. Environ. Microbiol. 41, 9–16.

Giambrone, G.P., 2013. National cost of hospitalization for Legionnaires' disease, 2001–2009. CSTE Annual Conference, Pasadena, CA.

Gouvernement du Québec, 2014. Décret 454-2014, 21 mai 2014. Règlements et autres actes. Loi sur le bâtiment (chapitre B-1.1). Code de sécurité modification concernant le Règlement modifiant le Code de sécurité. Gazette officielle du Québec, 28 mai 2014 146 (22).

Greig, J.E., Carnie, J.A., Tallis, G.F., Ryan, N.J., Tan, A.G., Gordon, I.R., Zwolak, B., LJ, A., Guest, C.S., Hart, W.G., 2004. An outbreak of Legionnaires' disease at the Melbourne aquarium, April 2000: investigation and case-control studies. Med. J. Aust. 180, 556–572.

Greub, G., Raoult, D., 2004. Microorganisms resistant to free-living amoebae. Clin. Microbiol. Rev. 17, 413–433. https://doi.org/10.1128/cmr.17.2.413-433.2004.

Guerrieri, E., Bondi, M., Sabia, C., de Niederhäusern, S., Borella, P., Messi, P., 2008. Effect of bacterial interference on biofilm development by Legionella pneumophila. Curr. Microbiol. 57, 532–536.

Hampton, L.M., Garrison, L., Kattan, J., Brown, E., Kozak-Muiznieks, N.A., Lucas, C., Fields, B., Fitzpatrick, N., Sapian, L., Martin-Escobar, T., Waterman, S., Hicks, L.A., Alpuche-Aranda, C., Lopez-Gatell, H., 2016. Legionnaires' disease outbreak at a resort in Cozumel, Mexico. Open Forum Infect Dis 3, ofw170. https://doi.org/10.1093/ofid/ofw170.

Hechard, Y., Ferraz, S., Bruneteau, E., Steinert, M., Berjeaud, J.M., 2006. Isolation and characterization of a Staphylococcus warneri strain producing an anti-Legionella peptide. FEMS Microbiol. Lett. 252.

Hojo, F., Sato, D., Matsuo, J., Miyake, M., Nakamura, S., Kunichika, M., Hayashi, Y., Yoshida, M., Takahashi, K., Takemura, H., Kamiya, S., Yamaguchi, H., 2012. Ciliates expel environmental Legionella-laden pellets to stockpile food. Appl. Environ. Microbiol. 78, 5247–5257. https://doi.org/10.1128/AEM.00421-12.

Hwang, C., Ling, F., Andersen, G.L., LeChevallier, M.W., Liu, W.T., 2012. Microbial community dynamics of an urban drinking water distribution system subjected to phases of chloramination and chlorination treatments. Appl. Environ. Microbiol. 78, 7856–7865. https://doi.org/10.1128/AEM.01892-12.

Isozumi, R., Ito, Y., Ito, I., Osawa, M., Hirai, T., Takakura, S., Iinuma, Y., Ichiyama, S., Tateda, K., Yamaguchi, K., Mishima, M., 2005. An outbreak of Legionella pneumonia originating from a cooling tower. Scand. J. Infect. Dis. 37, 709–711. https://doi.org/10.1080/00365540510012143.

Katz, S.M., Hashemi, S., Brown, K.R., Habib, W.A., Hammel, J.M., 2009. Pleomorphism of Legionella Pneumophila. Ultrastruct. Pathol. 6, 117–129. https://doi.org/10.3109/01913128409018566.

Kim, B.R., Anderson, J.E., Mueller, S.A., Gaines, W.A., Kendall, A.M., 2002. Literature review-efficacy of various disinfectants against Legionella in water systems. Water Res. 36, 4443–44444.

Klaucke, D.N., Vogt, R.L., Larue, D., Witherell, L.E., Orciari, L.A., Spitalny, K.C., Pelletier, R., Cherry, B.W., Novick, L.F., 1984. Legionnaires' disease: the epidemiology of two outbreaks in Burlington Vermont. Am. J. Epidemiol. 119, 382–391.

van der Kooij, D., Veenendaal, H.R., Scheffer, W.J., 2005. Biofilm formation and multiplication of Legionella in a model warm water system with pipes of copper, stainless steel and cross-linked polyethylene. Water Res. 39, 2789–2798. https://doi.org/10.1016/j.watres.2005.04.075.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl. Environ. Microbiol. 79, 5112–5120. https://doi.org/10.1128/AEM.01043-13.

Kurtz, J.B., Bartlett, C.L.R., Newton, U.A., White, R.A., Jones, N.L., 1982. Legionella pneumophila in cooling water systems: report of a survey of cooling towers in London and a pilot trial of selected biocides. J. Hyg. 88, 369–381.

Kyritsi, M.A., Mouchtouri, V.A., Katsiafliaka, A., Kolokythopoulou, F., Plakokefalos, E., Nakoulas, V., Rachiotis, G., Hadjichristodoulou, C., 2018. Clusters of healthcare-associated Legionnaires' disease in two hospitals of Central Greece. Case Rep Infect Dis 2018, 2570758. https://doi.org/10.1155/2018/2570758.

Lau, H.Y., Ashbolt, N.J., 2009. The role of biofilms and protozoa in Legionella pathogenesis: implications for drinking water. J. Appl. Microbiol. 107, 368–378. https://doi.org/10.1111/j.1365-2672.2009.04208.x.

Leoni, E., Legnani, P.P., Bucci Sabattini, M.A., Righi, F., 2001. Prevalence of Legionella Spp. in swimming pool environment. Water Res. 35, 3749–3753.

Leoni, E., Catalani, F., Marini, S., Dallolio, L., 2018. Legionellosis associated with recreational waters: a systematic review of cases and outbreaks in swimming pools, spa pools, and similar environments. Int. J. Environ. Res. Public Health 15. https://doi.org/10.3390/ijerph15081612.

Levesque, S., Plante, P.L., Mendis, N., Cantin, P., Marchand, G., Charest, H., Raymond, F., Huot, C., Goupil-Sormany, I., Desbiens, F., Faucher, S.P., Corbeil, J., Tremblay, C., 2014. Genomic characterization of a large outbreak of Legionella pneumophila serogroup 1 strains in Quebec City, 2012. PLoS One 9, e103852. https://doi.org/10.1371/journal.pone.0103852.

Lin, Y.E., Lu, V.M., Huang, H.I., Huang, W.K., 2007. Environmental survey of Legionella pneumophila in hot springs in Taiwan. J Toxicol Environ Health A 70, 84–87.

Liu, Y., Zhang, W., Sileika, T., Warta, R., Cianciotto, N.P., Packman, A.I., 2011. Disinfection of bacterial biofilms in pilot-scale cooling tower systems. Biofouling 27, 393–402.

Llewellyn, A.C., Lucas, C.E., Roberts, S.E., Brown, E.W., Nayak, B.S., Raphael, B.H., Winchell, J.M., 2017. Distribution of Legionella and bacterial community composition among regionally diverse US cooling towers. PLoS One 12, e0189937. https://doi.org/10.1371/journal.pone.0189937.

McCoy, W.F., Downes, E.L., Leonidas, L.F., Cain, M.F., Sherman, D.L., Chen, K., Devender, S., Neville, M.J., 2012. Inacuracy in Legionella tests of building water systems due to sample holding time. Water Res. 46, 3497–3506.

McDade, J.E., Brenner, D.J., Bozeman, F.M., 1979. Legionnaires' disease bacterium isolated in 1947. Ann. Intern. Med. 90, 659–661.

Messi, P., Anacarso, I., Bargellini, A., Bondi, M., Marchesi, I., de Niederhäusern, S., Borella, P., 2011. Ecological behaviour of three serogroups of Legionella pneumophila within a model plumbing system. Biofouling 27, 165–172.

Miao, L., Wang, P., Hou, J., Yao, Y., Liu, Z., Liu, S., Li, T., 2019. Distinct community structure and microbial functions of biofilms colonizing microplastics. Sci. Total Environ. 650, 2395–2402.

Mitchell, E., O'Mahony, M., Watson, M.J., Lynch, D., Joseph, C., Quigley, C., Aston, R., Constable, G.N., Farrand, R.J., Maxwell, S., Hutchinson, D.N., Craske, J., Lee, V., 1990. Two outbreaks of Legionnaires' disease in Bolton Health District. Epidemiol. Infect. 159–170.

Moffat, J.F., Tompkins, L.S., 1992. A quantitative model of intracellular growth of Legionella pneumophila in Acanthamoeba castellanii. Infect. Immun. 60, 296–301.

Moran-Gilad, J., Lazarovitch, T., Mentasi, M., Harrison, T., Weinberger, M., Mordish, Y., Mor, Z., Stocki, T., Anis, E., Sadik, C., Grotto, I., 2012. Humidifier-associated paediatric Legionnaires' disease, Israel, February 2012. Euro Surveill 14.

Moritz, M.M., Flemming, H.C., Wingender, J., 2010. Integration of Pseudomonas aeruginosa and Legionella pneumophila in drinking water biofilms grown on domestic plumbing materials. Int. J. Hyg. Environ. Health 213, 190–197. https://doi.org/10.1016/j.ijheh.2010.05.003.

Nguyen, T.M., Ilef, D., Jarraud, S., Rouil, L., Campese, C., C, D., Haehebaert, S., Ganiayre, F., Marcel, F., Etienne, J., Desenclos, J.C., 2006. A community-wide outbreak of legionnaires disease linked to industrial cooling towers- how far contaminated aerosols spread? J. Infect. Dis. 193, 102–111.

Ortiz-Roque, C., Hazen, T.C., 1987. Abundance and distribution of Legionellaceae in Puerto Rican waters. Appl. Environ. Microbiol. 53, 2231–2236.

Paranjape, K., Bedard, E., Whyte, L., Ronholm, J., Prevost, M., Faucher, S.P., 2020. Presence of Legionella spp. in cooling towers: the role of microbial diversity, Pseudomonas, and continous chlorine application. Water Res., 115252 https://doi.org/10.1101/540302.

Pereira, R.P.A., Peplies, J., Hofle, M.G., Brettar, I., 2017. Bacterial community dynamics in a cooling tower with emphasis on pathogenic bacteria and Legionella species using universal and genus-specific deep sequencing. Water Res. 122, 363–376. https://doi.org/10.1016/j.watres.2017.06.011.

Piao, Z., Sze, C.C., Barysheva, O., Iida, K.-C., Yoshida, S., 2006. Temperature-regulated formation of mycelial mat-like biofilms by Legionella pneumophila. Appl. Environ. Microbiol. 72, 1613–1622.

Proctor, C.R., Gächter, M., Kötzsch, S., Rölli, F., Sigrist, R., Walser, J.-C., Hammes, F., 2016. Biofilms in shower hoses – choice of pipe material influences bacterial growth and communities. Environmental Science: Water Research & Technology 2, 670–682. https://doi.org/10.1039/c6ew00016a.

Proctor, C.R., Dai, D., Edwards, M.A., Pruden, A., 2017. Interactive effects of temperature, organic carbon, and pipe material on microbiota composition and Legionella pneumophila in hot water plumbing systems. Microbiome 5, 130. https://doi.org/10.1186/s40168-017-0348-5.

Proctor, C.R., Reimann, M., Vriens, B., Hammes, F., 2018. Biofilms in shower hoses. Water Res. 131, 274–286.

Rhoads, W.J., Pruden, A., Edwards, M.A., 2017. Interactive effects of corrosion, copper, and chloramines on Legionella and mycobacteria in hot water plumbing. Environ Sci Technol 51, 7065–7075. https://doi.org/10.1021/acs.est.6b05616.

Rogers, J., Keevil, C.W., 1992. Immunogold and fluorescein immunolabelling of Legionella pneumophila within an aquatic biofilm visualized by using episcopic differential interference contrast microscopy. Appl. Environ. Microbiol. 58, 2326–2330.

Rogers, J., Dowsett, B.A., Dennis, P.J., Lee, J.V., Keevil, C.W., 1994a. Influence of temperature and plumbing material selection on biofilm formation and growth of Legionella pneumophila in a model potable water system containing complex microbial Flora. Appl. Environ. Microbiol. 60.

Rogers, J., Dowsett, B.A., Dennis, P.J., Lee, V., Keevil, C.W., 1994b. Influence of plumbing materials on biofilm formation and growth on Legionella pneumophila in potable water systems. Appl. Environ. Microbiol. 60, 1842–1851.

Rowbotham, J., 1980. Preliminary report on the pathogenicity of Legionella pneumophila for freshwater and soil amoebae. J. Clin. Pathol. 33, 1179–1183. https://doi.org/10.1136/jcp.33.12.1179.

Saleem, M., Fetzer, I., Harms, H., Chatzinotas, A., 2013. Diversity of protists and bacteria determines predation performance and stability. The ISME journal 7, 1912–1921.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75, 7537–7541. https://doi.org/10.1128/AEM.01541-09.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garett, W.S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12.

Serrano-Suarez, A., Dellunde, J., Salvado, H., Cervero-Arago, S., Mendez, J., Canals, O., Blanco, S., Arcas, A., Araujo, R., 2013. Microbial and physicochemical parameters associated with Legionella contamination in hot water recirculation systems. Environ. Sci. Pollut. Res. Int. 20, 5534–5544.

Shaheen, M., Scott, C., Ashbolt, N.J., 2019. Long-term persistence of infectious Legionella with free-living amoebae in drinking water biofilms. Int. J. Hyg. Environ. Health 222, 678–686.

Sheehan, K.B., Henson, J.M., Ferris, M.J., 2005. Legionella species diversity in an acidic biofilm Community in Yellowstone National Park. Appl. Environ. Microbiol. 71, 507–511.

Shelton, B.G., Flanders, W.D., Morris, G.K., 1994. Legionnaires' disease outbreaks and cooling towers with amplified Legionella concentrations. Curr. Microbiol. 28, 359–363.

Simões, M., Simões, L.C., Vieira, M.J., 2010. A review of current and emergent biofilm control strategies. LWT- Food Science and Technology 43, 573–583.

Smith, S.S., Ritger, K., Samala, U., Black, S.R., Okodua, M., Miller, L., Kozak-Muiznieks, N.A., Hicks, L.A., Steinheimer, C., Ewaidah, S., Presser, L., Siston, A.M., 2015. Legionellosis outbreak associated with a hotel fountain. Open Forum Infect Dis 2, ofv164. https://doi.org/10.1093/ofid/ofv164.

Springston, J.P., Yocavitch, L., 2017. Existence and control of Legionella bacteria in building water systems: a review. J. Occup. Environ. Hyg. 14, 124–134. https://doi.org/10.1080/15459624.2016.1229481.

Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M.D.M., Breiner, H., Richards, T.A., 2010. Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. Mol. Ecol. 19, 21–31. https://doi.org/10.1111/j.1365-294X.2009.04480.x.

Stout, J.E., Yu, V.L., Yee, Y.C., Vaccarello, S., Diven, W., Lee, T.C., 1992. Legionella pneumophila in residential water supplies: environmental surveillance with clinical assessment for Legionnaires' disease. Epidemiol. Infect. 109, 49–57.

Taravaud, A., Ali, M., Lafosse, B., Nicolas, V., Feliers, C., Thibert, S., Levi, Y., Loiseau, P.M., Pomel, S., 2018. Enrichment of free-living amoebae in biofilms developed at upper water levels in drinking water storage towers: an inter- and intra-seasonal study. Sci. Total Environ. 633, 157–166. https://doi.org/10.1016/j.scitotenv.2018.03.178.

Taylor, M., Ross, K., Bentham, R., 2013. Spatial arrangement of legionella colonies in intact biofilms from a model cooling water system. Microbiol Insights 6, 49–57. https://doi.org/10.4137/MBI.S12196.

Thomas, V., Bouchez, T., Nicolas, V., Robert, S., Loret, J.F., Levi, Y., 2004. Amoebae in domestic water systems: resistance to disinfection treatments and implication in Legionella persistence. J. Appl. Microbiol. 97, 950–963. https://doi.org/10.1111/j.1365-2672.2004.02391.x.

Thomas, V., Loret, J.F., Jousset, M., Greub, G., 2008. Biodiversity of amoebae and amoebae-resisting bacteria in a drinking water treatment plant. Environ. Microbiol. 10, 2728–2745. https://doi.org/10.1111/j.1462-2920.2008.01693.x.

Tison, D.L., Pope, D.H., Cherry, B.W., 1980. Growth of Legionella pneumophila in association with blue-green algae (cyanobacteria). Appl. Environ. Microbiol. 39, 456–459.

Tsao, H.F., Scheikl, U., Herbold, C., Indra, A., Walochnik, J., Horn, M., 2019. The cooling tower water microbiota: seasonal dynamics and co-occurrence of bacterial and protist phylotypes. Water Res. 159, 464–479. https://doi.org/10.1016/j.watres.2019.04.028.

Turetgen, I., Cotuk, A., 2007. Monitoring of biofilm-associated Legionella pneumophila on different substrata in model cooling tower system. Environ. Monit. Assess. 125, 271–279. https://doi.org/10.1007/s10661-006-9519-8.

Wadowsky, R.M., Yee, R.B., 1983. Satellite growth of Legionella pneumophila with an environmental isolate of Flavobacterium breve. Appl. Environ. Microbiol. 46, 1447–1449.

Wadowsky, R.M., Wolford, R., McNamara, A.M., Yee, R.B., 1985. Effect of temperature, pH, and oxygen level on the multiplication of naturally Occuring Legionella pneumophila in potable water. Appl. Environ. Microbiol. 49, 1197–1205.

Walser, S.M., Gerstner, D.G., Brenner, B., Höller, C., Liebl, B., Herr, C.E., 2014. Assessing the environmental health relevance of cooling towers–a systematic review of legionellosis outbreaks. Int. J. Hyg. Environ. Health 217, 145–154.

Wang, H., Masters, S., Edwards, M.O., Falkinham, J.O., Pruden, A., 2014. Effect of disinfectant, water age and pipe materials on bacterial and eukaryotic community structures in drinking water biofilm. Environ Sci Technol 48, 1426–1435. https://doi.org/10.1021/es402636u.

Whiley, H., 2016. Legionella risk management and control in potable water systems: argument for the abolishment of routine testing. Int. J. Environ. Res. Public Health 14. https://doi.org/10.3390/ijerph14010012.

Whiley, H., Keegan, A., Fallowfield, H., Ross, K., 2014. Uncertainties associated with assessing the public health risk from Legionella. Front. Microbiol. 5, 501. https://doi.org/10.3389/fmicb.2014.00501.

White, P.S., Graham, F.F., Harte, D., Baker, M., Ambrose, C., Humphrey, A., 2013. Epidemiological investigation of a Legionnaires' disease outbreak in Christchurch,New Zealand: the value of spatial methods for practical public health. Epidemiol. Infect. 141, 789–799.

WHO, 2007. In: Bartram, J., Chartier, Y., Lee, J., Pond, K., Surman-Lee, S. (Eds.), Legionella and the Prevention of Legionellosis. World Health Organization.

Wingender, J., Flemming, H.C., 2011. Biofilms in drinking water and their role as reservoir for pathogens. Int. J. Hyg. Environ. Health 214, 417–423.

Yamamoto, Y., Sugiura, M., Kusunoki, S., Ezaki, T., Ikedo, M., Yabbuchi, E., 1992. Factors stimulating propagation of legionellae in cooling tower water. Appl. Environ. Microbiol. 58, 1394–1397.

Zhang, C., Li, C., Zheng, X., Zhao, J., He, G., Zhang, T., 2016. Effect of pipe materials on chlorine decay, trihalomethanes formation, and bacterial communities in pilot-scale water distribution systems. Int. J. Environ. Sci. Technol. 14, 85–94. https://doi.org/10.1007/s13762-016-1104-2.

Zwart, G., Crump, B., Kamst-van Agterveld, M.P., H., F., Han, S.-K., 2002. Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquat. Microb. Ecol. 28, 141–155.

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Legionella pneumophila levels and sequence-type distribution in hospital hot water samples from faucets to connecting pipes

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ABSTRACT

Recent studies have reported increased levels of *Legionella pneumophila* (*Lp*) at points of use compared to levels in primary and secondary components of hot water systems, suggesting possible selection by environmental conditions. In this study, concentrations of *Lp* in a hospital hot water system were evaluated by profile sampling, collecting successive water samples to determine the prevalence at the faucet (distal) and upstream piping before and after a system intervention to increase temperature. *Lp* strain diversity was compared between different points of use and different areas of the hot water system (i.e., tap, intermediate piping and main upflow piping). In total, 47 isolates were recovered from 32 positive hot water samples collected from designated taps, showers and recirculation loops; these isolates were subsequently analyzed by sequence-based typing (SBT). *Lp* levels were comparable between first draw (500 mL) and flushed (2 and 5 min) samples, whereas a decrease was observed in the amount of culturable cells (1 log). Two sequence types (STs) were identified throughout the system. ST378 (sg4/10) was present in 91% of samples, while ST154-like (sg1) was present in 41%; both STs were simultaneously recovered in 34% of samples. Isolated STs displayed comparable tolerance to copper (0.8 −5 mg/L) and temperature (55 °C, 1 h) exposure. The ability to replicate within THP1 cells and *Acanthamoeba castellanii* was similar between the two STs and a comparative environmental outbreak strain. The low *Lp* diversity and the detection of both *Lp* sequence types in repeated subsequent samples collected from positive faucets in a hospital wing suggest a minimal impact of the distal conditions on strain selection for the sampled points, as well as a possible adaptation to stressors present in the system, leading to the predominance of a few strains.

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1. Introduction

A marked increase in *Legionella pneumophila* infections has been reported over the last decade, as shown by the 286% increase in cases of legionellosis observed in the US between 2000 and 2014 (Garrison et al., 2016). Similarly, the number of Legionnaires' disease cases in Europe steadily increased between 2011 and 2016, with 81% of these due to *L. pneumophila* (European Centre for Disease Prevention and Control (ECDC) (2017)). An estimated mortality rate of 8% has been associated with legionellosis (Centers for Disease Control and Prevention (CDC) 2017, European Centre for Disease Prevention and Control (ECDC) 2017), reaching as high as 25% in healthcare-associated outbreaks (Soda et al., 2017). In the United States, *Legionella* was the most-reported cause of outbreaks associated with drinking water from 2013 to 2014, causing the majority of hospitalizations (88%) and all deaths associated with drinking water outbreaks (Benedict et al., 2017). *Legionella* is known to proliferate in engineered water systems, such as cooling towers and large-building water distribution systems (Buse et al., 2012). Although cooling-tower associated outbreaks generally

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result in larger case clusters, potable water is nevertheless the most frequent reported source of exposure resulting in an infection by *L. pneumophila* (Garrison et al., 2016).

Opportunistic microbial pathogens are present and can be amplified in the plumbing system of large buildings, posing a health risk for vulnerable individuals. Conditions present in the plumbing of large buildings, such as elevated stagnation, sporadic water use, variable hydraulic regimes, large surface-to-volume ratios, biofilm formation and variable temperatures can provide favorable conditions for *L. pneumophila* (Flemming and Bendinger, 2014). In healthcare facilities, hot water systems feeding taps and showers are reported to have a higher prevalence of *L. pneumophila* relative to other *Legionella* species (Bargellini et al., 2011; Boppe et al., 2016; Marchesi et al., 2011). High levels of contamination measured at the point of utilization suggests a distal amplification of *L. pneumophila* of up to 100-fold compared to levels in the hot water system (Boppe et al., 2016; Cristina et al., 2014). Similarly, heterotrophic plate counts (HPCs) can increase 1 to 3 log-fold in distal volume samples compared to levels found in 2–5-min flushed water, depending on the system configuration and prior stagnation (Bagh et al., 2004; Bédard et al., 2018; Cristina et al., 2014; Lautenschlager et al., 2010). The source of *Legionella* at distal points of the system, such as the faucet and its immediate connecting pipes, is primarily the hot water system (Bédard et al., 2016b; Cristina et al., 2014) and possibly the cold water system (Donohue et al., 2014; Marciano-Cabral et al., 2010; Pryor et al., 2004). Several potential causes of *L. pneumophila* amplification in hot water systems have been identified, including materials favorable to biofilm growth (Lu et al., 2014; Moritz et al., 2010), stagnation (Lu et al., 2017; Rhoads et al., 2015), and (most frequently) temperature and copper concentrations (Boppe et al., 2016; Dai et al., 2018; Lu et al., 2014). In a large building hot water system, these factors generally vary across the system, especially environmental factors like residual oxidants, copper concentrations and temperature, which often closely reflect stagnation. Furthermore, the selective amplification of distinct *L. pneumophila* strains between the faucet, its connection piping and the hot water system has not been established. Additionally, it is not known whether different *L. pneumophila* sequence types can be recovered in distal vs. flushed samples, or if strain selection varies from one faucet to another.

Municipal and building water systems can be colonized by multiple *L. pneumophila* sequence types (STs). Several studies have reported a low number of dominant environmental strains within a system (Byrne et al., 2018; David et al., 2017; Levesque et al., 2014; Oberdorfer et al., 2008; Qin et al., 2014). The prevalence of one ST can be driven by its superior adaptation to the specific conditions within its environment. Adaptation to new man-made environmental niches may be responsible for the recent independent geographical emergence of a few dominant disease-causing STs (David et al., 2016). Strains exposed to drinking water stressors, such as nutrient-poor conditions, high temperatures, and high copper and chlorine levels may adapt to these conditions and thrive in this environment over time (Al-Bana et al., 2014; Allegra et al., 2011; Boppe et al., 2016; Cervero-Arago et al., 2015). The infectivity of such environmental strains is often unknown, especially in the absence of detected clinical cases (Sharaby et al., 2018; Sousa et al., 2018). Conversely, the presence of host cells and the capacity of *L. pneumophila* strains to multiply within these cells may increase levels of contamination and risk of infection.

The main objective of this study was to compare *L. pneumophila* levels of contamination and strain diversity between the faucet and the hot water system in a hospital wing with elevated *L. pneumophila* contamination. Understanding the distribution of *L. pneumophila* contamination from faucets to system piping and if certain sequence types are specific to the faucet volume, will allow the optimization of corrective measures. The secondary objectives were to: 1) quantify the presence of *L. pneumophila* in various sections of the hot water system using profile sampling; 2) evaluate the impact of a system intervention to increase temperature on the STs recovered in distal and flushed samples; 3) evaluate the tolerance of prevalent STs to copper and control temperature exposure; and 4) verify the potential for infectivity of the prevalent STs.

2. Methods

2.1. Environmental sampling

This study was performed in the summer of 2016 in a ten-story, 450-bed hospital fed by chlorinated surface-filtered drinking water. The mean incoming municipal water temperature was 26.2 °C, with a measured residual chlorine level of 0.4 mg Cl_2/L, an average heterotrophic plate count of 9.5 CFU/mL, and 1.8×10^3 viable cells/mL. The mean water temperature directly out of the boiler feeding into the hot water system was 61.6 °C, with very low residual chlorine concentrations ($\leq$0.1 mg Cl_2/L). Hot water was produced by a flash water heater, fed to the main horizontal flow and return loops, distributed to each wing through horizontal secondary flow and return loops, and fed to the point of use through 9 to 21 vertical risers per wing, as previously described (Boppe et al., 2016, Fig. 1). Following an environmental investigation of the hot water system, a high incidence of *L. pneumophila* was detected in Wing 3. In this wing, the mean maximum hot water temperature measured at the point of use (49.6 °C) was below the required 55 °C (Boppe et al., 2016; RBQ, 2014). All 18 sampled points of use were positive for *L. pneumophila*. As a result, an intervention was conducted on the system to improve hydraulics and increase temperature at the faucet (Boppe et al., 2016). Briefly, the main recirculating pump efficiency was improved, and flow restriction was implemented on hydraulically advantaged wings, resulting in an increased water recirculation velocity in Wing 3.

Three positive faucets located in Wing 3 with the highest *L. pneumophila* contamination were selected for in-depth investigation to understand whether the *L. pneumophila* contamination was distal or systemic. Initial positivity was assessed via 500 mL first-draw hot water samples. The first faucet (F1) was fed by riser A, located close to the entrance of Wing 3 on the 6th floor (Fig. 1). The second and third faucets were each fed by riser B, located further into Wing 3 on the 2nd and 7th floor, respectively (Fig. 1). Sampling events were conducted before (April 26th to 28th, 2016) and after (May 2nd to 9th, 2016) the system intervention after an overnight stagnation. For each sampling at a faucet, three successive separate volumes were sampled immediately after opening the faucet in sterile propylene bottles: 1st volume of 500 mL, a 2nd volume of 500 mL and a 3rd volume of 1 L. Two additional 1 L samples were collected after 2 min and 5 min of flow. Sampling flow rates were between 3 and 5 L/min. A total of 30 water samples were collected and analyzed. Sample volumes and flush times were selected based on the studied system architecture in order to be representative of different sections within the building plumbing (Fig. 1): the first volume (500 mL) corresponds to the faucet and the section of plumbing specific to that faucet; the second (500 mL) and third (1 L) volumes represent water from the vertical flow and return loop adjacent to the sampled faucet; the fourth volume (2-min flush) represents the water in the vertical flow and return loop; and the last sample (5-min flush) is representative of the main horizontal pipe.

Water temperature, maximum hot water temperature, pH, turbidity and residual chlorine levels were also measured after 2 L and after 5 min for each sampling event at faucets, and after 0.5 L in

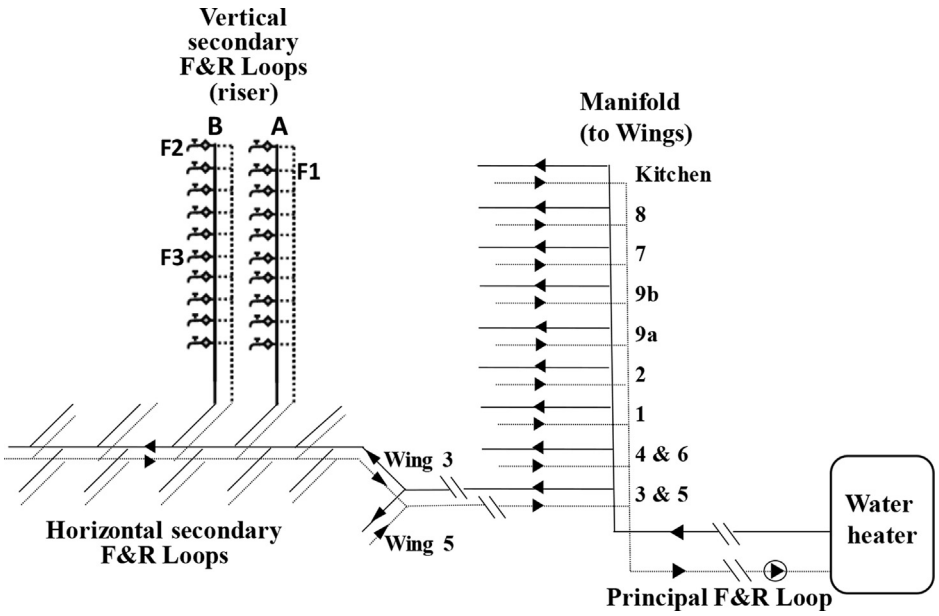

Figure 1. Hot water system schematic from the water heater to the studied faucets in Wing 3 (F1, F2, F3). F&R: Flow and return loop.

the additional locations. pH was measured using a Hach Multi-Parameter HQ40d tool with a pH probe PHC301, turbidity was measured with a Hach 2100Q (Hach, Loveland, CO, USA) and residual chlorine levels were measured using a Pocket Colorimeter™ II (Hach, Love-land, CO, USA).

2.2. Microbiological detection methods

All samples were analyzed within 24 h of sampling. Heterotrophic plate counts (HPCs) were performed according to method 9215-D on R2A agar plates at 22 °C with 7 days of incubation (American Public Health Association (APHA), 2005). Viable and total cell counts were determined by fluorescence microscopy following staining with a LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, USA) (Boulos et al., 1999). This kit differentiates intact (viable) cells from damaged cells using membrane integrity criteria. Briefly, 1 mL of sample was mixed with 3 µl of stain (propidium iodide and SYTO9), incubated in the dark for 15 min and filtered on a black 0.2 µm-pore diameter, 25 mm diameter polycarbonate filter (Millipore, Bedford, USA). Enumeration was performed with an epifluorescence microscope (Olympus, Japan) at 1000-fold magnification across ten fields of view. Total bacterial cells were defined as the sum of intact (green) and damaged (red) cells. When the cell count was too numerous to count, a dilution of the sample was performed in 0.85% sterile saline solution.

Water samples were cultured according to the quantitative method AFNOR NF T90-431 *Legionella* procedure, as previously described (Boppe et al., 2016), with a detection limit of 10 CFU/L. Briefly, volumes of water were filtered through sterile 0.45 µm membranes (Millipore, Germany), treated with acid (pH = 2; 5 min) and plated on Glycine Vancomycin Polymyxin Cycloheximide (GVPC)-selective agar (Biokar diagnostics, France). An untreated sample volume of 0.2 mL was plated directly. All plates were then incubated at 36 °C for up to 10 days. Typical colonies that developed after 4–10 days were sub-cultured on confirmation plates for 2–4 days at 36 °C. Resulting colonies that developed on Buffered Charcoal Yeast Extract (BCYE) agar, but not on BCYE without cysteine, were considered *Legionella* spp. The *Legionella* latex test (M45, Microgen bioproducts) was used for *L. pneumophila* confirmation. Confirmed *L. pneumophila* colonies were stored in 15% glycerol at −80 °C.

2.3. Sequence-based typing

In total, 41 *L. pneumophila* isolates recovered from the three studied faucets and six other isolates recovered from three first-draw 500 mL shower water samples and the hot water recirculation system (Boppe et al., 2016) were sequenced. The showers were located in different wings of the hospital, i.e., Wings 1, 2 and 6. All isolates were genotyped using sequence-based typing (SBT) according to the European Working Group for Legionella infections (EWGLI) protocol (Gaia et al., 2005). Briefly, genomic DNA was extracted from isolated colonies. Amplification of seven reference genes (*flaA, pilE, asd, mip, momps, proA, neuA*) was performed by PCR using primers as previously described (Mentasti and Fry, 2012). Amplicons were purified and sequenced by Genome Québec. Resulting sequences obtained via Sanger sequencing were trimmed and compared to previously assigned allele numbers using the EWGLI *Legionella pneumophila* Sequence-based Typing module. A 7-digit allelic profile was defined for each isolate, and Sequence Type (ST) was assigned using the EWGLI database. An isolate representative of each identified ST was serotyped using the agglutination test. For serogroups other than 1, further testing was performed in collaboration with the National Microbiology laboratory in Winnipeg, Canada, to determine the serogroup via agglutination test and direct fluorescence assay (Prolab, Richmond Hill On).

2.4. Copper and temperature resistance of L. pneumophila isolates

The tolerance of each representative ST strain to copper and temperature exposure was evaluated. Briefly, cells cultured in CYE were washed three times and starved for one week in synthetic water prior to inoculation in 20 mL of sterile synthetic water at pH 7.8 (estimated final concentration = 1×10^8 CFU/mL). Various copper concentrations (i.e., 0, 0.8, 1.3, 2 and 5 mg/L) were evaluated using copper chloride as the source of copper. A sample without

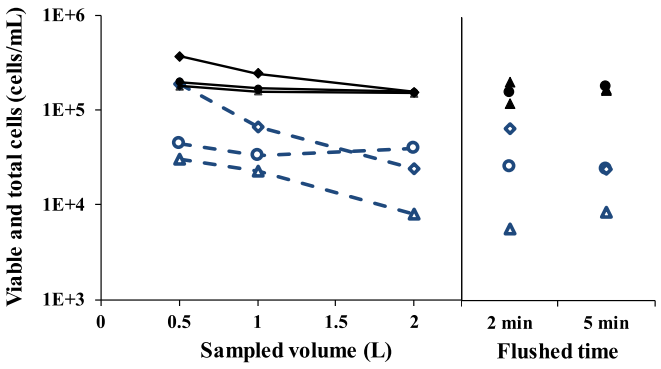

Fig. 2. Total (black line) and viable (dotted blue line) cell counts measured in water samples collected from three faucets, from first draw to 5-min flush. Cell counts were measured at 3 consecutive flushed volumes during the first draw and after flushing for 2 and 5 min. Faucets are represented by different symbols: F1 - triangles; F2 - circles; F3 - diamonds. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

copper was treated for 1 h at 55 °C to assess the impact of recirculating loop temperatures on these *L. pneumophila* strains. Samples were maintained at 36 °C after heat treatment or copper addition to simulate water temperatures at the faucet or in a shower. The survival of *L. pneumophila* isolates exposed to stress was monitored over time (at 1 h, 6 h, 24 h, 48 h, 72 h, 168 h, and 336 h) on BCYE plates. Water without cells was used as a negative control.

2.5. Intracellular replication

Intracellular replication of different strains of *L. pneumophila* was evaluated using THP1-derived human macrophages (ATCC TIB-202) and the amoeba *Acanthamoeba castellanii* (ATCC 30234). The laboratory wild-type strain JR32, derived from *L. pneumophila* strain Philadelphia-1, and a *dotA* mutant served as positive and negative controls, respectively. THP1 monocytes were cultured in 25 mL of RPMI (GIBCO) supplemented with L-glutamine and 10% heat-inactivated fetal bovine serum at 37 °C with 5% CO_2 for two

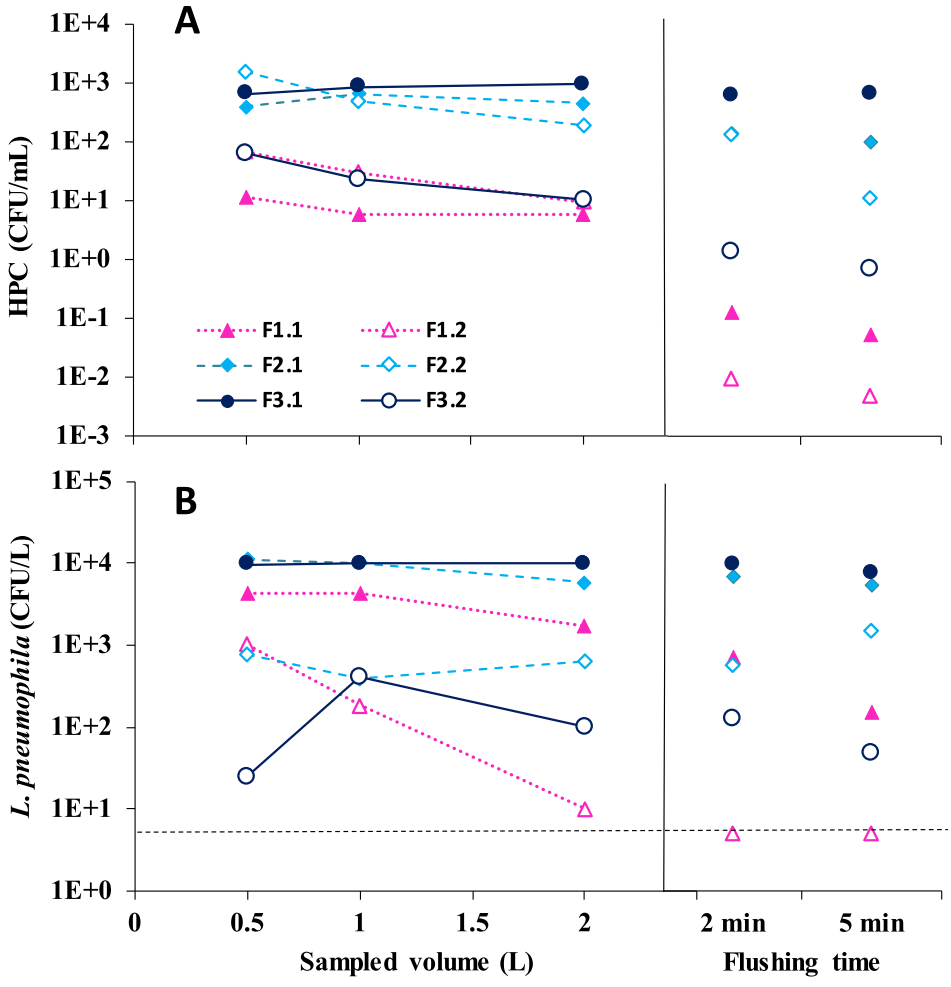

Fig. 3. Heterotrophic plate counts (HPC, A) and *L. pneumophila* culturable cells (B) detected in water samples from 3 faucets. Each faucet was sampled twice (filled symbols, before system intervention, and empty symbols, after system intervention). The results from the 3 faucets are shown by different lines and colors (F1 – pink dotted line; F2 – teal dashed line; F3 – navy full line). The dotted line in B represents the limit of detection for the *L. pneumophila* culture method (10 CFU/L). The results after the 2- and 5-min flushes for F1 after the intervention were below the limit of detection and are represented as 0.5 LD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

weeks. THP-1 cells were seeded in 24-well plates at a concentration of 5×10^5 cells/mL and differentiated into macrophages with 10^{-7} mol/L phorbol myristate acetate. After 48 h, the cells were washed, and fresh medium was added before infection. Amoeba were grown in 25 mL of Peptone Yeast Glucose (PYG) broth at 30 °C in T75 flasks and seeded in 24-well plates at a concentration of 2.5×10^5 cells/ml. Cells were infected after 24 h. Macrophages and amoeba were infected with 5×10^4 *L. pneumophila* cells/mL (multiplicity of infection: 0.1) and incubated in the same conditions as previously described. *L. pneumophila* levels were measured every day for four days using CFU counts on BCYE (buffered charcoal yeast extract) agar.

2.6. Statistical analysis

Statistical significance was assessed using the Mann-Whitney test and the Wilcoxon matched paired test. Statistica 13.3 and GraphPad Prism 7.04 were used for statistical calculations. The results were considered significant at $p < 0.05$.

3. Results

3.1. Microbial characterization of investigated faucets

Levels of viable and total cells were not significantly different between the different consecutive volumes sampled from the different faucets (Fig. 2). In general, HPC values in the first 2 L sampled were significantly different than those obtained after 2 and 5-min flushing ($p = 0.03$, Fig. 3). HPC levels and profiles were comparable before and after the system intervention for faucets F1 and F2. However, the HPC level was significantly different between F1 and F2 ($p = 0.005$), suggesting a source of contamination specific to the faucet. In faucet F3, HPC levels were significantly different between sampling events ($p = 0.008$, Fig. 3A). Faucet F3 had the largest temperature increase after the 5-min flush (>10 °C) compared to a temperature increase of 2 and 4 °C for faucets F1 and F2, respectively.

As opposed to HPC, *L. pneumophila* levels were not significantly higher in the distal volume compared to the flushed samples (Fig. 3B). No *L. pneumophila* was detected in faucet F1 after the system intervention for the 2 and 5-min flushed samples. Faucet F1 was fed by a separate vertical flow and return loop than faucets F2 and F3 (Fig. 1). In general, levels of *L. pneumophila* at each faucet were significantly lower ($p < 0.0001$) after the intervention (Fig. 4). Following these sampling campaigns, the hot water system temperature and hydraulics were further optimized, leading to a substantial reduction in *L. pneumophila* positivity and concentration in hot water (Boppe et al., 2016).

3.2. Spatial distribution of recovered L. pneumophila STs

The sequence type was determined for 41 isolates from the three studied faucets and 6 isolates from the shower and recirculation system samples (Table 1). The latter was performed to investigate the distribution of the sequence type found in Wing 3. Thirty-one isolates were identified as ST378, and one isolate was identified as ST378-like since *flaA* could not be amplified. These 32 isolates were serotyped as sg4/10. It was impossible to amplify the *flaA* gene with previously reported primers (Gaia et al., 2005) for the 15 other isolates (Table 1). They were identified as ST154-like based on a 6-allele profile. According to the EWGLI database, 43 of 51 entries harboring the same 6-allele profile were associated with ST154. The ST154-like isolates were serotyped as sg1 based on agglutination test results.

ST378 isolates were recovered from 90.6% of positive water samples compared to 40.6% for ST154-like isolates. In addition, ST378 was the lone isolate recovered in 56.3% of water samples compared to 6.3% for ST154-like isolates. Although ST378 (sg4/10) was dominant, both STs were shown to be detected in at least one sample from each of the 3 faucets and in shower S1 (Fig. 5, Table 1). There was no difference in ST distribution between first water volume of 500 mL and subsequent volumes for the studied faucets (Fig. 5), suggesting that the local conditions present within the faucet did not impact the presence or ratio of the STs established within the Wing 3 hot water system main horizontal pipe. In addition, the intervention did not change the prevalence of the STs (Fig. 5). The presence of both STs in showers located in three other wings of the building suggest that those STs were not limited to the studied wing. Furthermore, despite a low positivity rate (≤250 UFC/L), an isolate identified as ST154-like was recovered from water sampled directly from the main recirculation loop.

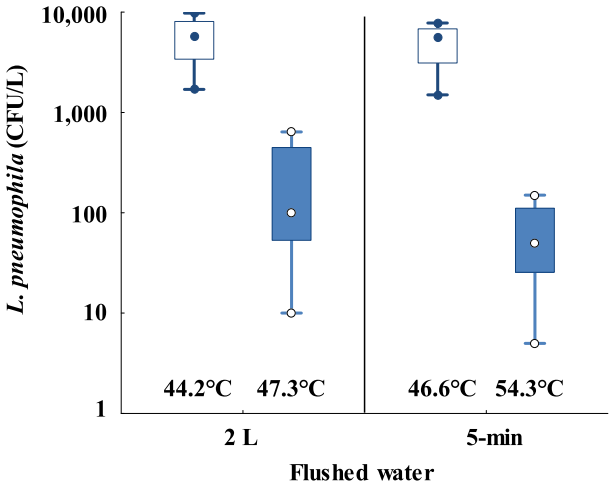

Fig. 4. Measured concentrations of *L. pneumophila* in water in samples collected after 2 L and after 5-min flushes before (filled circles) and after (open circles) the system intervention. Mean water temperature for each condition is shown below the data points. Whiskers: min-max; Box: standard error. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. Adaptation of isolated strains to drinking water stressors and their capacity to replicate within host cells

The prevalence of the two STs suggests that they are the best sequence type able to thrive in the environmental conditions present in this building. The copper piping within the hot water distribution system led to high levels of copper in the water after stagnation, with an average level of 478 μg/L and a maximum value of 743 μg/L measured at a tap. In comparison, an average value of 175 μg/L was measured after 5-min flushing. Exposure of representative strains from both STs to copper levels between 0.8 and 5 mg/L had a low impact on the ability to culture representative isolates from each ST (Fig. 6). After two weeks, less than one log-fold reduction was observed for both strains (Fig. 6). The tolerance of both STs to the recommended hot water recirculation temperature (55 °C) was also evaluated to assess whether an increase in temperature within the system could cause a shift in the prevalence of the STs. The examined isolates showed approximately 2 log-fold reductions after exposure to 55 °C temperatures for 1-h (Fig. 6). Of note, the reduction observed immediately after exposure was maintained over the two following weeks. These results suggest that the particular strains present in a hot water system may depend on the stress factors present and the ability of

Table 1
Sequence-based typing results for 47 isolates recovered from 32 hot water *Lp* culture samples.

Device	Sample	Sampling vs. intervention	Isolate	Sequence type	Allele number						
					flaA	pilE	asd	mip	momps	proA	neuA
F1	0.5L	Before	10	378	3	13	1	17	14	9	7
F1	0.5L	After	3	378	3	13	1	17	14	9	7
F1	0.5L	After	4	378	3	13	1	17	14	9	7
F1	1L	Before	11	378	3	13	1	17	14	9	7
F1	1L	Before	12	154-like	NA	14	16	16	15	13	2
F1	1L	After	1	378	3	13	1	17	14	9	7
F1	1L	After	2	378	3	13	1	17	14	9	7
F1	2L	Before	52	378	3	13	1	17	14	9	7
F1	2L	Before	51	154-like	NA	14	16	16	15	13	2
F1	2L	After	5	378	3	13	1	17	14	9	7
F1	2minF	Before	53	154-like	NA	14	16	16	15	13	NA
F1	5minF	Before	21	378	3	13	1	17	14	9	7
F2	0.5L	Before	13	378	3	13	1	17	14	9	7
F2	0.5L	After	34	378	3	13	1	17	14	9	7
F2	0.5L	After	35	154-like	NA	14	16	16	15	13	2
F2	1L	Before	14	378	3	13	1	17	14	9	7
F2	1L	After	26	378	3	13	1	17	14	9	7
F2	1L	After	24	154-like	NA	14	16	16	15	13	2
F2	1L	After	25	154-like	NA	14	16	16	15	NA	2
F2	2L	Before	41	378	3	13	1	17	14	9	7
F2	2L	After	27	154-like	NA	14	16	16	15	13	2
F2	2L	After	28	378	3	13	1	17	14	9	7
F2	2minF	Before	42	378	3	13	1	17	14	9	7
F2	2minF	After	15	378	3	13	1	17	14	9	7
F2	5minF	Before	47	378	3	13	1	17	14	9	7
F2	5minF	After	16	378	3	13	1	17	14	9	7
F2	5minF	After	17	154-like	NA	14	16	16	15	13	2
F3	0.5L	Before	23	378	3	13	1	17	14	9	7
F3	0.5L	Before	22	154-like	NA	14	16	16	15	13	2
F3	0.5L	After	6	378	3	13	1	17	14	9	7
F3	0.5L	After	7	154-like	NA	14	16	16	15	13	2
F3	1L	Before	29	378	3	13	1	17	14	9	7
F3	1L	After	9	378	3	13	1	17	14	9	7
F3	2L	Before	31	378	3	13	1	17	14	9	7
F3	2L	After	18	378	3	13	1	17	14	9	7
F3	2L	After	19	378	3	13	1	17	14	9	7
F3	2minF	Before	55	154-like	NA	14	16	16	15	13	2
F3	2minF	Before	56	378	3	13	1	17	14	9	7
F3	2minF	After	20	378	3	13	1	17	14	9	7
F3	5minF	Before	58	154-like	NA	14	16	16	15	13	2
F3	5minF	Before	57	378	3	13	1	17	14	9	7
S1	0.5L	Before	37	378	3	13	1	17	14	9	7
S1	0.5L	Before	36	154-like	NA	14	16	16	15	13	2
S1	0.5L	Before	49	154-like	NA	14	16	16	15	13	2
S2	0.5L	Before	38	378-like	NA	13	1	17	14	9	7
S3	0.5L	Before	43	378	3	13	1	17	14	9	7
Recirc	0.5L	Before	Q-0675	154-like	NA	14	16	16	15	13	2

NA: Not amplified; F: faucet; S: shower; Recirc: recirculation loop return prior to water heater.

the strains present in the in-feed water to survive those conditions.

Finally, the ability to survive and replicate within THP1 cells and *A. castellanii* was determined. Representative isolates for both STs were compared against an environmental epidemic strain (ID120292, ST62) associated with a previous legionellosis outbreak (Levesque et al., 2014). Isolates from both STs were not significantly different from the outbreak strain in their capacity to infect and survive in macrophages and host cells (Fig. 7).

4. Discussion

Distal amplification was observed for HPC and viable cells in the investigated faucets, as previously reported (Bédard et al., 2018; Cristina et al., 2014; Lautenschlager et al., 2010). The higher contamination of the distal point is generally attributable to prolonged stagnation at the point of use between usages, a large surface to volume ratio (promoting biofilm growth) and nonoptimal water temperatures (Bédard et al., 2018; Lautenschlager et al., 2010). In the principal and secondary recirculation loops, cold and hot water piping feed multiple points of use and therefore experience less stagnation and higher temperatures. In addition, materials used in the last few meters before the tap often include elastomeric materials, which are known to promote biofilm growth (Moritz et al., 2010). Distal amplification was therefore also expected for *L. pneumophila*, as previously reported by Cristina et al. (2014), where mean *L. pneumophila* concentrations were significantly higher at the outlet point compared to the hot water systems (Cristina et al., 2014). This was particularly true for *L. pneumophila* sg1, which had a 6-fold increase in percent positivity and 2-log-fold increase in levels at the point of use compared to the overall system (Cristina et al., 2014). In the present study, only faucet F1 displayed a decreasing profile as a function of flushed volume and time (Fig. 3B), although *L. pneumophila* levels were not significantly lower after flushing compared to the first draw volume. The comparable profiles detected in all five subsequent water samples from faucets F2 and F3 suggest that the secondary vertical flow and return loop water was also contaminated by *L. pneumophila* (Fig. 1). This is supported by the fact that both of these faucets were fed by

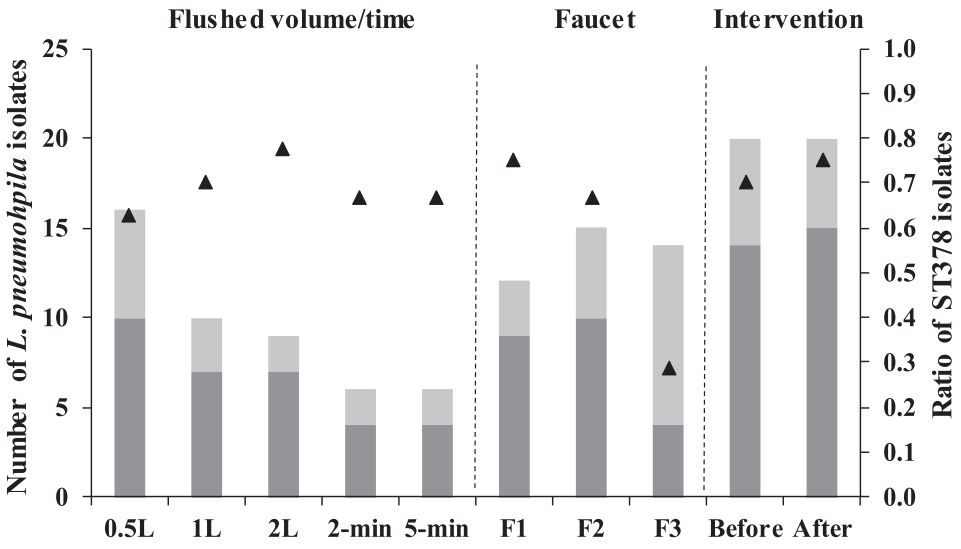

Fig. 5. Sequence type distribution (ST378: dark gray; ST154-like: light gray) as a function of flushed volume/time, faucet and system intervention. The ratio of ST378 is shown on the secondary y-axis (filled triangles).

	Stress	ST	Time (hours) 1	6	48	72	168	336
T°	55°C 1h	378	-2.0	-2.5	-2.9	-2.8	-2.4	-2.6
		154-like	-2.3	-2.1	-1.9	-2.5	-2.8	-2.5
Copper	0.8 mg Cu/L	378	-0.1	0.2	0.0	-0.4	-0.5	-0.2
		154-like	-0.1	-0.1	-0.2	-0.5	-0.4	-0.3
	1.3 mg Cu /L	378	0.2	0.1	-0.4	-0.5	-0.5	-0.2
		154-like	-0.1	-0.1	-0.4	-0.5	-0.5	-0.4
	2 mg Cu/L	378	0.0	-0.1	-0.4	-0.5	-0.6	-0.3
		154-like	-0.3	-0.4	-0.5	-0.6	-0.5	-0.6
	5 mg Cu/L	378	0.1	-0.2	-0.7	-0.8	-1.0	-0.8
		154-like	-0.6	-0.9	-1.0	-1.7	-0.7	-0.8

Fig. 6. Culturability of isolated STs after exposure to 55 °C for 1 h or copper concentrations between 0.8 and 5 mg/L. The results are expressed as log-fold reductions. Colors are on a continuous scale from green (2.8 log reduction) to red (no reduction). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the same vertical flow and return loop. These results suggest that in certain cases, the level of *L. pneumophila* contamination can be relatively homogeneous within the hot water system if it is operated at suboptimal temperatures, consistent with prior observations (Bédard et al., 2016a). The systemic contamination in the primary and secondary piping of this non-optimized hot water system may have acted as a reservoir to seed distal sites.

The system intervention performed to increase temperature and therefore reduce *L. pneumophila* contamination in the hot water system had a moderate impact on culturable cells (HPC) for faucets F1 and F2. Conversely, faucet F3 showed significantly lower HPC levels after intervention (Fig. 3A). It can be hypothesized that the larger increase in temperature observed in faucet F3 had an impact on culturable cells, which were adapted to a mean temperature of 41 °C prior to the intervention. The level of *L. pneumophila* contamination was greatly reduced by the temperature increase resulting from the system intervention (Figs. 2 and 3), consistent with previous reports (Arvand et al., 2011; Barna et al., 2016; Boppe et al., 2016; Völker and Kistemann, 2015). Culturable *L. pneumophila*

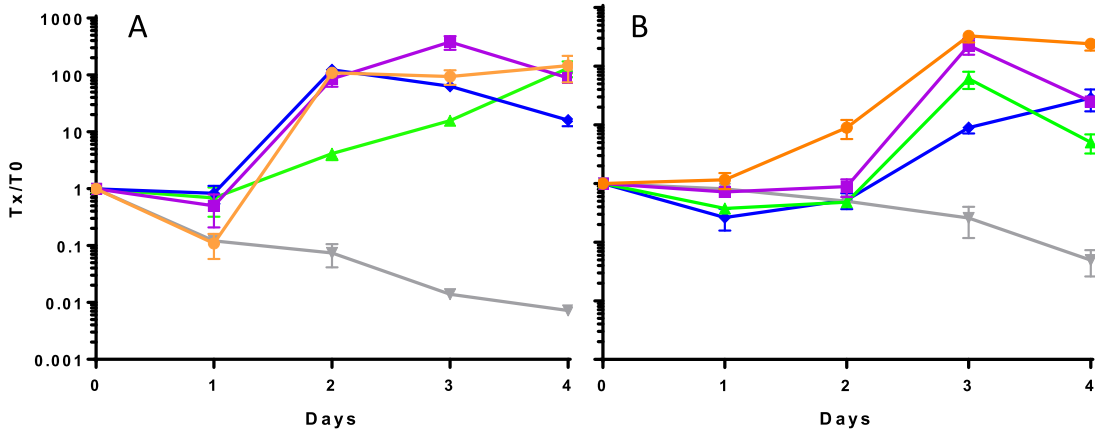

Fig. 7. Replication of *L. pneumophila* strains in THP1 cells (A) and *A. castellanii* (B). ST378 (purple square) and ST154-like (orange circle) were compared to JR32 (green triangle), dotA mutant (gray inverted triangle) and the Quebec ST62 outbreak strain (blue diamond). The results are expressed as the ratio of *L. pneumophila* cells at the time of measurement compared to initial levels. Mean and standard deviation of experiments performed in triplicate are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

levels measured from the water samples collected after 5-min flush were found to be inversely correlated with the water temperature ($R^2 = 0.90$), although the correlation was not as strong after the first 2 L ($R^2 = 0.69$). This is in line with previous observations by Dai et al., who concluded that an increase in hot water temperature had a greater impact on the recirculating system than on distal points of use (Dai et al., 2017). Despite the reduced levels of *L. pneumophila* after the system intervention (i.e., temperature increase), no change was observed in the ST378 ratio (Fig. 5), and the same STs were isolated (Table 1). These results, together with the observed ability of both STs to equally survive exposure to temperature and copper concentrations, suggest that both STs are well-adapted to the environmental stressors present in this system. These factors likely do not fully explain the dominance of ST378. The lower prevalence of sg1 observed is in line with the lower prevalence of sg1 previously reported in UK environmental samples (42.7%) compared to its widespread clinical presence (88%) (Harrison et al., 2007).

Only two distinct STs (ST378 and ST154-like) were recovered through the culture method used. Low *L. pneumophila* diversity was previously observed in hospital hot water systems following an outbreak investigation (Bédard et al., 2016b; David et al., 2017; Oberdorfer et al., 2008). In Canada, an environmental investigation revealed that 46 isolates recovered from clinical and environmental sources (e.g., water and biofilm from the hot water system) were >85% similar by PFGE profiling, and a subset of 12 isolates analyzed by sequence-based typing were associated with ST1427 (Bédard et al., 2016b). In Germany, a study conducted in six hospitals over 10 years concluded that each hospital had one to four dominant PFGE types that were stable over time (Oberdorfer et al., 2008). Recently, following a Legionnaires' disease outbreak in South Michigan, 18 environmental strains from plumbing and 33 clinical strains were analyzed by sequence based-typing (Byrne et al., 2018). Two dominant STs from one clonal complex were identified in premise plumbing samples, whereas four clonal complexes (including 10 STs) were recovered from clinical samples (Byrne et al., 2018). Finally, the use of whole genome sequencing in a broad study of 229 ST1 or ST1-derived sequence types isolated from environmental and clinical samples from 17 hospitals revealed a long-term persistence of highly similar or identical strains in several hospitals (David et al., 2017). However, David et al. also observed an important level of diversity in other hospitals, showing evidence of microevolution within wards (David et al., 2017). Despite the low diversity reported in the present study, environmental samples may still harbor more than one strain at very low prevalence rates, which may not be recovered by culture during a single sampling event or by single colony picking (in contrast to clinical investigations showing that one colony pick for patient samples is sufficient due to low sample diversity recovered) (David et al., 2018). The identification of all STs within a system is particularly relevant when considering environmental investigations and matching with clinical isolates.

Despite a reported higher pathogenicity of sg1 isolates (Garcia-Nunez et al., 2009), no significant differences were observed concerning the ability of ST378 (sg4/10) to replicate within macrophages and amoeba cells compared to ST154-like (sg1) isolates. The two environmental STs isolated have previously been associated with clinical cases in Canada, suggesting their capacity to infect humans (Levesque et al., 2016; Reimer et al., 2010). Four sporadic cases in Québec were associated with ST378, two of which were associated with an environmental source (Levesque et al., 2016). However, the EWGLI database shows a majority of environmental isolates are associated with this ST, primarily identified as sg 4 or sg10. Reimer et al. identified a water distribution system in Québec as a source of ST378 sg4 (Reimer et al., 2010). Sequence-type ST154 is among the most frequent environmental sequence types isolated in the United States in association with outbreaks (Kozak-Muiznieks et al., 2014). Eight sporadic legionellosis cases in Québec were associated with ST154 between 1993 and 2015 (Levesque et al., 2016; Reimer et al., 2010). Therefore, the infectious potential of the two STs suggested by our intracellular replication results is consistent with the documented reports of clinical cases associated with those STs.

Our study presents certain limitations. First, since the study focused on the in-depth investigation of highly contaminated *L. pneumophila* faucets in a problematic hospital wing to understand the prevalence of *L. pneumophila* in different sections of the upflow system, it was not possible to make a definitive determination regarding the dominance of these two STs throughout the hospital. However, the recovery of the same two STs and no new ST in the showers sampled in the three other wings and in the main recirculation loop indicate that they are not only present in Wing 3. Second, isolates were collected from water samples, since it was not possible to collect biofilm from different sections of piping within the context of the study. Despite these limitations, these results show evidence of water contamination by *L. pneumophila* from faucets to the main pipe in deficient building system. Understanding the relative importance of the faucet in promoting high levels of *L. pneumophila* in hot water systems is important for the identification of effective corrective actions. The recovery of the same STs independent of the pipe section or the faucet sampled suggest that those STs are best adapted to this specific plumbing system environment. The low diversity within a building and the differences in predominant STs from building to building could be linked to specific stressors, water quality, water usage pattern and operating conditions for each building, selecting for the STs that are the best suited to survive in the specific environment.

5. Conclusions

- *L. pneumophila* contamination was detected at similar concentrations throughout the hot water system of the examined hospital wing, from the faucet to the main horizontal flow and return loop. Contamination was not only distal but also associated with secondary flow and return loops, reflecting deficient temperature control across this wing.
- Only two STs (ST378 and ST154-like) were recovered from the study samples. The dominance of the non-sg1 ST378 was observed consistently between faucet and system samples and from one faucet to another, suggesting minimal impact of the local conditions on strain selection and ST distribution. Recirculation or stagnation did not influence the dominant STs present.
- Hot water temperature increase as a result of the system intervention was a determining factor for *L. pneumophila* levels measured in water; however, it did not affect the presence and the ratio of the two STs already present within the system in the short term.
- The comparable ability of both STs to tolerate copper and high temperature exposure and to multiply within host cells rule out these factors as determinants of the dominance of the non-sg1 ST378 type.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal

relationships which may be considered as potential competing interests:

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References

Al-Bana, B.H., Haddad, M.T., Garduno, R.A., 2014. Stationary phase and mature infectious forms of *Legionella pneumophila* produce distinct viable but non-culturable cells. Environ. Microbiol. 16 (2), 382–395.

Allegra, S., Grattard, F., Girardot, F., Riffard, S., Pozzetto, B., Berthelot, P., 2011. Longitudinal evaluation of the efficacy of heat treatment procedures against *Legionella spp.* in hospital water systems by using a flow cytometric assay. Appl. Environ. Microbiol. 77 (4), 1268–1275.

American Public Health Association (APHA), American Water Works Association (AWWA) and Water Environment Federation (WEF), 2005. Standard Methods for the Examination of Water and Wastewater, 21th Edition (Washington, DC, USA).

Arvand, M., Jungkind, K., Hack, A., 2011. Contamination of the cold water distribution system of health care facilities by *Legionella pneumophila*: do we know the true dimension? Euro Surveill. 16 (16), 6.

Bagh, L.K., Albrechtsen, H.J., Arvin, E., Ovesen, K., 2004. Distribution of bacteria in a domestic hot water system in a Danish apartment building. Water Res. 38 (1), 225–235.

Bargellini, A., Marchesi, I., Righi, E., Ferrari, A., Cencetti, S., Borella, P., Rovesti, S., 2011. Parameters predictive of *Legionella* contamination in hot water systems: association with trace elements and heterotrophic plate counts. Water Res. 45 (6), 2315–2321.

Barna, Z., Kadar, M., Kalman, E., Scheirich Szax, A., Vargha, M., 2016. Prevalence of *Legionella* in premise plumbing in Hungary. Water Res. 90, 71–78.

Bédard, E., Boppe, I., Kouamé, S., Martin, P., Pinsonneault, L., Valiquette, L., Racine, J., Prévost, M., 2016a. Combination of heat shock and enhanced thermal regime to control the growth of a persistent *Legionella pneumophila* strain. Pathogens 5 (2), 35.

Bédard, E., Laferrière, C., Déziel, E., Prévost, M., 2018. Impact of stagnation and sampling volume on water microbial quality monitoring in large buildings. PLoS One 13 (6), e0199429.

Bédard, E., Lévesque, S., Martin, P., Pinsonneault, L., Paranjape, K., Lalancette, C., Dolcé, C.-É., Villion, M., Valiquette, L., Faucher, S.P., Prévost, M., 2016b. Energy conservation and the promotion of *Legionella pneumophila* growth: the probable role of heat exchangers in a nosocomial outbreak. Infect. Control Hosp. Epidemiol. 37 (12), 1475–1480.

Benedict, K., Reses, H., Vigar, M., Roth, D.M., Roberts, V.A., Mattioli, M., Cooley, L.A., Hilborn, E.D., Wade, T.J., Fullerton, K., Yoder, J., Hill, V.R., 2017. Surveillance for waterborne disease outbreaks associated with drinking water — United States, 2013–2014. Weekly 66 (44), 1216–1220.

Boppe, I., Bédard, E., Taillandier, C., Lecellier, D., Nantel-Gauvin, M.-A., Villion, M., Laferrière, C., Prévost, M., 2016. Investigative approach to improve hot water system hydraulics through temperature monitoring to reduce building environmental quality hazard associated to *Legionella*. Build. Environ. 108, 230–239.

Boulos, L., Prévost, M., Barbeau, B., Coallier, J., Desjardins, R., 1999. LIVE/DEAD® *Bac*Light™: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J. Microbiol. Methods 37 (1), 77–86.

Buse, H.Y., Schoen, M.E., Ashbolt, N.J., 2012. *Legionellae* in engineered systems and use of quantitative microbial risk assessment to predict exposure. Water Res. 46 (4), 921–933.

Byrne, B.G., McColm, S., McElmurry, S.P., Kilgore, P.E., Sobeck, J., Sadler, R., Love, N.G., Swanson, M.S., Shuman, H.A., 2018. Prevalence of infection-competent serogroup 6 Legionella pneumophila within premise plumbing in Southeast Michigan. mBio 9 (1).

Centers for Disease Control and Prevention (CDC), 2017. Active Bacterial Core Surveillance (ABCs) Report Emerging Infections Program Network Legionellosis, 2015.

Cervero-Arago, S., Rodriguez-Martinez, S., Puertas-Bennasar, A., Araujo, R.M., 2015. Effect of common drinking water disinfectants, chlorine and heat, on free *Legionella* and *Amoebae*-associated *Legionella*. PLoS One 10 (8), e0134726.

Cristina, M.L., Spagnolo, A.M., Casini, B., Baggiani, A., Del Giudice, P., Brusaferro, S., Poscia, A., Moscato, U., Perdelli, F., Orlando, P., 2014. The impact of aerators on water contamination by emerging gram-negative opportunists in at-risk hospital departments. Infect. Control Hosp. Epidemiol. 35 (2), 122–129.

Dai, D., Prussin 2nd, A.J., Marr, L.C., Vikesland, P.J., Edwards, M.A., Pruden, A., 2017. Factors shaping the human exposome in the built environment: opportunities for engineering control. Environ. Sci. Technol. 51 (14), 7759–7774.

Dai, D., Rhoads, W.J., Edwards, M.A., Pruden, A., 2018. Shotgun metagenomics reveals taxonomic and functional shifts in hot water microbiome due to temperature setting and stagnation. Front. Microbiol. 9 (2695).

David, S., Afshar, B., Mentasti, M., Ginevra, C., Podglajen, I., Harris, S.R., Chalker, V.J., Jarraud, S., Harrison, T.G., Parkhill, J., 2017. Seeding and *Establishment* of *Legionella pneumophila* in hospitals: implications for genomic investigations of nosocomial legionnaires' disease. Clin. Infect. Dis. 64 (9), 1251–1259.

David, S., Mentasti, M., Parkhill, J., Chalker, V.J., 2018. Low genomic diversity of *Legionella pneumophila* within clinical specimens. Clin. Microbiol. Infect. 24 (9), 1020.e1-1020.e4.

David, S., Rusniok, C., Mentasti, M., Gomez-Valero, L., Harris, S.R., Lechat, P., Lees, J., Ginevra, C., Glaser, P., Ma, L., Bouchier, C., Underwood, A., Jarraud, S., Harrison, T.G., Parkhill, J., Buchrieser, C., 2016. Multiple major disease-associated clones of *Legionella pneumophila* have emerged recently and independently. Genome Res. 26 (11), 1555–1564.

Donohue, M.J., Vesper, S.J., Mistry, J., King, D., Kostich, M., Pfaller, S., O'Connell, K., 2014. Widespread molecular detection of *Legionella pneumophila* serogroup 1 in cold water taps across the United States. Environ. Sci. Technol. 48 (6), 3145–3152.

European Centre for Disease Prevention and Control (ECDC), 2017. Surveillance Report. Annual Epidemiological Report for 2015 Legionnaires' Disease, p. 7 (Stockholm).

Flemming, H.C., Bendinger, B., 2014. In: van der Kooij, D., van der Wielen, P.W. (Eds.), Microbial Growth in Drinking-Water Distribution Systems. Problems, Causes, Prevention and Research Needs. IWA Publishing, London, UK, pp. 207–238.

Gaia, V., Fry, N.K., Afshar, B., Luck, P.C., Meugnier, H., Etienne, J., Peduzzi, R., Harrison, T.G., 2005. Consensus sequence-based scheme for epidemiological typing of clinical and environmental isolates of *Legionella pneumophila*. J. Clin. Microbiol. 43 (5), 2047–2052.

Garcia-Nunez, M., Pedro-Botet, M.L., Ragull, S., Sopena, N., Morera, J., Rey-Joly, C., Sabria, M., 2009. Cytopathogenicity and molecular subtyping of *Legionella pneumophila* environmental isolates from 17 hospitals. Epidemiol. Infect. 137 (2), 188–193.

Garrison, L.E., Kunz, J.M., Cooley, L.A., Moore, M.R., Lucas, C., Schrag, S., Sarisky, J., Whitney, C.G., 2016. Vital signs: deficiencies in environmental control identified in outbreaks of Legionnaires' disease — North America, 2000–2014. MMWR (Morb. Mortal. Wkly. Rep.) 65 (22), 576–584.

Harrison, T.G., Doshi, N., Fry, N.K., Joseph, C.A., 2007. Comparison of clinical and environmental isolates of Legionella pneumophila obtained in the UK over 19 years. Clin. Microbiol. Infect. 13 (1), 78–85.

Kozak-Muiznieks, N.A., Lucas, C.E., Brown, E., Pondo, T., Taylor Jr., T.H., Frace, M., Miskowski, D., Winchell, J.M., 2014. Prevalence of sequence types among clinical and environmental isolates of *Legionella pneumophila* serogroup 1 in the United States from 1982 to 2012. J. Clin. Microbiol. 52 (1), 201–211.

Lautenschlager, K., Boon, N., Wang, Y., Egli, T., Hammes, F., 2010. Overnight stagnation of drinking water in household taps induces microbial growth and changes in community composition. Water Res. 44 (17), 4868–4877.

Levesque, S., Lalancette, C., Bernard, K., Pacheco, A.L., Dion, R., Longtin, J., Tremblay, C., 2016. Molecular typing of *Legionella pneumophila* isolates in the province of Quebec from 2005 to 2015. PLoS One 11 (10), e0163818.

Levesque, S., Plante, P.L., Mendis, N., Cantin, P., Marchand, G., Charest, H., Raymond, F., Huot, C., Goupil-Sormany, I., Desbiens, F., Faucher, S.P., Corbeil, J., Tremblay, C., 2014. Genomic characterization of a large outbreak of *Legionella pneumophila* serogroup 1 strains in Quebec city, 2012. PLoS One 9 (8), e103852.

Lu, J., Buse, H., Struewing, I., Zhao, A., Lytle, D., Ashbolt, N., 2017. Annual variations and effects of temperature on *Legionella* spp. and other potential opportunistic pathogens in a bathroom. Environ. Sci. Pollut. Control Ser. 24 (3), 2326–2336.

Lu, J., Buse, H.Y., Gomez-Alvarez, V., Struewing, I., Santo Domingo, J., Ashbolt, N.J., 2014. Impact of drinking water conditions and copper materials on downstream biofilm microbial communities and *Legionella pneumophila* colonization. J. Appl. Microbiol. 117 (3), 905–918.

Marchesi, I., Marchegiano, P., Bargellini, A., Cencetti, S., Frezza, G., Miselli, M., Borella, P., 2011. Effectiveness of different methods to control legionella in the water supply: ten-year experience in an Italian university hospital. J. Hosp. Infect. 77 (1), 47–51.

Marciano-Cabral, F., Jamerson, M., Kaneshiro, E.S., 2010. Free-living amoebae, Legionella and Mycobacterium in tap water supplied by a municipal drinking water utility in the USA. J. Water Health 8 (1), 71–82.

Mentasti, M., Fry, N.K., 2012. Sequence-based Typing Protocol for Epidemiological Typing of *Legionella pneumophila*. European Society of Clinical Microbiology and Infectious Diseases (ESGLI) Version 5.0.

Moritz, M.M., Flemming, H.C., Wingender, J., 2010. Integration of *Pseudomonas aeruginosa* and *Legionella pneumophila* in drinking water biofilms grown on domestic plumbing materials. Int. J. Hyg Environ. Health 213 (3), 190–197.

Oberdorfer, K., Mussigbrodt, G., Wendt, C., 2008. Genetic diversity of *Legionella pneumophila* in hospital water systems. Int. J. Hyg Environ. Health 211 (1–2), 172–178.

Pryor, M., Springthorpe, S., Riffard, S., Brooks, T., Huo, Y., Davis, G., Sattar, S.A., 2004. Investigation of opportunistic pathogens in municipal drinking water under different supply and treatment regimes. Water Sci. Technol. 50 (1), 83–90.

Qin, T., Zhou, H., Ren, H., Guan, H., Li, M., Zhu, B., Shao, Z., 2014. Distribution of sequence-based types of legionella pneumophila serogroup 1 strains isolated from cooling towers, hot springs, and potable water systems in China. Appl. Environ. Microbiol. 80 (7), 2150–2157.

Régie du bâtiment du Québec (RBQ), 2014. Modifications du Québec applicables au

Code national de la plomberie Canada 2010, p. 67.
Reimer, A.R., Au, S., Schindle, S., Bernard, K.A., 2010. Legionella pneumophila monoclonal antibody subgroups and DNA sequence types isolated in Canada between 1981 and 2009: laboratory Component of National Surveillance. Eur. J. Clin. Microbiol. Infect. Dis. 29 (2), 191–205.
Rhoads, W.J., Ji, P., Pruden, A., Edwards, M.A., 2015. Water heater temperature set point and water use patterns influence *Legionella pneumophila* and associated microorganisms at the tap. Microbiome 3 (67), 1–13.
Sharaby, Y., Rodríguez-Martínez, S., Pecellin, M., Sela, R., Peretz, A., Höfle, M.G., Halpern, M., Brettar, I., 2018. Virulence traits of environmental and clinical *Legionella pneumophila* MLVA genotypes. Appl. Environ. Microbiol. 84 (10).
Soda, E.A., Barskey, A.E., Shah, P.P., Schrag, S., Whitney, C.G., Arduino, M.J., Reddy, S.C., Kunz, J.M., Hunter, C.M., Raphael, B.H., Cooley, L.A., 2017. Vital signs: health care–associated legionnaires' disease surveillance data from 20 states and a large metropolitan area — United States, 2015. MMWR (Morb. Mortal. Wkly. Rep.) 66 (2), 584–589.
Sousa, P.S., Silva, I.N., Moreira, L.M., Veríssimo, A., Costa, J., 2018. Differences in virulence between *Legionella pneumophila* isolates from human and non-human sources determined in galleria mellonella infection model. Front. Cell. Infect. Microbiol. 8 (97).
Völker, S., Kistemann, T., 2015. Field testing hot water temperature reduction as an energy-saving measure – does the Legionella presence change in a clinic's plumbing system? Environ. Technol. 36 (16), 2138–2147.

ORIGINAL ARTICLE

Energy Conservation and the Promotion of *Legionella pneumophila* Growth: The Probable Role of Heat Exchangers in a Nosocomial Outbreak

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OBJECTIVE. To determine the source of a *Legionella pneumophila* serogroup 5 nosocomial outbreak and the role of the heat exchanger installed on the hot water system within the previous year.

SETTING. A 400-bed tertiary care university hospital in Sherbrooke, Canada.

METHODS. Hot water samples were collected and cultured for *L. pneumophila* from 25 taps (baths and sinks) within wing A and 9 taps in wing B. Biofilm (5) and 2 L water samples (3) were collected within the heat exchangers for *L. pneumophila* culture and detection of protists. Sequence-based typing was performed on strain DNA extracts and pulsed-field gel electrophoresis patterns were analyzed.

RESULTS. Following 2 cases of hospital-acquired legionellosis, the hot water system investigation revealed a large proportion of *L. pneumophila* serogroup 5 positive taps (22/25 in wing A and 5/9 in wing B). High positivity was also detected in the heat exchanger of wing A in water samples (3/3) and swabs from the heat exchanger (4/5). The outbreak genotyping investigation identified the hot water system as the source of infections. Genotyping results revealed that all isolated environmental strains harbored the same related pulsed-field gel electrophoresis pattern and sequence-based type.

CONCLUSIONS. Two cases of hospital-acquired legionellosis occurred in the year following the installation of a heat exchanger to preheat hospital hot water. No cases were reported previously, although the same *L. pneumophila* strain was isolated from the hot water system in 1995. The heat exchanger promoted *L. pneumophila* growth and may have contributed to confirmed clinical cases.

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Each year, hospital-acquired legionellosis cases result in prolonged hospitalization with elevated mortality rates.[1] These cases are predominantly associated with *Legionella pneumophila* serogroup 1 (sg1) strains present in the hospital hot water systems.[2–4] A few outbreaks and isolated cases have been related to *L. pneumophila* (*Lp*) serogroup 5 (sg5).[5–7] Several factors can contribute to *Legionella* growth and persistence within hospital water systems: temperature, stagnation, biofilm, material, disinfectant, and water quality.[8–12] Key measures to control *Lp* in hot water systems are to maintain elevated water temperatures throughout the system and to minimize stagnation through optimal water circulation.[13–16] Although an infectious dose has not been determined, several countries have established action levels between 1,000 and 10,000 colony-forming units (CFU)/L, and a concentration higher than 10,000 CFU/L requires immediate corrective actions.[13,15,17–19]

At the same time, healthcare facilities are encouraged to implement energy and water conservation devices to meet accreditation requirements, such as Leadership in Energy and Environmental Design certification. The use of waste heat recovery systems to preheat hospital hot water prior to the water heater is an option offered to hospitals to increase energy efficiency. However, operational practices and ideal growth

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conditions associated with these devices can promote the development and persistence of *Lp* unless a thorough risk assessment is performed. In this study, we report a nosocomial outbreak of *Lp* sg5 in the year following the installation of 2 heat exchangers in 2 distinct hospital wings as part of an energy conservation upgrade. The objectives of our study were to determine the source of the outbreak and to understand the role of the heat recovery systems.

METHODS

Two nosocomial cases of *Lp* were reported in August 2014 within a wing (wing A) of a 400-bed tertiary care university hospital in Sherbrooke, Canada. Clinical samples were cultured on buffered charcoal-yeast extract (BCYE) medium, and isolates were sent to the Laboratoire de Santé Publique du Québec for identification and serogroup confirmation as described previously.[20] Following the reported cases, 250 mL of first flush hot water were collected from 25 taps (baths and sinks) within wing A (300 beds) and from 9 taps within wing B (100 beds, supplied by a separate hot water system). The heat exchangers from wings A and B were investigated in June and July 2015, respectively. Biofilm samples and water (2 L) were collected from 3 locations: at the water inflow pipe, inside the heat exchanger, and at the water outflow pipe. Two additional biofilm samples were recovered within the heat exchanger. Environmental samples were cultured according to the Association Française de Normalisation NF T90-431 method[21] with the addition of a 1-mL filtration. Briefly, different volumes were filtered through sterile 47 mm diameter 0.45 µm mixed ester cellulose membranes (Millipore) and an untreated sample volume of 0.2 mL was plated on glycine-vancomycin-polymyxin-cycloheximide selective agar (Biokar Diagnostics) and incubated at 36°C for 10 days. Before plating, acid treatment was applied to filtered samples (pH, 2; 5 min). Typical colonies that developed after 4 to 10 days were subcultured on confirmation plates (BCYE agar without and with cysteine) for 2 to 4 days, at 36°C. Resulting colonies that developed on BCYE agar, but not on BCYE without cysteine, were considered as *Legionella* spp. The *Legionella* latex test (DR0800; Oxoid) was used for *Lp* confirmation. The calculated detection limit for the culture method was 10 CFU/L for both *Legionella* spp. and *Lp*. Pulsed-field gel electrophoresis (PFGE) and sequence-based typing (SBT) were performed as described previously.[20] In some cases, nested-SBT protocol was performed on DNA extracts obtained from water sampling.[22] PFGE patterns were analyzed according to the criteria of Tenover et al[23] and with BioNumerics software, version 6.5 (Applied Maths), by the unweighted pair-group method with arithmetic average clustering method using the Dice coefficient with both position tolerance and optimization of 1%. Biofilm and water samples from the heat exchanger were analyzed for the presence of protists through direct microscopy and 18s polymerase chain reaction amplification using the following primers: Euk1A (5′-CTGGTTGATCCTG CCAG-3′); Euk516r (5′-ACCAGACTTGCCCTCC-3′).

Statistical analysis (*Z*-test and Kruskal-Wallis) was performed with Statistica, version 10 (Dell), to compare the percent positivity and level of *Lp* in wing A and wing B. The significance level was set at $P = .05$.

RESULTS

Nosocomial legionellosis was diagnosed in 2 patients hospitalized in wing A (August 2014), and clinical specimens were positive for *Lp* sg5. The first case occurred in the oncology ward, on the seventh floor of wing A. The patient was admitted on July 12 with acute myeloid leukemia. The first signs of pneumonia appeared on August 1. A sample was collected through bronchoalveolar lavage and the presence of *Lp* sg 2–14 was confirmed. The second case occurred in a patient admitted on July 25 to the medical intensive care unit (ninth floor, wing A) for a third-degree atrioventricular block. His main comorbidity was a chronic obstructive pulmonary disease for which he took prednisone. He had a permanent pacemaker placement on July 26 and was transferred to the surgical ward (eighth floor, wing A), where he stayed from July 27 to August 5. Significant coronary artery disease was discovered and he underwent coronary artery bypass graft surgery on August 5. Following this intervention, he was transferred to the surgical intensive care unit (third floor, wing B), where he remained for the rest of his stay. The first signs of pneumonia appeared on August 8 and clinical samples were recovered through bronchoscopy. The presence of *Lp* sg 2–14 was also confirmed. Provided the incubation time of 2 to 10 days, the patient was possibly exposed during the 3-day stay in wing B (intensive care unit) but most probably during the 10-day stay in the surgical ward in wing A.

Environmental investigation from the hot water systems revealed a large proportion of *Lp* sg5 positive taps with high levels of contamination (88% in wing A and 56% in wing B; Table 1), whereas there was no *Lp* detected in the cold water feeding into the hospital.[9] The percentage of positive taps and the level of contamination by *Lp* were significantly higher in wing A compared with wing B. A copper-silver ionization treatment was present on both hot water systems at the time of the outbreak. Disinfection by heat shock (≥60°C for

TABLE 1. *Legionella pneumophila* Positivity and Levels Measured in Hot Water Sampled From Taps in Wing A and Wing B at the Time of the Outbreak (August 2014)

***L. pneumophila* positivity**	**Wing A**	**Wing B**
Number of sampled taps	25	9
Number of *L. pneumophila* positive taps	22	5
Mean *L. pneumophila,* CFU/L	27,200	1,700
Standard deviation, CFU/L	18,921	1,857
Median *L. pneumophila,* CFU/L	18,000	1,000
Maximum *L. pneumophila* level, CFU/L	80,000	5,000

NOTE. CFU, colony-forming units.

≥7 minutes at each tap) was conducted as previously described[9] in wing A (August 2014) and in wing B (September 2014). The disinfection was followed by the implementation of a higher temperature set point at the water heater outlet (≥60°C) in both wings. Following temperature corrective measures, no cases of legionellosis were reported despite written directives asking hospital physicians to obtain *Legionella* cultures of respiratory specimens for all cases of nosocomial pneumonia. No *Lp* were detected in any of the water and biofilm heat exchanger samples from wing B (including the feed and outflow water samples), whereas high positivity for *Lp* sg5 was detected in wing A in water samples (3/3) and swabs from the inner surface of the heat exchanger (4/5). A gradient of *Lp* was observed in water samples from wing A heat exchanger: 510 CFU/L in the feed water, 5,000 CFU/L in the heat exchanger water, and 88,000 CFU/L in the outflow water, prior to the water heater. Protists were not readily observed by microscopy in any of the collected samples. Polymerase chain reaction also failed to detect protists in the biofilm swabs and in water samples. The heat exchangers were stopped at the time of the sampling and have not been back in service since.[9]

The hospital hot water was preheated with residual energy from the building heating system loop with single-pass heat exchangers (34 plates in wing A and 21 plates in wing B). The available surfaces were estimated at 15 m^2 (wing A) and 5.5 m^2 (wing B), with water volumes of 11.4 L and 3.8 L, respectively, resulting in a very high surface-to-volume ratio of 14 cm^{-1}. Temperatures within the heat exchangers ranged from 9°C to 46°C, and prolonged stagnation was observed during the night, resulting in no flow for 48% and 51% of the time in wing A and wing B, respectively. The average flow rates during daily operation were estimated to be 18 L/min in wing A and 43.8 L/min in wing B, well below the maximum designed flow rate of 230 L/min. Figure 1 illustrates the flow diagram of the heat exchangers for both wings. The hot water temperature feeding into both distribution systems was less than 55°C before the outbreak.

In total, 34 clinical and environmental isolates (wing A), 7 environmental isolates (wing B), and 4 environmental isolates collected in 1995 (wing A) were typed by PFGE and SBT (Figure 2). Environmental isolates dating from 1995 were collected from the hot water system in wing A as part of a previous case investigation, where the source of infection was found to be unrelated to this hospital. Genotyping results revealed that all isolated environmental strains (1995 and 2014) harbored the same related PFGE pattern as the outbreak strain (Figure 2). All typed isolates were also from the same SBT type (ST-1427). Partial SBT profiles were obtained from 12 additional hot water sample DNA extracts using the nested-SBT protocol (Table 2), collected a year after the outbreak.[9] The obtained alleles were similar to ST-1427 for 11 of the 12 DNA extracts, suggesting the presence of the same strain in those water samples.

DISCUSSION

The environmental investigation indicated the hot water system as the most probable source of the outbreak. Although *Lp* was detected in both systems investigated, the level of contamination was significantly higher in wing A. Despite the observed reduction in hot water contamination levels after the heat shock disinfection,[9] *Lp* was still detected by culture in

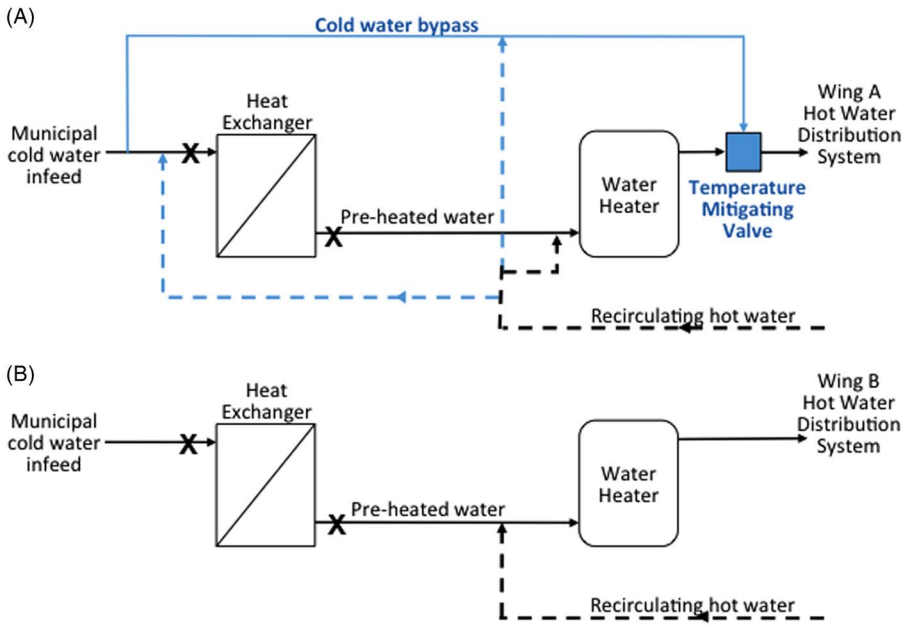

FIGURE 1. Hot water production unit flow diagram for wing A (A) and wing B (B). Differences between the 2 systems are highlighted in a different color; the letter X indicates sampling locations before and after the heat exchangers.

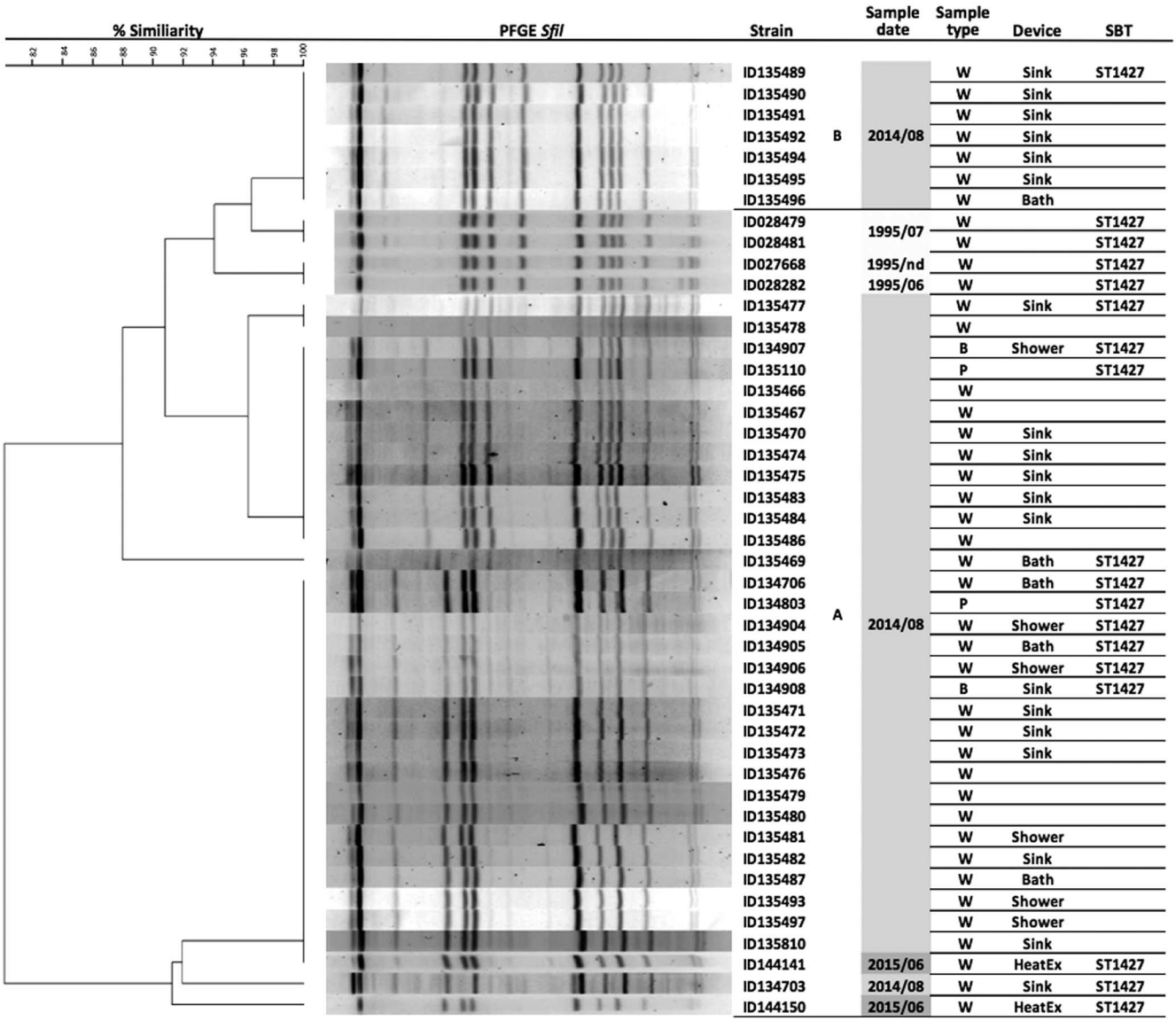

FIGURE 2. Pulsed-field gel electrophoresis (PFGE) patterns and sequence-based typing (SBT) types of *Legionella pneumophila* serogroup 5 isolated from clinical and environmental samples.

more than 45% of faucets and in the recirculation water for both wings in February 2015, 6 months after the implementation of corrective measures. The high level of *Lp* outbreak strain detected in all water samples from the heat exchanger in wing A at the time of sampling suggests its potential colonization and role at the time of the outbreak. Although no protists could be isolated, their presence in heat exchangers should also be monitored in light of the manufacturer's warning of biofouling risk due to organisms such as protozoa, a natural reservoir for *Lp*.[24]

The relatedness of the environmental strains (n = 4 in 1995; n = 39 in 2014–2015) and patient strains (n = 2) confirms a system-wide contamination with the established *Lp* sg5 strain and suggests its persistence over a period of 20 years in the hot water system. The heat exchanger in wing A promoted the *Lp* sg5 genotype present in the system, whereas no colonization of the wing B heat exchanger was identified. Detailed investigation of the flow diagrams and onsite validation showed important differences between the design and operation of the 2 hot water systems, including the heat exchanger configuration (Fig. 1). The heat exchanger from wing A was fed by a combination of cold makeup water and recirculated hot water depending on demand, and up to 48% of the recirculated water did not transit through the flash water heater. The risk of *Lp* proliferation in heat exchangers is exacerbated by (1) the prevailing environmental conditions (eg, temperature, surface area, surface-to-volume ratio, materials); (2) operational conditions (eg, low flow, stagnation); and (3) the microbial load and presence of *Lp* in the feed

TABLE 2. Sequence-Based Typing (SBT) Results From 12 Hot Water Sample DNA Extracts Collected in Wing A and Wing B in July 2015[9]

		SBT allele sequence number							
Sample no.	**Location**	***flaA***	***pilE***	***asd***	***mip***	***mompS***	***proA***	***neuA*h**	**ST**
ID145265	Wing A (Tap)	3	12	1	6	14	9	F	1427-*like*
ID145266	Wing A (Tap)	3	12	1	6	14	9	F	1427-*like*
ID145267	Wing A (Tap)	3	F	F	6	14	9	F	1427-*like*
ID145268	Wing A (Tap)	3	12	1	6	14	F	F	1427-*like*
ID145269	Wing A (Tap)	3	12	1	6	14	F	F	1427-*like*
ID145270	Wing A (Tap)	3	12	NEW	6	F	NEW	F	New profile
ID145329	Wing B (Tap)	3	12	1	6	14	9	F	1427-*like*
ID145330	Wing B (Recirc)	3	12	1	6	14	9	F	1427-*like*
ID145331	Wing B (Tap)	3	12	1	6	14	9	F	1427-*like*
ID145332	Wing B (Tap)	3	12	1	6	14	9	F	1427-*like*
ID145333	Wing A (Heat exchanger water infeed)	3	12	1	6	14	9	F	1427-*like*
ID145334	Wing A (Heat exchanger water infeed)	3	12	1	6	14	9	F	1427-*like*
Outbreak strain		3	12	1	6	14	9	220	1427

NOTE. F, polymerase chain reaction amplification failed; NEW, allele number pending; ST, sequence type.

water, which was the case in wing A for the recirculated water feed. The presence of the outbreak strain in the system for the past years combined with feeding contaminated recirculated water into a heat exchanger providing ideal growth conditions likely culminated in the higher *Lp* loads measured in wing A and a higher risk of infection. The contamination observed in wing B was associated with areas having recirculation deficiencies, preventing the hot water temperature from being maintained in these areas. Resolution of the identified deficiencies contributed to the reduction in *Lp* contamination observed.[9]

The physical characteristics and operating conditions of heat exchangers provide ideal conditions for biofilm formation and the development of opportunistic pathogens. In the present study, although physical characteristics of the heat exchangers were similar in the 2 wings, the piping diagram and operating conditions were different. Design and operation of hot water system should prevent *Lp* proliferation and prevent the conditions in which amoebae-hosting biofilms develop. Furthermore, hot water system operators should not rely on the passage of water through the water heater to inactivate *Lp* from the recirculating stream and makeup preheated water. Short exposure to elevated temperatures may not be sufficient to inactivate certain strains of *Lp* shown to resist high temperatures (70°C) for prolonged periods (60 minutes) and to develop heat resistance after shock treatment.[25,26]

Our study was subjected to a number of limitations. First, although the same strain was isolated in the system in 1995, we could only hypothesize that it was present continuously in the system over the 20 years preceding the outbreak in 2014 since there was no monitoring of *Lp* performed in the hot water system during those years. Second, PFGE could not be performed on strains isolated from the hot water system samples 1 year after the outbreak. Third, owing to the urgency to apply corrective measures, the observed decrease in system contamination cannot be attributed to a specific corrective measure but rather to the sum of actions that were taken to eradicate the contamination. Finally, sampling of the heat exchangers was not performed at the time of the outbreak because they were not suspected initially.

In healthcare facilities serving patients more vulnerable to legionellosis, the risk associated with the installation of such devices needs to be carefully evaluated with respect to the important costs associated with legionellosis hospitalization (US $34,000/episode) and the elevated mortality rate.[1] Estimated energy savings in the present case study ranged from US $700 to US $1,700 per month. The addition of energy conservation devices and operational procedures should be evaluated by the water safety committee together with the infection prevention and control team, and weighed against the risk of exposing patients and the burden of preventive monitoring.

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REFERENCES

1. Collier SA, Stockman LJ, Hicks LA, Garrison LE, Zhou FJ, Beach MJ. Direct healthcare costs of selected diseases primarily or partially transmitted by water. *Epidemiol Infect* 2012;140: 2003–2013.

2. Demirjian A, Lucas CE, Garrison LE, et al. The importance of clinical surveillance in detecting Legionnaires' disease outbreaks: a large outbreak in a hospital with a *Legionella* disinfection system—Pennsylvania, 2011–2012. *Clin Infect Dis* 2015;60:1596–1602.
3. Bartley PB, Ben Zakour NL, Stanton-Cook M, et al. Hospital-wide eradication of a nosocomial *Legionella pneumophila* serogroup 1 outbreak. *Clin Infect Dis* 2015;62:273–279.
4. Durando P, Orsi A, Alicino C, et al. A fatal case of nosocomial Legionnaires' disease: implications from an extensive environmental investigation and isolation of the bacterium from blood culture. *Infect Control Hosp Epidemiol* 2015;36:1483–1485.
5. Perola O, Kauppinen J, Kusnetsov J, Karkkainen UM, Luck PC, Katila ML. Persistent *Legionella pneumophila* colonization of a hospital water supply: efficacy of control methods and a molecular epidemiological analysis. *APMIS* 2005;113:45–53.
6. Montagna MT, Ricci C, Napoli C, et al. *Legionella pneumophila* serogroup 5 infection in the presence of multiple environmental contamination: the importance of a bacteriological diagnosis. *Ital J Public Health* 2007;4.
7. Mineshita M, Nakamori Y, Seida Y, Hiwatashi S. *Legionella pneumonia* due to exposure to 24-hour bath water contaminated by *Legionella pneumophila* serogroup-5. *Intern Med* 2005;44:662–665.
8. Buse HY, Schoen ME, Ashbolt NJ. *Legionellae* in engineered systems and use of quantitative microbial risk assessment to predict exposure. *Water Res* 2012;46:921–933.
9. Bédard E, Boppe I, Kouamé S, et al. Combination of heat shock and enhanced thermal regime to control the growth of a persistent *Legionella pneumophila* strain. *Pathogens* 2016;5:35.
10. Marchesi I, Marchegiano P, Bargellini A, et al. Effectiveness of different methods to control *Legionella* in the water supply: ten-year experience in an Italian university hospital. *J Hosp Infect* 2011;77:47–51.
11. Declerck P. Biofilms: the environmental playground of *Legionella pneumophila*. *Environ Microbiol* 2010;12:557–566.
12. Moritz MM, Flemming HC, Wingender J. Integration of *Pseudomonas aeruginosa* and *Legionella pneumophila* in drinking water biofilms grown on domestic plumbing materials. *Int J Hyg Environ Health* 2010;213:190–197.
13. Health and Safety Executive (HSE). *Legionnaires' Disease: Technical Guidance.* Part 2: The control of *Legionella* bacteria in hot and cold water systems. United Kingdom: HSE Books; 2013.
14. American Society of Heating, Refrigerating and Air-Conditioning Engineers (ASHRAE). *Legionellosis: Risk Management for Building Water Systems*. Atlanta: ASHRAE; 2015:22.
15. République Française. Circular DGS/EA4/2010/448, December 21st 2010 regarding the implementation of February 1st 2010 Ministerial Order Legionella surveillance in sanitary hot water production, storage and distribution installations [in French]. http://www.sante.gouv.fr/fichiers/bo/2011/11-01/ste_20110001_0100_0130.pdf. Published 2010. Accessed August 21, 2016.
16. Centre scientifique et technique du bâtiment (CSTB). Technical guide – controlling the risk of *Legionella* in sanitary hot water distribution systems—Maîtrise du risque de développement des légionelles dans les réseaux d'eau chaude sanitaire—faults and recommandations [in French]. http://www.ars.rhonealpes.sante.fr/fileadmin/RHONE-ALPES/RA/Direc_sante_publique/Protection_Promotion_Sante/Environnement_Sante/Legionelle/guideCSTB-312.pdf. 2012.
17. Government of South Australia, Health Protection Programs, Public Health Services, Public Health and Clinical Systems, Department for Health and Ageing, on the recommendation of the Chief Public Health Officer. Guidelines for the control of *Legionella* in manufactured water systems in South Australia 2008. Rundle, Australia. 2013. Report no. 978174 2435169.
18. DVGW German Technical and Scientific Association for Gas and Water. Technical Rule: Code of Practice W 551. Drinking water heating and drinking water piping systems; technical measures to reduce *Legionella* growth; design, construction, operation and rehabilitation of drinking water installations. 2004.
19. Austrian Standards Institute. Hygienerelevante Planung, Ausführung, Betrieb, Wartung, Überwachung und Sanierung von zentralen Trinkwasser-wärmungsanlagen. 2007:44.
20. Levesque S, Plante PL, Mendis N, et al. Genomic characterization of a large outbreak of *Legionella pneumophila* serogroup 1 strains in Quebec city, 2012. *PLOS ONE* 2014;9:e103852.
21. Association Française de Normalisation (AFNOR). Qualité de l'eau—Recherche et dénombrement de *Legionella* spp et *de Legionella pneumophila*—Méthode par ensemencement direct et après concentration par filtration sur membrane ou centrifugation. La Plaine Saint-Denis, France, 2014:34.
22. Ginevra C, Lopez M, Forey F, et al. Evaluation of a nested-PCR-derived sequence-based typing method applied directly to respiratory samples from patients with Legionnaires' disease. *J Clin Microbiol* 2009;47:981–987.
23. Tenover FC, Arbeit RD, Goering RV, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995;33:2233–2239.
24. Lau HY, Ashbolt NJ. The role of biofilms and protozoa in *Legionella* pathogenesis: implications for drinking water. *J Appl Microbiol* 2009;107:368–378.
25. Allegra S, Berger F, Berthelot P, Grattard F, Pozzetto B, Riffard S. Use of flow cytometry to monitor *Legionella* viability. *Appl Environ Microbiol* 2008;74:7813–7816.
26. Allegra S, Grattard F, Girardot F, Riffard S, Pozzetto B, Berthelot P. Longitudinal evaluation of the efficacy of heat treatment procedures against *Legionella* spp. in hospital water systems by using a flow cytometric assay. *Appl Environ Microbiol* 2011;77:1268–1275.