Study of the microbiome of a model cooling tower harboring *Legionella pneumophila*

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Abstract

Legionella pneumophila is a waterborne pathogen and the causative agent of Legionnaires disease (LD), a severe pneumonia, contracted by the inhalation of contaminated aerosols. Cooling towers are considered to be the main sources of outbreaks. Because of this, there is a growing awareness of the conditions that impact *Lp* colonization of water systems and efforts to elucidate the risk factors have been made. The microbiota seems to affect the presence of *Legionella* as well but the relationship between the microbial community within water distribution systems and *Lp* is not well understood. Moreover, the studies conducted to analyze the microbiota in water distribution systems more often do not take into account the microbial populations within the biofilm, just studying the microbiota in the water. A model cooling tower was built and filled with water from a real cooling tower. The temperature in the system represented the real temperature found in these heat-exchanging systems. It was inoculated with *Vermamoeba Vermiformis*, a natural host of *Lp*. *Lp* was further inoculated in the system. Water samples were taken throughout the duration of the experiment and biofilms were extracted at the dismantlement of the system after 3 months. 16S rRNA and 18S rRNA genes amplicon sequencing was carried out to analyze the bacterial communities and the host cells communities. The effect of the material (PVC and an elastomer) and the temperature of the pipes (22.7°C and 30.7°C) on the microbial communities in biofilm samples was studied. There were significant differences between the bacterial communities in biofilms growing on the different materials. Moreover, bacterial communities growing in biofilms in the same material but at different temperatures were also different. This was not the case for the eukaryotic communities. Eukaryotic

communities present in biofilms growing in PVC were affected by the temperature while the communities present in the elastomer were not affected. Our results showed that *Lp* was mostly present in water samples while the host cells were present in the biofilm. This suggests that Lp might have grown in the biofilm and was released into the water after the lysis of the host cells. Moreover, our study showed that a better understanding of the dynamics of *Legionella* proliferation in amoebae present in biofilms is needed.

Résumé

Legionella pneumophila (*Lp*) est une bactérie de transmission aquatique et l'agent pathogène de la légionellose, une sévère pneumonie contractée par inhalation d'aérosols contaminés. Les tours de refroidissement sont considérées comme la principale cause d'éclosions de Légionellose. C'est pour cela qu'une connaissance des conditions qui impactent la présence de *Lp* dans des tours de refroidissement est de plus en plus développée et des efforts pour élucider des facteurs de risques sont faits. Le microbiote semble avoir un impact sur la présence de *Lp* mais la relation entre la communauté microbienne dans des systèmes d'approvisionnement d'eau et *Lp* n'est pas claire. De même, des études effectuées pour analyser le microbiote dans des systèmes d'approvisionnement d'eau, ne prennent souvent pas en compte la population microbienne du biofilm et étudient simplement le microbiote de l'eau.

Un modèle de tour de refroidissement a été construit et rempli avec de l'eau d'une vraie tour. La température dans le système a représenté la température réelle de ces systèmes d'échange de chaleur. Le système a été inoculé avec *Vermamoeba vermiformis*, un hôte naturel de *Legionella. Lp* a été ensuite inoculée dans le système. Des échantillons d'eau ont été pris régulièrement pendant tout le long de l'expérience et le biofilm a été extrait lors du démantèlement du système trois mois après. Un séquençage d'amplicon des gènes 16S rRNA et 18S rRNA a été effectué pour analyser les communautés bactériennes et des cellules hôtes. Les matériaux (PVC et un élastomère) et les différentes températures dans le système (22.7 et 30.7°C) ont eu un impact sur les communautés du biofilm. Il est intéressant de noter qu'il y a eu des différences significatives entre les communautés bactériennes des biofilms dans les différents

matériaux. De même, les communautés bactériennes dans les biofilms d'un même matériel mais exposées à des températures différentes sont dissimilaires . Ceci n'est pas le cas pour les eucaryotes. La température semble uniquement affecter les communautés présentes dans le PVC et pas les communautés dans l'élastomère. Nos résultats montrent que *Lp* est présente dans les échantillons d'eau tandis que les cellules hôtes sont présentes dans le biofilm. Ceci suggère que *Lp* aurait pu pousser dans le biofilm et être relâchée dans l'eau lors de la lyse des cellules hôtes. En outre, notre étude a montré qu'une meilleure compréhension de la dynamique de prolifération de *Lp* en relation avec les cellules hôtes dans le biofilm des tuyaux est nécessaire.

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Abbreviations

CDC: Centers for Disease Control

CFU: Colony Forming Unit

Dot: Defective in organelle trafficking

EPA: Environmental Protection Agency

ER: Endoplasmic Reticulum

GVPC: Glycine-Vancomycin-Polymyxin-Cycloheximide Medium

HPC: Heterotrophic plate count

LCV: Legionella-containing vacuole

LD: Legionnaires' disease

Lp: *Legionella pneumophila*

*Lp*1: *Legionella pneumophila* serogroup 1

PCR: Polymerase chain reaction

PVC: Polyvinyl chloride

PEX: Cross-linked polyethylene

USA: United States of America

USD: United States Dollars

VBNC: Viable but non-culturable

V.v: *Vermamoeba vermiformis*

Introduction

Legionella pneumophila (*Lp*) is a Gram negative waterborne bacterium, first described after an outbreak of pneumonia following the American Legion convention in Philadelphia in 1976 (Fraser et al. 1977; McDade 1977; Brenner et al. 1978; Brenner et al. 1979; McDade et al. 1979). Nowadays, *Lp* is known for causing Legionnaires' disease (LD), a severe pneumonia contracted by the inhalation of contaminated aerosols (Buse et al. 2012). *Lp* is the main cause of waterborne disease in the USA and the costs related to the hospitalization due to LD exceed $716 million USD per year (Giambrone 2013; Whiley et al. 2014).

Like other waterborne pathogens, *Lp* is ubiquitous in aquatic ecosystems (Fliermans et al. 1981; Fliermans 1996). In addition, this bacterium is also naturally present in artificial water systems (Fields et al. 2002a; Farhat et al. 2009). In fact, hot water distribution systems, cooling towers, pools and fountains can promote favorable conditions for *Lp* colonization and proliferation (Buse et al. 2012). Water stagnation, temperatures between 25 and 50 °C, the presence of amoebae or biofilm and poor management of the systems are some of the conditions that promote the presence of the bacteria (Kruse et al. 2016). In water systems, *Lp* can be found in two states: a planktonic motile state, and in biofilm. Nonetheless, the bulk of *Legionella* population, like other microorganisms, is found within biofilms (Murga et al. 2001; Di Gregorio et al. 2017). In addition, some organisms can promote the growth of *Lp* while some others can inhibit it (Tison et al. 1980; Wadowsky and Yee 1983; Hechard et al. 2006; Guerrieri et al. 2008; Messi et al. 2011).

It is common to detect legionella in water distribution systems. However, not all detection events are linked to LD outbreaks. This suggests that the presence of

Legionella is not enough to cause an outbreak (Mitchell et al. 1990; Alary and Roy 1992; Marrie et al. 1994; Wullings and van der Kooij 2006; Mathys et al. 2008; Yu et al. 2008; Hampton et al. 2016; Llewellyn et al. 2017). The bacteria can proliferate under permissive environmental conditions and reach levels that present a health risk. Even though it has been established that abiotic and biotic factors affect the presence of *Lp* in cooling towers, the dynamics of its proliferation and its location within cooling towers are not yet well understood. A study conducted by Paranjape et al. (2019) showed that the interplay between abiotic factors such as chlorination and biotic factors like the presence of *Pseudomonas* in cooling tower had a detrimental effect on the presence of *Lp* in some water systems. Indeed, to individually select and study parameters linked with *Lp* growth is not sufficient. The multitude of factors that affect *Lp* proliferation do not act independently from each other. Both the biotic and abiotic factors that promote *Lp* presence in a cooling tower are closely related to one another.

Therefore, the work presented makes use of a model cooling tower to study the dynamics of *Lp* colonization in both water and in the biofilm. This model does not only focus on one factor impacting *Lp* growth. Instead, we propose a study where our model cooling tower is a microbial biosphere. Indeed, the evolution of the microbiota in the water and in the biofilm depends on well-established and controlled conditions. The objective of the study was to determine the microbial community in the model cooling tower model in biofilm and in water and relate it to the dynamics of *Lp* colonization. In addition, special emphasis was made to understand the impact of the temperature and material of the surface on the microbial communities in the biofilm

Chapter 1: Literature Review

Establishment of the genus *Legionella*

In 1976, during an American Legion convention in Philadelphia, 182 people contracted an apparent pneumonia, 147 people were hospitalized and 29 (16%) of them died (Fraser et al. 1977). During the following months, an extensive investigation was carried out to determine what was the etiologic agent of the illness called Legionnaires' disease. Initial reports indicated that the bacterium appeared to be airborne and that the person to person transmission was unlikely (Fraser et al. 1977). Then, a gram-negative bacterium was isolated from the lungs of six patients (McDade 1977). Inoculation of the bacterium in guinea pigs produced a similar disease and the genus *Legionella* was established (McDade 1977; Brenner et al. 1978; Cherry et al. 1978).

Nowadays, the genus *Legionella* comprises more than 50 species, encompassing at least 70 serogroups (Newton et al. 2010). *Legionellae* are aerobic alphaproteobacteria, rod shaped, Gram-negative, with polar or lateral flagella with a width of 0.3-0.9 µm and a length of 2 to 20µm length (filamentous form) (Fliermans 1996). *Legionella* displays pleomorphism influenced by the environment, showing coccoid, bacillary or long filamentous forms (McDade 1977; Mauchline et al. 1992; Katz et al. 2009; Mercante and Winchell 2015). *Legionella*e are fastidious bacteria, catalase positive, chemoorganotrophic and use amino acids as carbon sources, and most strains are auxotrophs for seven amino acids that they acquire from the host cell (Pine et al. 1979; George et al. 1980; Tesh et al. 1983; Fliermans 1996; Eylert et al. 2010; Price et al. 2014). *Legionella*e are unable to reduce nitrate or use ammonium as nitrogen source (Keen and

Hoffman 1984) and do not oxidize or ferment carbohydrates (George et al. 1980; Fields 1996; Fields et al. 2002b; Steinert et al. 2002). Even though more than half of *Legionella* species have been associated with human disease, *Legionella pneumophila* is the most studied bacterium of its genus since it is responsible for over 90% of reported Legionellosis cases (Muder and Yu 2002; Newton et al. 2010; Pearce et al. 2012; Mercante and Winchell 2015). *Lp* serogroup 1 (*Lp*1) is responsible for 70-90% of human infections (Doleans et al. 2004; Thomas et al. 2015).

Legionnaires disease.

Legionellosis is a respiratory illness caused by inhalation of *Legionella*-contaminated aerosols (Fields et al. 2002b; Mercante and Winchell 2015). Legionellosis presents as two distinct clinical entities, Legionnaires Disease (LD) and Pontiac fever (Fraser et al. 1977; Glick et al. 1978). LD is a severe pneumonia and has an incubation time of 2-14 days. On the other hand, Pontiac fever is a benign flu-like form and usually resolves itself without medical treatment. The early symptoms of LD include fever, cough, headache, myalgia, malaise and gastrointestinal symptoms (Tsai et al. 1979; Steinert et al. 2002; Cunha 2010; Cunha et al. 2016; Cunha and Cunha 2017). The later manifestations of the disease include high fever and alveolitis. At this stage, severe lung damage featuring patchy infiltrated regions can be observed by X-ray radiography (Steinert et al. 2002). This atypical pneumonia distinguishes itself from other community acquired pneumonias by the failures of other organs characterized by cardiac, hepatic and renal abnormalities (Cunha 2006; Cunha et al. 2016). The risks of contracting Legionellosis are higher among individuals that have underlying medical conditions,

smokers, men, older than 50 years and immunocompromised individuals (Farnham et al. 2014; Parr et al. 2015; Cunha et al. 2016).

The incidence of outbreaks of Legionnaires disease 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). It is one of the most common causes of pneumonia in the USA and its mortality rate is estimated to be 5% to 30% (Marston et al. 1994; Parr et al. 2015). In Europe, between 5,500 and 6,500 cases are reported annually. The incident rate is around one LD case per 100,000 inhabitants and there are 450 deaths per year (Beauté 2013; 2017). Approximately 70% of the reported cases were community-acquired, 20% travel associated and 10% healthcare-related. Between 2011 and 2015, Austria, Czech Republic, Germany, Italy and Norway reported increased rates of reported cases of LD (Beauté 2017).

Moreover, the incidence of LD is related to seasonal variations and weather conditions, specially rainfall (Garcia-Vidal et al. 2013). A study conducted Fisman et al. (2005) suggested that the acute occurrence of the disease is predicted by wet humid weather. This was confirmed by the results of a study conducted by Hicks et al. (2007), where the association between monthly Legionellosis incidence and monthly rainfall totals from 1990 to 2003 was analyzed. An increased rainfall was associated to an increased risk of Legionellosis of 14.6%.

Diagnosis and management of LD require the identification of *Legionella* spp. in clinical samples. The success of detecting *Legionella* depends on the severity of the disease, the integrity of the specimen and the efficacy of the method of detection. The

detection of *Legionella* infection can be performed on a multitude of specimens such as lung tissue, blood serum, urine and respiratory tract secretions (Mercante and Winchell 2015). Even though culture diagnosis remains the standard method for detecting *Legionella* in clinical specimens, a multitude of methods have been developed to diagnose LD. For instance, the Urinary Antigen Test allows a rapid diagnostic and quick response detecting specifically *Lp*1 and represent 82 to 97% of the diagnostic tool used for LD confirmation in Europe and the United States (Mercante and Winchell 2015). Molecular techniques such as nucleic acid amplification-based methods are also used to detect specific species and serogroups of *Legionella* (Cunha 2010; Cunha et al. 2016).

Association of *Lp* with protozoa

Legionella lives in low nutrient freshwater systems; however, the levels of nutrients that the bacterium requires to grow and proliferate are rarely found in aquatic systems. Quickly after its identification, it was found that *Legionella* is an intracellular parasite of Free Living Amoebas (Rowbotham 1980). Protozoans serve as host cells, required for the intracellular replication of *Legionella* species (Abu Kwaik et al. 1998; Atlas 1999; Murga et al. 2001). The bacterium can replicate intracellularly in 14 species of amoebas, two species of ciliates and one species of mold (Abu Kwaik et al. 1998; Solomon et al. 2000; Fields et al. 2002b). In freshwater, *Lp* is a parasite of *Naegleria*, *Acanthamoeba*, and *Vermamoeba* (Rowbotham 1980; Tyndall and Domingue 1982; Holden et al. 1984; Newsome et al. 1985; Moffat and Tompkins 1992; Douterelo et al. 2014; Ashbolt 2015). In water distribution systems, high concentrations of *Legionella* are correlated with the

presence of amoeba and ciliates (Barbaree et al. 1986; Paszko-Kolva et al. 1991; Scheikl et al. 2016).

Host cells also protect *Legionella* from harsh environments (Steinert et al. 2002; Martinez et al. 2018). Intracellular *Legionella* are more resistant to biocides and antibiotics than free living cells (Barker et al. 1995; Dupuy et al. 2011). In addition, amoeba promote resuscitation of viable but non-culturable (VBNC) *Legionella* cells (Garcia et al. 2007). After intracellular growth, *Legionella* is released into the water by the lysis of amoeba and can then infect another host cell (Greub and Raoult 2004; Lau and Ashbolt 2009). Moreover, the bacterium can be expelled in cysts from ciliates such as a O*ligohymenophorea* species like *Tetrahymena* as well as amoebas such as *Acanthamoeba* (Bouyer et al. 2007; Berk et al. 2008; Hojo et al. 2012) . These cysts provide protection from the environment, temperature and biocides (Kilvington and Price 1990). Bacteria released through these vesicles are viable for up to 6 months and are more resistant to heat and disinfection (Bouyer et al. 2007). Vesicles expelled from amoeba may contain from 20 to 200 Legionella cells. Berk et al. (1998) demonstrated that *A. polyphaga* is able to expel 25 *Lp* filled vesicles over a 24h period. Moreover, Buse and Ashbolt (2012) showed that in a drinking water distribution system, the maximum number of *Lp* released from *A. polyphaga* and *Naegleria fowleri* was respectively 1348 and 385 CFU per amoeba.

Association with biofilm

Legionella can be found in the water in two states; a planktonic state, motile in the water or in a sessile state within the biofilm. Biofilm is composed of an assemblage of

surface attached bacteria and an extracellular polymeric substance that encapsulates and protects them from deleterious conditions (Donlan 2002). The formation of biofilm consists of three phases: bacterial attachment, biofilm maturation and bacterial dispersal (Declerck 2010). *Legionella*, like other microorganisms, is mostly surface associated within water distribution systems (Lau and Ashbolt 2009). This is because biofilms provide shelter, protecting bacteria from harsh environments (Berjeaud et al. 2016). *Legionella* present in biofilm are extremely resistant to biocides such as chlorine (Kim et al. 2002; Borella et al. 2005a; Berjeaud et al. 2016). Indeed, chlorination is an appropriate treatment in water distribution systems for removal of free living bacteria; nonetheless, this treatment remains insufficient for the removal of sessile bacteria (Cooper and Hanlon 2010; Simões et al. 2010). In addition, multispecies biofilms are more resistant to disinfection than single-species biofilms because of the presence of nitrifying bacteria that contribute to the depletion of disinfectant residuals (Berry et al. 2006; Berjeaud et al. 2016).

Generally, propagation of *Legionella* within water distributions systems occur through its interactions with protozoa in biofilms (Rogers et al. 1994b; Lau and Ashbolt 2009). This is due to the fact that protozoa graze on bacteria present in the biofilm (Abdel-Nour et al. 2013; Berjeaud et al. 2016). Consequently, *Lp* presence in biofilm is correlated with the biomass of protozoa (Abdel-Nour et al. 2013). Interestingly, *Legionella* is also able to survive in biofilms without the presence of amoeba. This suggests that certain sessile bacteria can provide sufficient nutrients for *Legionella* to grow extracellularly in biofilms (Rogers and Keevil 1992). For instance, *Flavobacterium* and *Cyanobacteria* can promote *Lp* growth within biofilm (Tison et al. 1980; Wadowsky and Yee 1983).

To understand this interplay between *Lp* and biofilm, several studies have been conducted in order to determine the capacity of the bacterium to form biofilm (Rogers et al. 1994a; Wery et al. 2008; Farhat et al. 2009; Moritz et al. 2010; Buse et al. 2014; Buse et al. 2017; Rhoads et al. 2017). Formation of biofilm by *Lp in vitro* requires rich media (Mampel et al. 2006; Piao et al. 2006; Declerck 2010). This suggests that under environmental circumstances, *Legionella* incorporates in pre-established biofilms as a secondary colonizer (Declerck 2010).Instead of attaching to surfaces and growing biofilm, the bacterium will form an association with other microbes that previously developed biofilm (Watnick and Kolter 2000).

Moreover, the presence of *Lp* within biofilm varies based on the material of the surface (Rogers et al. 1994b; Armon et al. 1997; Moritz et al. 2010; Wang et al. 2012; Lin et al. 2013; Rozej et al. 2015). A study showed that *Lp* was able to integrate and persist for up to 4 weeks in drinking water biofilms grown on cross-liked polyethylene and copper surfaces. However, the recovery of cultivable *Lp* from copper coupons was low (Moritz et al. 2010). Another study compared *Lp* growth on one-year old biofilms grown on copper and PVC coupons in the presence of *A. polyphaga*. *Lp* appeared to colonize copper-grown biofilm more efficiently in proportion to the total cells present (Buse et al. 2014). In addition, the bacterium has a better capacity of colonizing biofilm grown on PVC than biofilm grown on glass (Armon et al. 1997). Moreover, temperature affects *Lp* cultivability in biofilm (Rogers et al. 1994b; Buse et al. 2017). Integration of *Legionella* into biofilms is therefore affected by the temperature of the water, surface material, water quality and biofilm age (Buse et al. 2017). The morphology of biofilm associated cells also seem to be affected by temperature. This was evidenced by Piao et al. (2006) who studied the

ability of *Lp* to form biofilm on glass, polypropylene and polystyrene at different temperatures. Biofilm grown at 25°C displayed rod shaped *Lp* cell and its arrangement was a typical mushroom like structure. Biofilms formed at higher temperatures, displayed a greater cell density.

Legionella intracellular cycle

As stated previously, *Legionella pneumophila* is able to multiply intracellularly in protozoan hosts like amoeba. However, the bacterium is also able to replicate in a human monocytes and alveolar macrophages. This polyvalent host specificity is due to the fact that these hosts cells share many similarities (Horowitz and Silverstein 1986; Rowbotham 1986; Oliva et al. 2018). Furthermore, *Lp* uses similar strategies to grow intracellularly in both hosts (Abu Kwaik 1996; Gao et al. 1997; Fields et al. 2002b; Hilbi et al. 2007). This similarity suggests a possible co-evolution of *Legionella* within protozoa that allowed the bacterium to be able to colonize mammalian cells (Oliva et al. 2018). Several studies revealed that certain species of Legionella such as *Lp* and *L. longbeachae* have acquired genes coding for proteins with eukaryotic-like properties (Cazalet et al. 2004; Cazalet et al. 2010; Gomez-Valero et al. 2011; Oliva et al. 2018).

Lp enters the host cell either by conventional or coiling phagocytosis (Horowitz and Maxfield 1984; Bozue and Johnson 1996; Fields et al. 2002b; Escoll et al. 2013). The infection cycle of *Lp* consists of a series of events triggered by the action of one or multiple effector proteins secreted by the Dot/Icm type IV secretion system (Molmeret et al. 2004). After phagocytosis, *Lp* forms a specialized vacuole, the *Legionella* Containing Vacuole (LCV) that allows its replication within the host. There, the bacterium avoids lysosome-

mediated degradation. This vacuole differs from the phagosome containing non-pathogenic bacteria due to the fact that it does not undergo acidification and the phagosomal membranes are different (Horowitz 1983; Horowitz and Maxfield 1984; Newton et al. 2010; Xu and Luo 2013; Oliva et al. 2018). Additionally, the LCV does not fuse with lysosomes. Following the uptake of *Lp* by phagocytic cells, the LCV becomes surrounded by small vesicles originating from the rough endoplasmic reticulum (ER) (Isberg et al. 2009; Newton et al. 2010; Xu and Luo 2013; Oliva et al. 2018). Other organelles such as mitochondria are also recruited in proximity to the LCV (Molmeret et al. 2004). The phagosomal membrane undergoes a transformation, resembling rough ER and becomes surrounded by ribosomes (Horowitz and Maxfield 1984). Within this ER-like compartment the bacterium starts to multiply and eventually causes the lysis of the host cell (Figure 1.1).

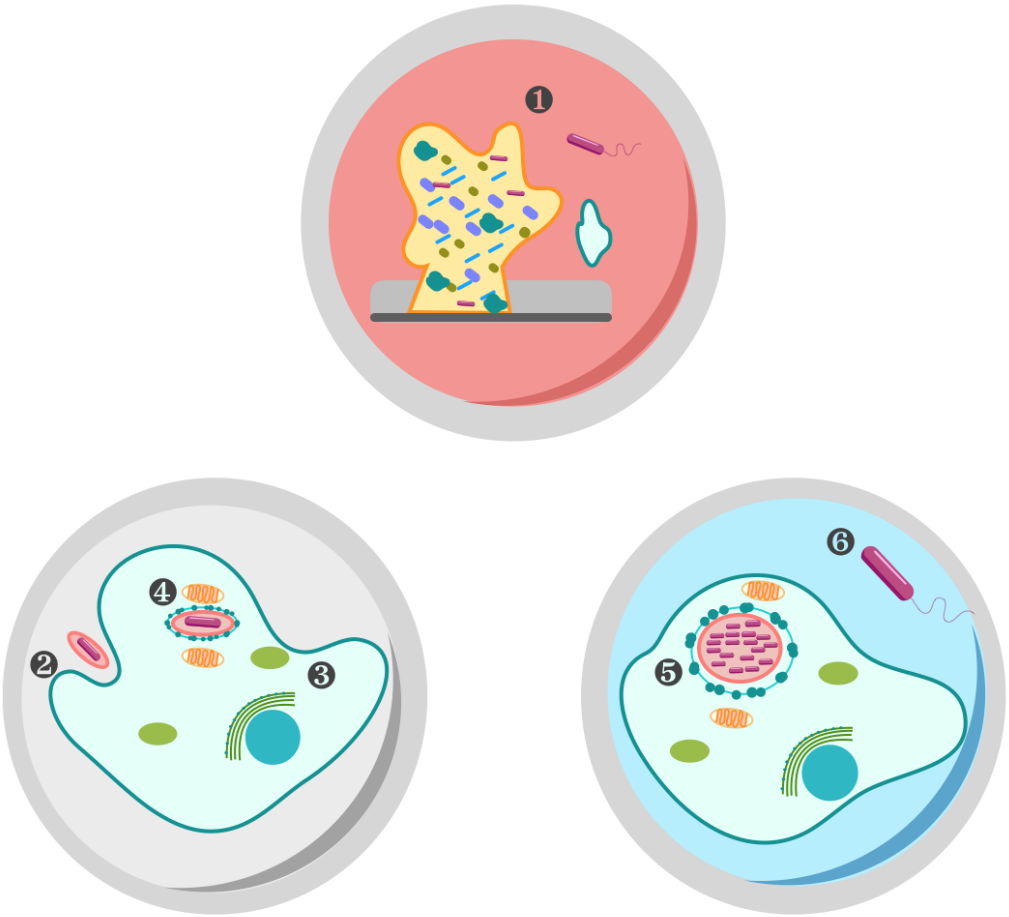

Figure 1.1 *Legionella* intracellular life cycle

Lp can replicate intracellularly in amoeba and ciliates present in water distribution systems (1). After phagocytosis, *Lp forms* Legionella containing vacuole (LCV) (2). This LCV does not fuse with lysosomes (3). The LCV recruits organelles such as mitochondria and ER from which acquires nutrients (4). The phagosomal membrane undergoes a transformation, resembling rough ER and becomes surrounded by ribosomes. The bacterium replicates (5). This replication causes eventually the lysis of the host cell and the bacteria are released back into the water (6).

Environmental sources

Natural Sources

Legionellae are ubiquitous waterborne bacteria, found at low concentrations in fresh water sources, representing less than 1% of the resident bacterial population (Borella et al. 2005a). The exception is *L. longbeachae* which resides mainly in soil (Fliermans et al. 1981). In several studies conducted to assess the presence of *Legionella* in freshwater systems, *Legionella* has been detected in up to 40% of freshwater systems by culture and in 80% by PCR (Fields 1996; Borella et al. 2005a). Several species have been isolated from different aquatic environments such as lakes, groundwater, ocean water and sewage as well as in extreme environments such as polar lakes and hypersaline lakes (Fliermans et al. 1981; Cherry et al. 1982; Ortiz-Roque and Hazen 1987; Palmer et al. 1993; Sheehan et al. 2005; Lin et al. 2007; Carvalho et al. 2008; Gast et al. 2011).

Anthropogenic Water Systems

Legionella can be distributed from fresh water sources at low concentrations into man-made water distribution systems. These systems allow for optimal survival and growth conditions for the proliferation of the bacteria (Steinert et al. 2002; Borella et al. 2005a). The proliferation in water distribution systems that create aerosols may lead to the inhalation of contaminated aerosols and cause LD (Figure 1.2).

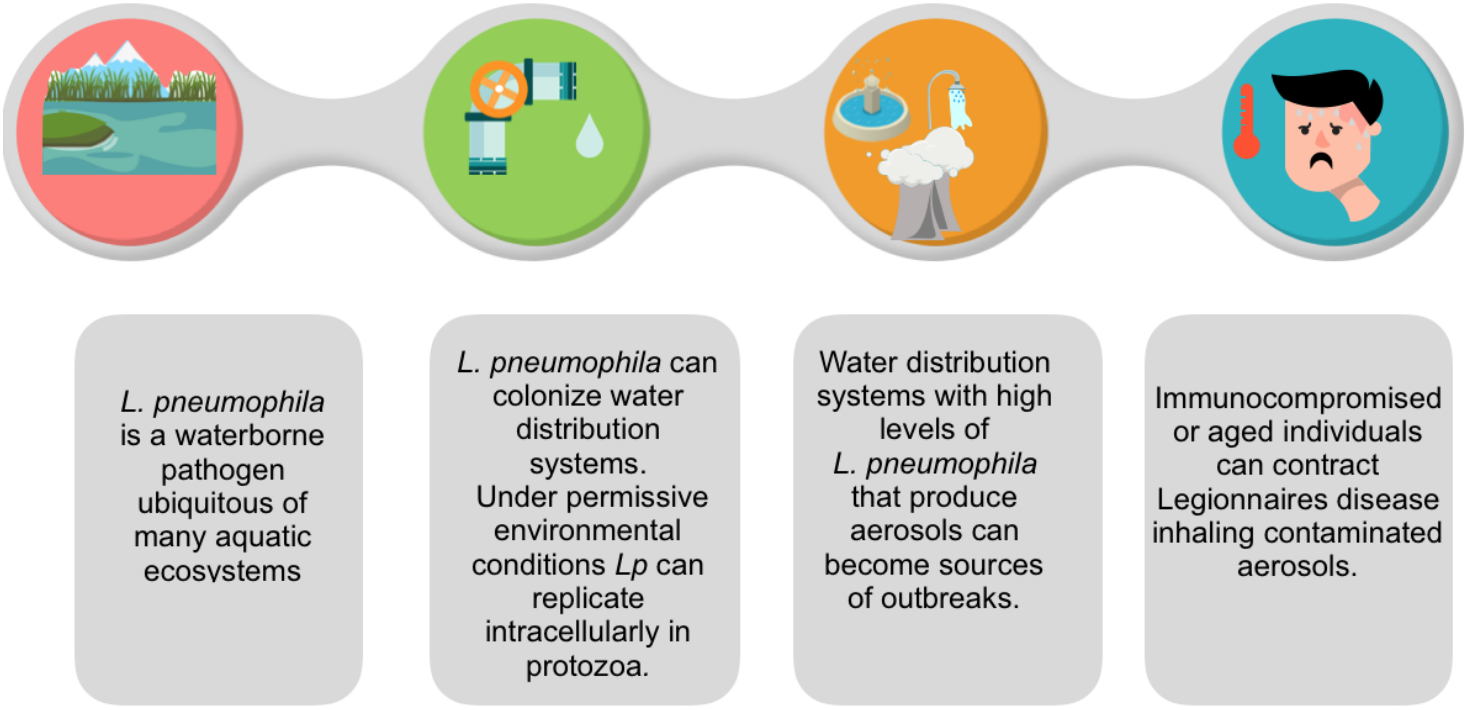

Figure 1.2 Transmission of Legionellosis through water distribution systems

Legionella has been detected in pools (Leoni et al. 2001; Leoni et al. 2018), water fountains (Smith et al. 2015), dental units (Atlas et al. 1995), humidifiers (Moran-Gilad et al. 2012), cooling towers (Cappabianca et al. 1994; Brown et al. 1999; Mouchtouri et al. 2010; Fitzhenry et al. 2017; Llewellyn et al. 2017; Paranjape et al. 2019), domestic potable water distribution systems (Stout et al. 1992), hospitals (Bedard et al. 2016; Kyritsi et al. 2018) and hotels (Hampton et al. 2016) among other systems. A study conducted in Quebec, Canada, in which the presence of *Legionella was* assessed in domestic water, showed that *Legionella* was recovered from water samples of 32.7% of the houses that participated in the survey (Alary and Roy 1992). Another Canadian study that compared the prevalence of *Legionella* in water distribution systems of single-family houses and multiple dwelling apartments revealed that 6.2% of water samples from single family

residences were *Legionella* positive while 25% of the multiple dwelling apartments harbored *Legionella* (Marrie et al. 1994). The conditions inside of hot water distribution systems from hospitals and hotels favor the presence of *Legionella*. The temperatures in these systems are usually bellow 50°C and the conditions of the distribution systems might favor the stagnation of water and formation of sludge (Hart and Makin 1991). Between 27.3 to 75% of health facilities and 75% of water distribution systems within hotels are reportedly contaminated with *Legionella* (Borella et al. 2005b; Mouchtouri et al. 2007; Mavridou et al. 2008; Fragou et al. 2012; Velonakis et al. 2012).

Even though cooling towers are the major source of LD outbreaks and 28% of LD sporadic cases are linked to these, sporadically occurring LD may also be linked to potable water distribution systems (Straus et al. 1996; Marston et al. 1997; von Baum et al. 2008; Cassell et al. 2018). These cases constitute between 3% and 7% of the community acquired pneumonia (Cassell et al. 2018). Water distribution systems harboring *Lp* are an important source of community acquired LD (Stout et al. 1985; Atlas 1999). Several studies have linked LD outbreaks to water distribution systems (Mitchell et al. 1990; Mathys et al. 2008; Yu et al. 2008; Hampton et al. 2016).

Legionella in 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 (Figure 1.3).

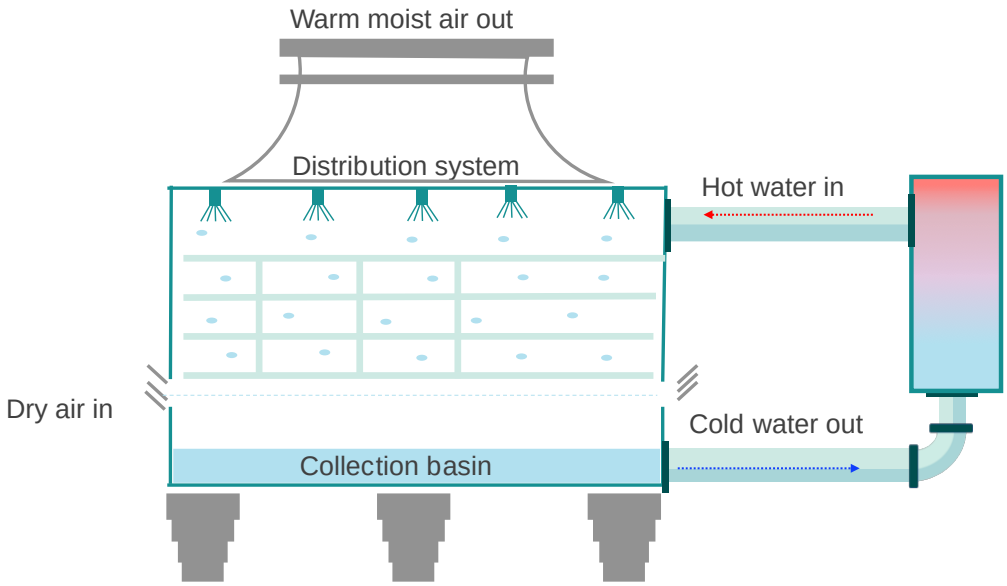

Figure 1.3: Schematic representation of a cooling tower.

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.

The source of the outbreak of 1979 in Philadelphia that made 182 people sick was a cooling tower (Kurtz et al. 1982). Since then, several studies have reported the links between outbreaks of Legionellosis and cooling towers (Addiss et al. 1989; Breiman et

al. 1990; Mitchell et al. 1990; Shelton et al. 1994; Bell et al. 1996; Brown et al. 1999; Greig et al. 2004; Isozumi et al. 2005; Wang et al. 2014; Fitzhenry et al. 2017).

Aerosols

Cooling towers are important generators of aerosols. Thus, they can become sources of outbreaks if they harbour high levels of *Legionella*. It is estimated that inhalation occurs when particles containing *Legionella* cells have an aerosol size <10 μm (Schoen and Ashbolt 2011). These can be dispersed over long distances and potentially infect a multitude of exposed individuals. Indeed, aerosols from cooling towers have been reported to travel long distances. A study conducted to evaluate the dispersion of LD in Sheboygan, Wisconsin, suggested a distance of airborne transmission between 1 and 2 miles from the cooling tower (Addiss et al. 1989). Another study conducted by Klaucke et al. (1984) suggested a distance of airborne transmission of at least 150 m. Nguyen et al. (2006) investigated a community-wide outbreak of legionnaires disease in Pas-de-Calais, France, found that the distance of transmission was at least 6 km. Bhopal et al. (1991) studied the source of sporadic cases LD comparing the locations of the patients' homes in relation to the location of cooling towers. The study revealed that the risk decreased with increasing distance. The population living within 0.5 km of any cooling tower had a relative risk of contracting LD three times higher that of the people living more than 11 km away. Another example of this is an outbreak in Quebec City in 2014 where people living within 11 km from the cooling tower responsible for the outbreak were affected (Levesque et al. 2014). Moreover, the distance of airborne transmission is affected by meteorological conditions such as high humidity, low pressure and rain events (Fisman et al. 2005;

Gleason et al. 2016). It is recommended that cooling towers be located away from building air intakes, from other building openings and from areas of public access (WHO 2007) and that the systems have technical counter measures such as drift protectors to avoid the emission of small droplets.

Abiotic factors that affect *Legionella* colonization in cooling towers

Awareness of the conditions that have an impact on *Lp* in water systems and cooling towers is growing and efforts are made to elucidate the risk factors linked to outbreaks. Some characteristics of the cooling towers such as the water temperature and the materials within the system as well as the management of the equipment have an impact in the presence of *Legionella*.

Temperature

In a cooling tower system, the temperature of the water is typically between 29°C and 35°C at the output of the heat exchanger, and between 22°C and 28°C at the output of the tower (American Society of Heating Refrigerating and Air-Conditioning Engineers 2000). As stated previously, the optimal temperature for proliferation and growth of *Legionella* in water systems is 35°C (Katz et al. 2009). As an illustration of this, a survey of cooling tower water from 40 towers in Japan showed that counts of *Legionella*e correlated positively with increases in water temperature, reaching higher density of *Legionella* at water temperatures between 25 °C and 35°C (Yamamoto et al. 1992).

Due to the need of the presence of free-living protozoa in water for the proliferation of *Legionella*, studies have been conducted to assess the impact of temperature on the

prevalence of protozoa in water. High temperatures, above 50°C, impact negatively their presence in water (Canals et al. 2015). Free living amoebae usually grow at temperatures between 10 and 30°C (Rodriguez-Zaragoza 1994; Cervero-Arago et al. 2013). However, some species are thermotolerant such as *Naegleria flowleri* and *Vermamoeba vermiformis* that can grow above 40°C (Rhoads et al. 2015). Furthermore, the temperature affects the ability of *Lp* to infect protozoa. For instance, *Lp* infects *Acanthamoeba* at temperatures over 25°C. In contrast, at temperatures below 20°C *Lp* is digested, and eliminated from the host within cysts (Ohno et al. 2008). In addition, temperature shifts of the water, greater than 10°C trigger the reduction of the *Legionella* species in cooling towers (Pereira et al. 2017).

In these systems, it is hard to alter the temperature in order to control the presence of *Legionella*. However, it should be noted that in other water distribution systems, the augmentation of the temperature in the water is often used as a strategy to control the presence of *Legionella* (Bedard et al. 2015). Thermal disinfection consists in flushing all outlets, faucets and shower heads for more than 30 min at temperatures above 70°C(HSE 2013). High temperatures can promote the differentiation of *Legionella* into a VBNC state (Chang et al. 2007; Allegra et al. 2011). Moreover, at increased temperatures, *Legionella* cells have a reduced efficacy at infecting host cells, requiring prolonged co-incubation times with the hosts (Cervero-Arago et al. 2019). Prolonged exposure to higher temperatures (>60°C) in water systems can be effective against cultivable *Lp* but also, in the long term, against VBCN cells (Cervero-Arago et al. 2019).

Material

In a cooling tower, different materials are used in different parts of the system. In medium and small installations, steel with galvanized zinc is greatly employed. Fittings and tubing material are usually made of brasses and bronzes while stainless steel is often used for the drive shafts and hardware in harsh environments. Cast iron is employed for several components such as housing and valves while stainless steel is also a popular material for the construction of the cold-water collectors. Plastics are becoming more and more popular because of their low cost and durability. Materials such as PVC, fiberglass reinforced plastics, polypropylene and acrylonitrile butadiene styrene (ABS) are greatly found in these systems (American Society of Heating Refrigerating and Air-Conditioning Engineers Inc 2008).

The pipe material has an impact in the growth of *Legionella.* This was illustrated by Rogers et al. (1994b) who assessed the influence of eight different plumbing materials on the growth of *Legionella pneumophila* in potable water systems. Latex seemed to be the material that promoted the most the development of biofilm and growth of *Lp*. Interestingly, that was attributed to the leaching of nutrients from the material. In addition, biofilms formed in PVC, cross-linked polyethylene (PEX) and ethylene-propylene-diene-monomer are able to support *Lp* (van der Kooij et al. 2005). Cast iron supports better biofilm development than polycarbonate and copper. In addition, the presence of cast iron rust in drinking water systems creates a permissive environment for the growth of *Legionella* (Zhu et al. 2014; Van der Lugt et al. 2017; Mraz and Weir 2018). Stainless has also been shown to be a material that supports *Legionella* growth (van der Kooij et al. 2005).

Another interesting interaction is the relationship with the water temperature and the surface materials. For instance, at higher temperatures, the release of copper irons responsible for the antimicrobial character of copper pipes is decreased (Proctor et al. 2017). Moreover, plastic and elastomeric materials are known for their capacity of leaching nutrients such as organic carbon, and it has been observed that the leaching of these nutrients is enhanced by the augmentation of water temperature (Rogers et al. 1994a; van der Kooij et al. 2005; Moritz et al. 2010; Proctor et al. 2017).

These findings reveal that there is an interaction between the factors promoting *Legionella pneumophila* in cooling towers.

Disinfection

Several oxidizing agents are known for being effective in potable water disinfection, such as chlorine, chlorine dioxide, chloramines and ozone (Kim et al. 2002). Chlorination is the most common disinfection method in cooling towers and other water distribution systems. Low levels of chlorine as well as the use of tributyltin oxide do not eliminate the presence of *Lp* suggesting that some biocides might be more effective for the prevention of *Legionella* in cooling towers (Kurtz et al. 1982). For instance, *Legionella* is more resistant to chlorine than *E. coli* and other coliforms (Jakubek et al. 2013). The survival of *Legionella* in water treated with chlorine can be explained by the presence of *Legionella* within biofilms. Water systems treated with chlorine are more likely to have been linked to an outbreak of LD than water systems disinfected with monochloramine (Flannery et al. 2006; Moore et al. 2006). As a matter of fact, monochloramine penetrates more efficiently into biofilm and its residual remains for longer distances within water

distribution systems which makes it an effective disinfectant for the control of *Legionella* (Kool et al. 2000; Flannery et al. 2006). In addition, interventions by chlorine dioxide have a strong impact on the *Legionella* community composition. This compound is known to be an effective disinfectant against waterborne pathogens and biofilm. (Pereira et al. 2017). However, due to the corrosion of iron pipes, its use is not recommended in cooling towers (Springston and Yocavitch 2017). Several non-oxidizing compounds are also used for the disinfection of cooling towers. These include heterocyclic ketones, guanidines, isothiazolines, carbamates, glutaraldehyde and methylene bisthiocyanate among others (Levorino et al. 2017).

Microbiota of water distribution systems

The microbiota present in the system plays an important role in the proliferation of the bacterium. Moreover, both the biotic and abiotic factors that promote *Lp* presence in a cooling tower are closely related to one another.

For instance, there is an interplay between pipe materials and microbial composition. Different materials can promote the presence of different bacterial species and are closely related to the microbiota composition (Douterelo et al. 2016; Proctor et al. 2016). Even though there is a lack of information about the influence of the materials in the microbial composition in cooling towers, a multitude of studies have been carried out in water distributions systems from which we can extrapolate. For instance, it has been shown that chlorinated poly vinyl chloride displays a low microbial diversity in biofilm whereas steel pipes exhibit high bacterial diversity (Yu et al. 2010; Jang 2011). Proctor et al. (2016) carried out a study to analyze bacterial growth and microbial communities in

biofilms in different flexible polymeric materials. Their results showed that different materials have different capacities to support biomass. Moreover, different taxa were enriched in different materials according to their capacity to support biomass. For instance, *Actinomycetales* and *Solirubrobacterales* were mostly prevalent in low-biomass supporting materials such as PEX and silicone while *Bdellovibrio* and *Sediminibacterium* were found in high-biomass supporting materials such as PVC.

Furthermore, the choice of the disinfectant as well as the frequency of its application has also a great impact in the presence of *Legionella* and the microbiota composition in cooling towers. Even though oxidizing biocides are characterised by their non-selective attack on microorganisms, it seems that this disinfection method can promote the growth of certain microorganisms (Jakubek et al. 2013). Indeed, continuous chlorine application in cooling towers reduce microbial diversity and promote the presence of *Pseudomonas*, which may have inhibitory effect on *Legionella* (Paranjape et al. 2019). Hwang et al. (2012) carried out a 2 years-study to examine the impact of chlorination and chloramination in microbial communities in an urban drinking water system. Pyrosequencing technology was employed to study the community composition. The results revealed that *Cyanobacteria, Methylobacteriaceae, Sphingomonadaceae,* and *Xanthomonadaceae* were more abundant in chlorinated water while *Methylophilaceae, Methylococcaceae*, and *Pseudomonadacea*e were more abundant in chloraminated water, supporting the notion that the choice of disinfectant has an impact on shaping the microbiome.

The microbial community inside water systems has an impact on the presence of *Legionella*. It has been suggested that high HPC (heterotrophic plate counts) in water

distribution systems increase the odds of colonization of *Lp* (Messi et al. 2011; Serrano-Suarez et al. 2013). However, it has been found that cooling towers with high HPC do not always harbour *Lp,* suggesting that some cooling towers may host a microbial population resistant to *Legionella* colonization (Duda et al. 2015). In the last decades, the interactions between different species of a microbiota has gained attention and the dependence of *Legionella pneumophila* on other aquatic bacteria has been shown (Surman et al. 1994). As an illustration of this, an association between *Lp* and *Cyanobacterium* was demonstrated by Tison et al. (1980), indicating that *Lp* might use extracellular products as carbon and energy sources. Moreover, positive interactions between *Lp* and other species such as *Flavobacterium* and *Brevundimonas* have also been observed (Wadowsky and Yee 1983; Paranjape et al. 2019). Other bacteria present in water distribution systems may be a sources of additional nutriments that can be metabolized by *Lp* (Wadowsky and Yee 1983).Conversely, some species can inhibit the growth of *Legionella.* Guerrieri et al. (2008) conducted a study to investigate the interactions between *Legionella* and other aquatic bacteria. Eighty bacteria were screened for the production of bacteriocin-like substances (BLS) and 66.2 % of these were active against *Lp.* The results suggested that *Pseudomonas fluorescens* was the best producer of BLSs and that it has a strong negative effect on biofilm integration of *Lp*, enhancing the detachment of *Legionella*. Messi et al. (2011) conducted a study to investigate the relationship of the microbiota in water samples and the growth of *Lp*. Among the 14 bacteria consistently isolated from the samples, five produced BLSs. Through the production of different metabolites, some species are able to restrain the presence of *Legionella*. For instance, *Staphylococcus warneri* was able to inhibit *Legionella* growth by

producing a highly hydrophobic peptide (Hechard et al. 2006). Another example of this is a study by Paranjape et al. (2019) were the microbiota of different cooling towers in the province of Quebec was analyzed. The results suggested that *Legionella* populations in cooling towers were affected by the level of *Pseudomonas*. The presence of *Legionella* in the systems was negatively correlated with the *Pseudomonas* population.

A better understanding of the abundance of species in aquatic microbial communities has been acquired along with the development of new molecular profiling approaches. Indeed, a complete microbial profiling of microbial communities can be achieved through high throughput DNA sequencing techniques using 16S and 18S rRNA amplicon sequencing (Fuhrman et al. 2006; Bautista-de los Santos et al. 2016; Scheikl et al. 2016; Llewellyn et al. 2017; Pereira et al. 2017; Stamps et al. 2018; Tsao et al. 2019). DNA sequencing allows us to have a snapshot of the microbiome of water systems as well as enabling us to study the dynamics of the different species and identify different factors driving the changes in the taxa abundance (Pereira et al. 2017). Next Generation Sequencing platforms can produce thousands of shorts reads in a single run detecting a multitude of members of microbial communities (Metzker 2010). The study of the microbiome in water distribution systems has been used to assess the efficacy of water sanitation methods (Kwon et al. 2011; Hwang et al. 2012). In cooling towers, microbial profiling has been used to study the dynamics of colonization of *Lp* as well as to correlate its presence with several factors. Wery et al. (2008) demonstrated that the bacterial community in a cooling tower was perturbed periodically by chemical treatment and biofilm detachment. Interestingly, the structure of the *Legionella* Spp. population changed at different periods. During the proliferation of *Legionella pneumophila* in the system the

diversity of *Legionella* species decreased significantly. Other microbiome studies showed that the microbial communities depend on the climate regions and the water source (Llewellyn et al. 2017; Paranjape et al. 2019). In addition, the microbial community composition is highly dynamic and subject to seasonal change (Tsao et al. 2019).Co-occurrence analysis of bacterial and protozoan communities using both 18S and 16S amplicon sequencing in cooling towers is helpful to predict bacteria-host interactions in cooling towers (Scheikl et al. 2016; Tsao et al. 2019).

To study the microbial ecology of water systems, it is important to sample water and biofilm since studies have shown that the composition of the microbial communities is different in the biofilm and in the water (Wang et al. 2014; Di Gregorio et al. 2017). While several studies have been conducted to analyse the microbial ecology in water, biofilm is not included in most of the microbiome studies. This is due to the fact that pipes are not easily accessible and sampling of biofilm in functional water distribution systems can become logistically impossible (Douterelo et al. 2014). In order to study biofilm in real pipes networks, there are two approaches. Firstly, the use of cut out pipes, where entire sections of pipes are removed from the system to extract the biofilm (Wingender and Flemming 2004) The protocols for this method are expensive, destructive and labour intensive (Douterelo et al. 2014). The other method for biofilm sampling involves the use of coupons that can be inserted inside the pipes. Biofilm formed in these surfaces can be easily extracted from the network. However, these devices can distort the hydrological conditions in pipes (Douterelo et al. 2014). Turetgen and Cotuk (2007) developed a model cooling tower to study the colonization of *Lp*. In biofilm in different materials. The system consisted of a recirculating water system and a heat source to promote evaporation. Pilot-

scale cooling tower models have been developed to study disinfection methods, (Liu et al. 2011; Farhat et al. 2012; Zhang et al. 2016), *Lp* growth and integration in biofilm (Taylor et al. 2013), corrosion, scaling, and biofouling (Chien et al. 2012).

Detection of *Legionella* in environmental samples

Cultivation in BCYE medium enriched with cysteine is the "gold standard method" of detection of *Legionella* (Steinert et al. 2002). The international standard ISO 11731 describes the standard culture method for the isolation, identification and enumeration of *Legionella* (Whiley 2016). This method is time consuming and has limitations. For instance, it is not possible to detect VBNC *Legionella* cells, resulting in an underestimation of *Legionella* populations. It is important to note that several factors such as temperature and disinfection agents affect the cultivability of *Legionella* (Chang et al. 2007; Alleron et al. 2008; Cervero-Arago et al. 2019). Borges et al. (2012) carried out the standard method for identification of *Legionella* in water distribution systems finding that the method led to false positive results. Water samples were plated in GVPC selective agar medium and morphologically characteristic isolates were sub-cultured in BCYE agar and in blood agar according to the standard method. Colonies identified as positive were identified by 16S rRNA. The results showed that none of the isolates belonged to the genus *Legionella*. Additionally, sample holding time prior to culturing for detection seems to impact *Legionella* enumeration (McCoy et al. 2012). Another method for detection of *Legionella* in water systems is real-time polymerase chain reaction (qPCR) analysis that allows rapid identification of the presence of *Legionella* DNA in samples. This method has a quick turnaround time and high specificity (Whiley et al. 2014). However, this method is not

selective enumerating live cells. Detection of both live cells and killed cells leads to an over estimation of *Legionella* populations (Delgado- Viscogliosi et al. 2009). Different alternative methods for the detection and quantification of viable *Legionella* cells have been developed based on direct fluorescent antibody, cytometry, oligonucleotides probes, biosensors and detection of specific enzymes (Aurell et al. 2004; Gruas et al. 2013; Su et al. 2018).

Control of *Legionella* in cooling towers and water distribution systems

Risk management strategies have an impact on the concentration of *Legionella* in engineered water systems. This is illustrated in a study by Mouchtouri et al. (2010) where they investigated the risk factors of *Legionella* contamination and assessed the effectiveness of control measures in 96 cooling towers of public buildings in Greece. The results showed that *Legionella* colonization was positively associated with a lack of knowledge on *Legionella* management, absence of regular *Legionella* testing, as well as poor disinfection strategies. Many countries have developed their own guidelines and regulations for the control of *Legionella* in water. The American Society of Heating, Refrigeration and Air-Conditioning Engineers' (ASHRAE) developed recommendations for prevention of Legionellosis in cooling towers and other water systems (American Society of Heating Refrigerating and Air-Conditioning Engineers 2000). These guidelines are intended for designers, operators, users, maintenance personnel and equipment manufacturers. It contains information on different water distribution systems and which characteristics of these systems contribute to the risk of Legionellosis (Whiley 2016). Most guidelines provide recommendations for the management of water distribution systems,

Legionella detection methods and define thresholds for safe *Legionella* levels in water. For instance, in Quebec, La Régie du bâtiment du Quebec establishes a limit of 10,000 CFU/L of *Legionella pneumophila* in cooling towers (Régie du bâtiment du Québec 2014a; b). Corrective measures are to be implemented when the concentration of *Lp* is between 10,000 to 1,000,000 CFU/L. When the concentration of *Legionella pneumophila* reaches a level of 1,000,000 CFU/L, decontamination procedures must be carried out immediately. Similar to Quebec's regulations, the European Union has multiple directives regarding bacterial contaminants in drinking and recreational water such as the EWGLINET (European Technical Guidelines for the Prevention, control and Investigation, of Infections Caused by *Legionella* species) (European Working Group for Legionella Infections 2011; Whiley 2016).

Chapter 2: Impact of materials and temperatures on *Legionella pneumophila*, *Vermamoeba vermiformis* and microbial diversity in the water and biofilm of a model cooling tower

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Contributions of authors: Adriana Torres Paniagua contributed to the experimental design, assembled the cooling tower, carried out the water sampling and DNA extraction, performed sequencing experiments, the bioinformatics data treatment and wrote the manuscript. Kiran Paranjape assisted with the sequencing and the bioinformatics. Mengqi Hu performed the HPC counts and assisted with DNA extraction. Emilie Bédard contributed to the experimental design. Sebastien Faucher contributed to the experimental design, the 18S amplicon sequencing and to the writing and editing the manuscript.

Abstract

Legionella pneumophila (Lp) is a waterborne bacterium known for causing Legionnaires' Disease, a severe pneumonia. Water systems, such as cooling towers, are major sources of infection, since they provide ideal conditions for *Lp* growth and produce aerosols. In such systems, *Lp* typically replicates inside host cells such as amoebas and ciliates. Several abiotic factors such as water temperature, pipe material, disinfection regime and

hydrodynamic conditions of the systems affect the colonization of cooling towers by *Lp.* However, the biotic factors promoting the growth of *Lp* in water systems are not well understood. Additionally, it is not clear where in the towers *Lp* preferentially grows. Therefore, we built a lab-scale cooling tower model to study the dynamics of *Lp* colonization in relationship to the resident microbiota and spatial distribution. The model was filled with water from an actual cooling tower harboring low levels of *Lp*. After 8 weeks the model was seeded with *Vermamoeba vermiformis*, a *Lp host.* The following week, the model was seeded with *Lp*. Two weeks later, the model was disassembled, the water was collected, and biofilm was extracted from the pipes. The microbiome was studied using 16S rRNA and 18S rRNA amplicon sequencing. The model cooling tower shaped the microbiota, displaying a microbial community significantly dissimilar from the microbial community of the initial water. The relative abundance of *Legionella* in water samples reached up to 11% whereas abundance in the biofilm was extremely low (≤0.5 %). The host cells were mainly present in the biofilm, while *Legionella* was not present in the biofilm at the time of sampling. Moreover, *Lp* may be growing intracellularly inside biofilm-associated amoeba and ciliates and released back into the water following host cell lysis. The surface material and pipe temperature had an impact on the composition of the biofilm's microbial communities. Beta diversity results showed a significant difference between the bacterial communities growing in PVC and in BTP. Furthermore, among these two materials, the bacterial communities were dissimilar in the surfaces that were at different temperatures in the system. Interestingly, the eukaryotic communities were also dissimilar in the two different materials. However, the temperature seemed to have an impact only in the biofilms growing in PVC.

Introduction

Legionella pneumophila (*Lp*) is a gram negative, intracellular waterborne pathogen known for causing Legionnaires disease (LD), a severe pneumonia, contracted by the inhalation of contaminated aerosols (McDade et al. 1979; Fields 1996; Fliermans 1996; Buse et al. 2012). *Lp* is the main cause of waterborne disease in the United States with an incident rate of 1.36 cases per 100,000 inhabitants (Centers for Disease Control and Prevention (CDC) 2013). 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 Legionnaires disease 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; 2017).

Lp is a natural inhabitant of many aquatic ecosystems such as lakes, hot springs and rivers (Fliermans et al. 1981; Ortiz-Roque and Hazen 1987; Borella et al. 2005b; Sheehan et al. 2005; Lin et al. 2007; Carvalho et al. 2008). There, it can be found as an intracellular parasite of free living amoeba and ciliates (Rowbotham 1980; Fields et al. 2002b). Importantly, *Legionella* is ubiquitous in potable water systems (Alary and Joy 1991). *Legionella* has been detected in pools (Leoni et al. 2001; Leoni et al. 2018) , water fountains (Smith et al. 2015), dental units (Atlas et al. 1995), humidifiers (Moran-Gilad et al. 2012), domestic potable water distribution systems (Stout et al. 1992), cooling towers (Llewellyn et al. 2017; Pereira et al. 2017; Paranjape et al. 2019) hospitals (Bedard et al. 2016; Kyritsi et al. 2018) and hotels (Hampton et al. 2016).

The first recognized outbreak of LD that sickened 182 people in 1976 in Philadelphia was associated to a contaminated cooling tower (McDade et al. 1979; Kurtz et al. 1982). Since then, cooling towers have been reported as the source of several outbreaks of LD (Addiss et al. 1989; Breiman et al. 1990; Mitchell et al. 1990; Shelton et al. 1994; Bell et al. 1996; Brown et al. 1999; Greig et al. 2004; Isozumi et al. 2005; Wang et al. 2014; Fitzhenry et al. 2017). Currently, cooling towers are the 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 than dispersed over long distances of up to 11 km (Klaucke et al. 1984; Bhopal et al. 1991; Fisman et al. 2005; Nguyen et al. 2006; Levesque et al. 2014; Cunha et al. 2016; Beauté 2017). In many countries, cooling towers are under surveillance and management plans are carried out to prevent the proliferation of *Legionella* (Whiley 2016; Springston and Yocavitch 2017).

Understanding the conditions affecting growth of *Lp* in water is critical to elucidate the risk factors linked to outbreaks and to 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. Several physical and chemical factors contributing to *Legionella* colonization have been identified. It is known that temperatures between 25°C and 50° are optimal for *Lp* growth and proliferation (Wadowsky et al. 1985; Yamamoto et al. 1992; Katz et al. 2009; Bedard et al. 2015). A long-term study conducted by Pereira et al. (2017) where the microbiome of the water of a cooling tower was analysed, suggested that shifts greater than 10°C in the temperature of the water of the cooling tower triggered the reduction or dominance of

Legionella. Moreover, the material of the pipes seems to greatly influence the abundance of *Legionella* in water distribution systems (Rogers et al. 1994b; van der Kooij et al. 2005; Moritz et al. 2010; Buse et al. 2014; Proctor et al. 2017). Several oxidizing agents can be employed to disinfect cooling towers, such as bromine, chlorine, chlorine dioxide, hydrogen peroxide, peracetic acid and ozone (Kim et al. 2002; WHO 2007; McCoy et al. 2012).

Biotic factors also affect the presence of *Lp* in cooling towers. High heterotrophic plate counts (HPC) in poorly managed water distribution systems seem to increase the odds of colonization of *Lp* (Messi et al. 2011; Serrano-Suarez et al. 2013). However, some cooling towers that have high HPC do not harbour *Lp*, suggesting that some cooling towers may host a microbial population resistant to *Legionella colonization* (Duda et al. 2015). The impact of bacterial species on the growth and proliferation of *Legionella pneumophila* has been studied previously. Some microorganisms, such as *Cyanobacteria* and *Flavobacterium*, were found to contribute to the growth of *Lp* (Tison et al. 1980; Wadowsky and Yee 1983). Interestingly, other bacteria such as *Pseudomonas* and *Staphylococcus warneri* (Hechard et al. 2006; Guerrieri et al. 2008; Paranjape et al. 2019) seem to have an antagonistic effect on the proliferation of *Legionella.*

Studies suggest that the microbial population residing in cooling towers is shaped by local climate and water sources (Llewellyn et al. 2017; Paranjape et al. 2019). Additionally, the microbiota is affected by the disinfectant residuals and application schedule (Hwang et al. 2012; Paranjape et al. 2019). These microbiome studies are often carried out with water samples; however, biofilm plays a crucial role in *Legionella*

proliferation and survival (Rogers and Keevil 1992; Flemming et al. 2002; Cooper and Hanlon 2010; Simões et al. 2010). In addition, the compositions of the microbial communities are different in the biofilm and in the water (Wang et al. 2014; Di Gregorio et al. 2017).

Pilot-scale models have been developed to study disinfection methods against *Legionella* (Liu et al. 2011; Farhat et al. 2012; Zhang et al. 2016), and *Lp* growth and integration in biofilm (Turetgen and Cotuk 2007; Taylor et al. 2013; Buse et al. 2014). To better understand the growth of *Lp* in cooling towers and its interaction with the resident microbiota, it is crucial to study the biofilm. It is difficult to perform such study on real cooling towers because 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 model to study the dynamics of *Lp* colonization in relationship to the resident microbiota and spatial distribution. This model consists of cold and warm water sections, and an aerated cooling device, filled with water from a real cooling tower. The objective of this study was to determine the microbial community in the model cooling tower in biofilm and in water and relate it with the dynamics of *Lp* colonization. In addition, special emphasis was made in the effect of the temperature and material of the surface on the microbial communities in the biofilm. This study is novel because the microbiota in biofilm and in water were both studied as well as the effect of the different temperatures and materials in the model.

Material and Methods

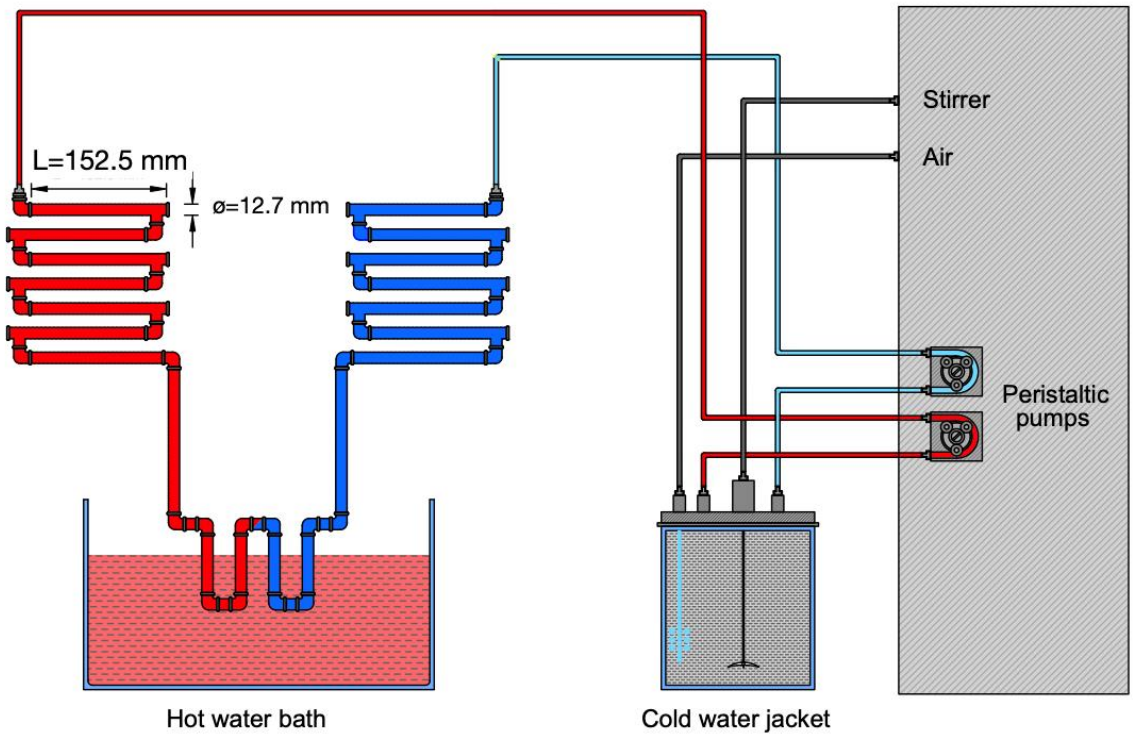
Cooling tower model

A lab-scale cooling tower model was designed to mimic critical components of real cooling tower (Figure 2.1). 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 inches and a diameter of 0.5 inch. A total volume of 1 L of water was pumped through the pipes by a peristaltic pump using BTP PharMed tubes, an elastomer Cole Parmer, USA) at a flow of 1L/hr. The temperatures of the pipes were constant during the whole experiment: 22.7 °C and 30.7 °C for the cold and warm water pipes respectively. Ambient air was pumped in the system at a flow of 3 L/min using 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 for two weeks, replenishing the chlorine solution every two days. Chlorine residual was measured before changing the chlorine solution using the DPD Colorimetric method. The system was deemed to have low biomass when chlorine residual was stable after two days in the system.

Following system disinfection, the model was rinsed with sterile distilled water for 24 hours and then filled with water from an actual cooling tower harboring low levels of *Lp* (t = 0) (*Lp* not detectable on *Legionella* screening tests.). An aliquot of water from this cooling tower was kept in a 10 L Polypropylene carboy (Nalgene, USA) at room temperature for three months as control water, to evaluate the effect of time on the water

microbiome. After a period of 9 weeks of running with cooling water tower (t=9w), the model was seeded with *Vermamoeba vermiformis (V.v)* (6×10^6 cells/L). After two more weeks (t =10 w), the model was seeded with *Legionella pneumophila* to a final concentration of 3.5×10^5 cells/L. The model was dismantled at t =13 w, three weeks after the inoculation with *Lp* (Figure 2.1).

A


L=152.5 mm
ø=12.7 mm
Stirrer
Air
Peristaltic pumps
Hot water bath
Cold water jacket

B

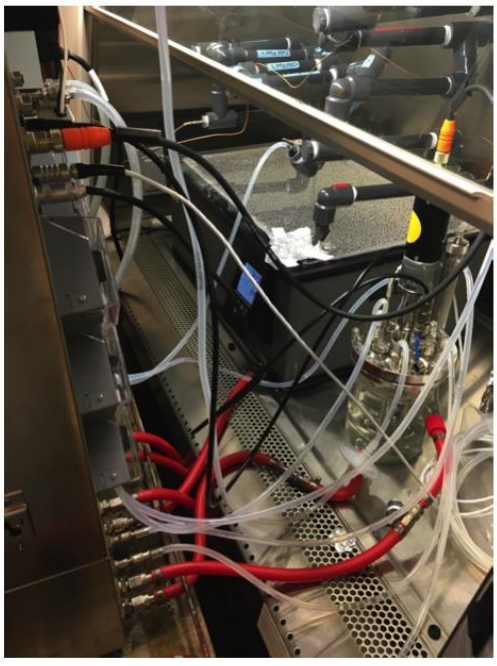


Figure 2. 1 Model cooling tower (A). Schematic representation of the model. This model is composed of a water-jacketed bioreactor a series of cold water pipes, a loop heated by a warm water bath and a series of warm water pipes. Water is pumped to the network of pipes and returned to the bioreactor with 2 peristaltic pumps. The bioreactor was maintained at 15°C while the water bath was set at 34.4°C. The direction of water flow is indicated with doted arrows. (B) Picture of the system.

Culture of *Legionella pneumophila*

Legionella pneumophila strain Philadelphia-1 (ATCC 33152) was inoculated in the model. The strain maintained at -80°C in 10% glycerol was grown on BCYE (ACES-buffered charcoal yeast extract) agar supplemented with 0.25 mg/ml L-cysteine and 0.4 mg/ml ferric pyrophosphate for 3 days. The colonies were suspended in a solution with filtered sterilized water from the cooling tower to a concentration of 3.5×10^5 cells/ mL. One mL of that solution was added to the model through the bioreactor.

Culture of *Vermamoeba vermiformis*

Vermamoeba vermiformis (ATCC 50237) was grown as monolayer in modified PYNFH medium at 30 °C (Fields 1990). Cells were passaged at a ratio of 1 in 5 when confluence was reached. For inoculation in the model cooling tower, the cells were harvested by centrifugation and washed three times in Page's Amoeba Saline.

Periodic water sampling

During the first eight weeks, a 20 mL sample of a volume of 20 mL was collected weekly for DNA extraction from the bioreactor. The last five weeks, three 20mL samples were taken each week to have biological triplicates. Additional samples were taken after inoculation with *V. v* and *Lp*. The volume loss was compensated by adding filtered sterilized water from the cooling tower. Water sampling was carried out from a bioreactor port. At the end of the experiment (t = 13w), water from the bioreactor and water filling the pipe network were collected separately. In total, 10 water samples were collected, including initial water from the cooling tower and control water. All water samples were filtered through a 0.45 µm pore size filter (Millipore, USA), and the filter were kept at -20°C until DNA extraction (S. Figure 1).

Model dismantlement and biofilm sampling

After 13 weeks of operation, the model was dismantled. The pipes were disassembled, and the attached biofilm was collected. Briefly, ten pipes of 15.25 cm of length (five pipes from the cold part and five from the warm part of the system) were unthreaded. Each end of pipe was plugged using a threaded cap (McMaster-Carr, USA) and filled with 10 mL of 3 mm sterile glass beads. The remaining volume was filled with water from the cooling tower that was previously filter-sterilized. The pipes were sonicated for 5 minutes in a sonication bath. (Cole Parmer, CANADA). Supernatant was collected, and the process was repeated 5 times. The resulting slurry was filtered through 0.45 µm nitrocellulose filters and kept at -20°C until DNA extraction. Biofilm from the tubes was

extracted directly by scraping the internal surface of these. The biofilm collected was directly treated for DNA extraction.

Heterotrophic plate count

Heterotrophic plate counts were performed on water samples on R2A agar two days a week for three months, as well as biofilm samples after the dismantlement. The plates were incubated at 30 °C for 48 hrs.

16S 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 amplicon sequencing was performed using the dual-index pair-ends approach described by Kozich et al. (2013). Twenty-four samples were analyzed in triplicate (Figure S 2.1) , including 7 water samples taken from the bioreactor, before and after inoculation with *V. v* and *Lp*, 1 sample taken from the initial water of the cooling tower (t=0) , 2 samples of aged water from the cooling tower left in a container at room temperature (t=13 weeks) (Control water), 10 samples of biofilm from the pipes, 2 samples of biofilm from peristaltic pump tubes and 2 samples of a combination of water and detached biofilm collected on the day of dismantlement of the system (Flocs). Due to the sampling methodology on the day of dismantlement, the sample of September 13 contained detached flocs. This is why this sample is included as a water sample and as a flocs sample. 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 a cycle that consisted of an initial denaturation step at 95°C for 2 min, 25 cycles of 95°C for 2 secs, 55 °C for 15 sec 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.

18S library preparation

18S amplicon sequencing was performed using a two-step PCR strategy. Seventeen samples were analyzed in triplicate (including 2 water samples taken from the model, before and after inoculation with both *V. v*, 1 sample taken from the initial water of the cooling tower (t=0), 10 samples of biofilm from the pipes, 2 samples of biofilm from tubes used to feed the bioreactor and the pipes and 2 sample of a combination of water and detached biofilm collected on the day of dismantlement of the system (Flocs).The V9 region of the 18S SSU 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

secs, 55 °C for 30 secs and 72°C for 30 secs, 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 minutes, 8 cycles of 95°C for 30 secs, 55 °C for 30 secs and 72°C for 30 secs, 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 amplicon sequencing.

Data Processing

The sequencing data was processed using the Mothur pipeline (Schloss et al. 2009). Paired-reads were first assembled into contigs. Contigs that presented ambiguous bases or that were longer than 275bp for the 16S sequencing and 373 bp for the 18S 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. For 16S sequencing, non-bacterial sequences such as Eukaryotes, Chloroplasts, Archaea and Mitochondria were cut out. For 18S sequencing, only Eukaryotic sequences were considered. Five of the replicates for the 16S analysis had significantly lower counts than the rest; hence, they were removed from the analysis (Warm pipe 7 c, Cold pipe 7 b, Aug 22 a, Aug 22 b, Aug 24 a). The rest of the samples were rarified to the next sample with the lower number of reads (3038 read counts). For the 18S sequencing one sample (Aug

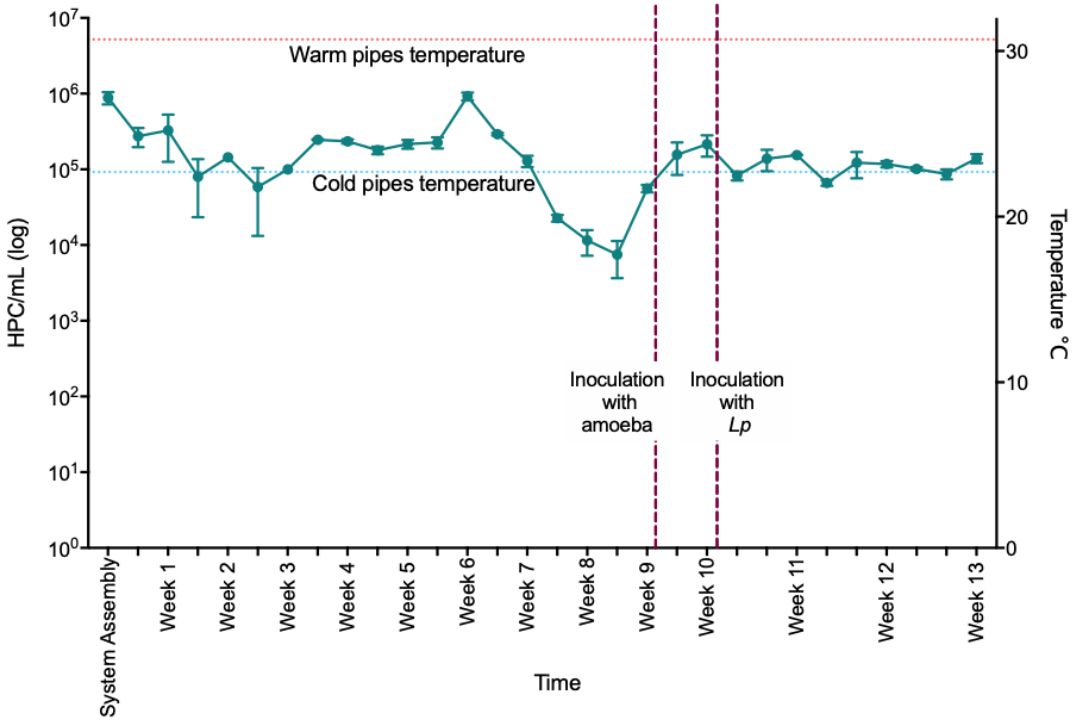
22a) was removed from the analysis due to its significantly lower read count. Operational Taxonomic Units were defined at an identity cut-off of 97%. Samples included in the analysis were rarified to the lowest read count sample (6325 read counts). The OTU data was analyzed with The MicrobiomeAnalyst web-based tool (Dhariwal et al. 2017) . Default parameters (at least 20% of the samples contain 2 counts or more) were used to filter OTU with low counts.

Beta diversity was calculated with the Bray-Curtis dissimilarity index to analyze differences between samples. Non-metric multidimensional scaling (NMDS) and Principal Component analysis (PCoA) plots were used to visualize the data. PERMANOVA analysis was performed to analyze the statistical significance and strength of the analysis.

Results

HPC were relatively stable during the first month of the experiment, having values between 10^5 and 10^6 CFU/ml (Figure 2.2). A decrease on the CFU was noticed at the end of July, following an increased in the volume of water collected from the reactor for DNA extraction. A rise of the CFU in the water was observed after inoculation with *Vermamoeba vermiformis* on August 16th. On the last day, there were 1.40 x 10^5 CFU/mL in the water, for an estimated total cultivable biomass of 1.47 x 10^8 CFU, assuming a volume of water of 1.05 L.

A


Warm pipes temperature
Cold pipes temperature
Inoculation with amoeba
Inoculation with Lp
HPC/mL (log)
Temperature °C
Time
System Assembly
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
Week 7
Week 8
Week 9
Week 10
Week 11
Week 12
Week 13

B

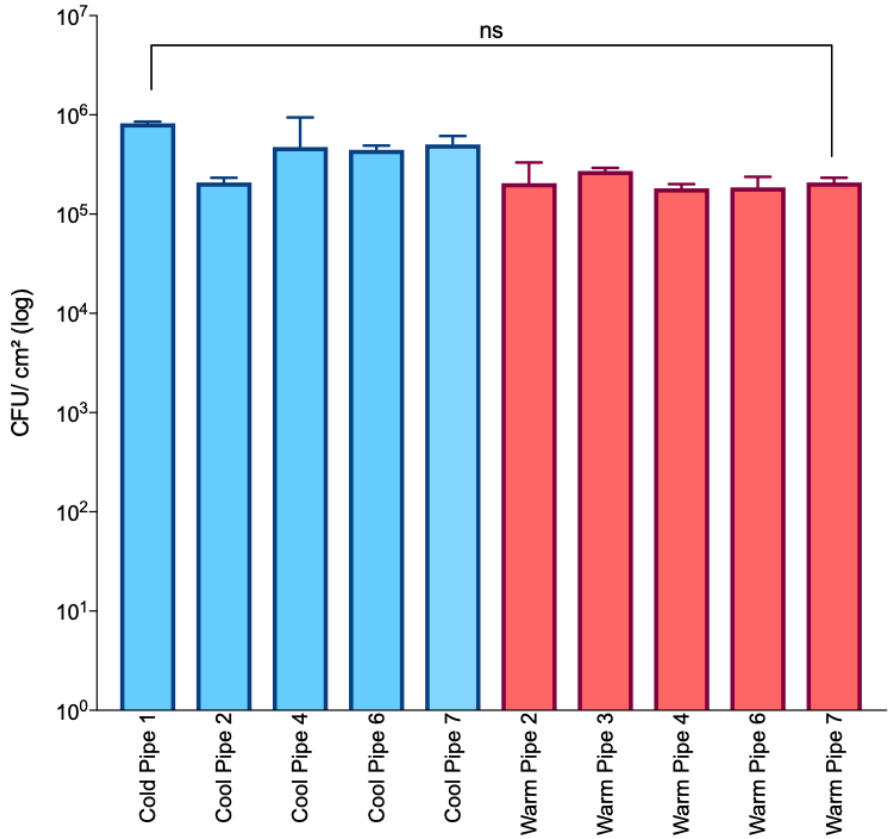

ns
CFU/ cm² (log)
Cold Pipe 1
Cool Pipe 2
Cool Pipe 4
Cool Pipe 6
Cool Pipe 7
Warm Pipe 2
Warm Pipe 3
Warm Pipe 4
Warm Pipe 6
Warm Pipe 7

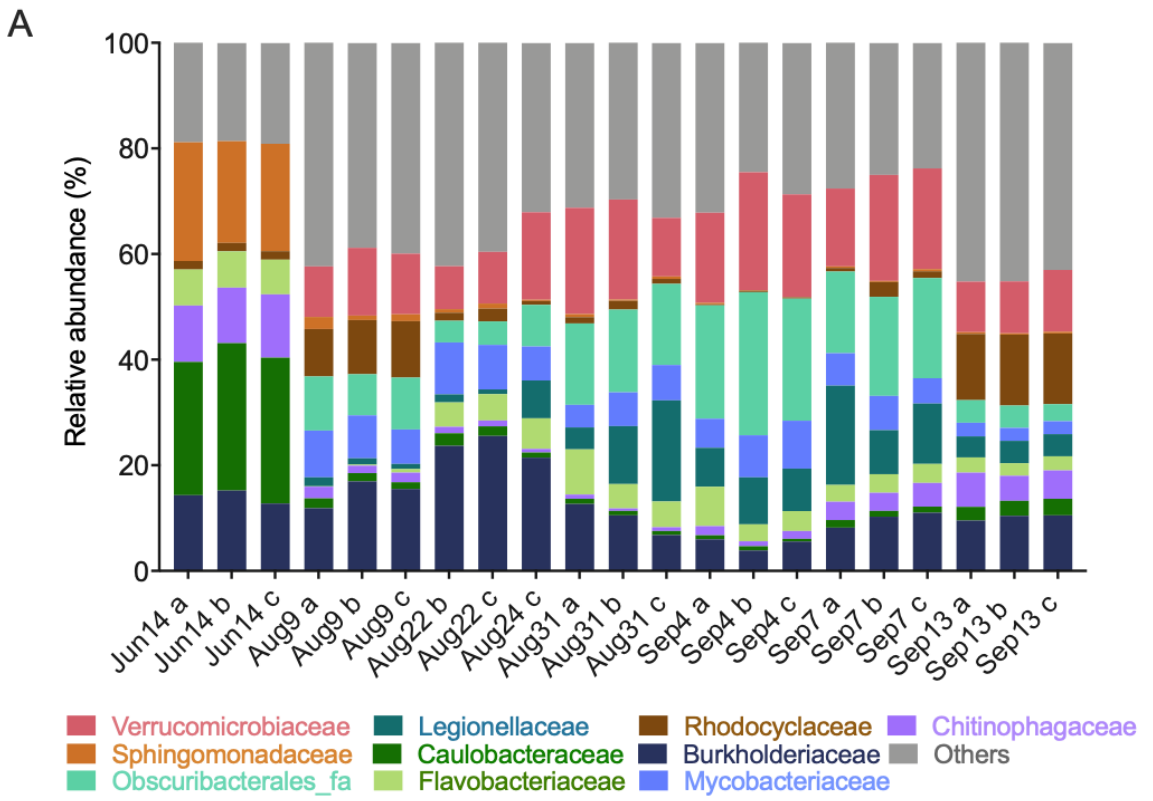
Figure 2.2: Heterotrophic plate counts for water and biofilm samples.

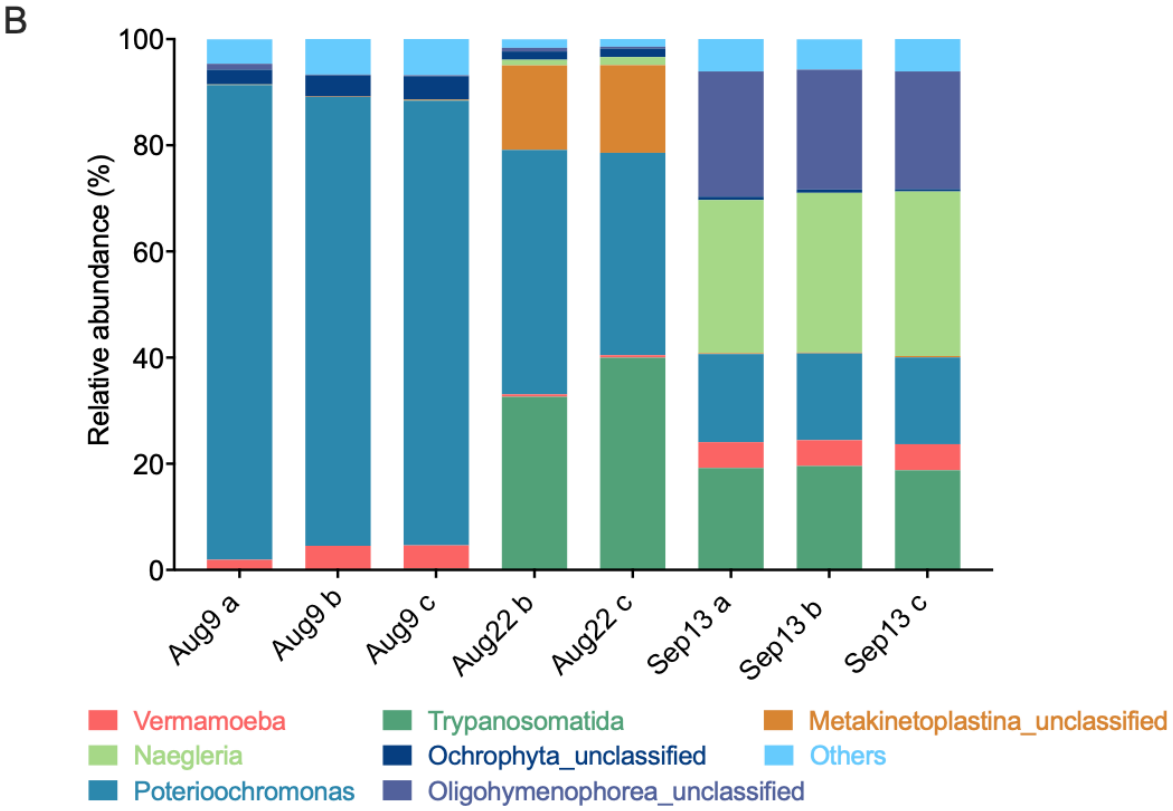
Water samples of 1mL were taken every 48 hours for 3 months (A) and HPC counts were performed on R2A agar. Data represents the mean for triplicate samples with standard deviation. The temperature in the cold pipes (blue dotted line) and warm pipes (red dotted line) are indicated. Biofilm samples from pipe segments with a length of 15.25 cm 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.

Flocs of detached biofilm were observed in the water collected from the pipes. Biofilm was observed on the walls of the pipes and the tubes. Biofilm was 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 CFU counts from the biofilm samples coming from the cold pipes and the ones taken from warm pipes (Figure 2.2 B). Based on colony morphology, there was no obvious difference in the microbial composition of the biofilm in the cold and warm pipes. Using an estimated surface of 2257.5 cm^2 for the pipe system, the biofilm density was estimated to 6.16×10^6 CFU/cm^2. This is on the high side of previously reported biofilm cultivable cell density in water distribution systems (Wingender and Flemming 2011). There were about 95 times more cultivable microorganisms in the biofilm than in the water, also in line with the literature, reporting that about 95% of bacterial cells in water systems are fixed on surfaces (Flemming et al. 2002).

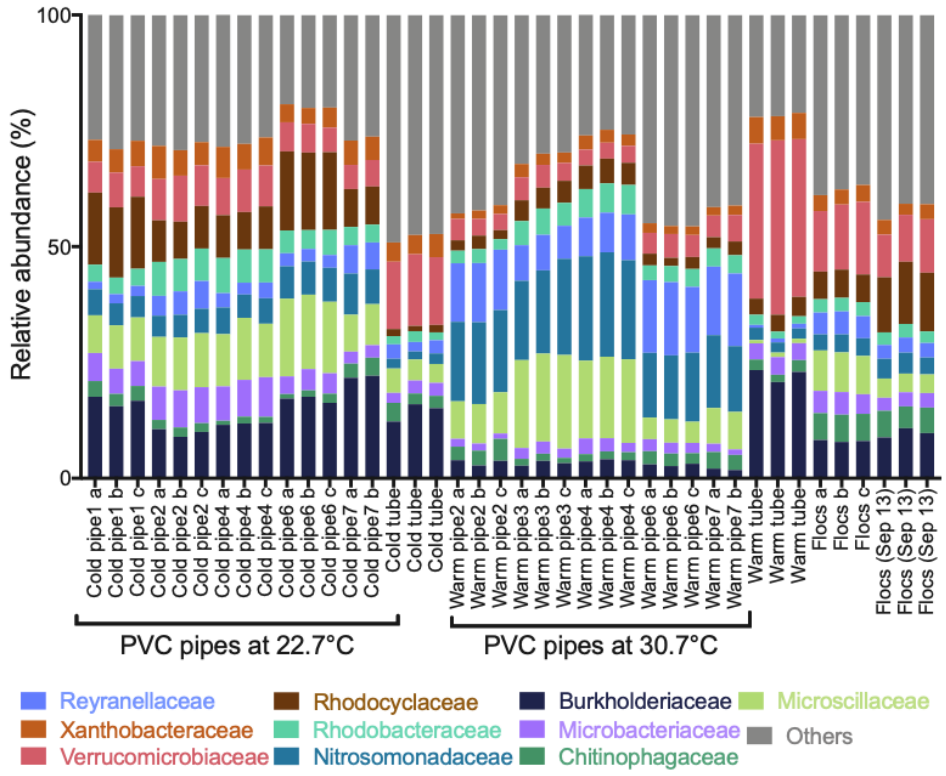
Characterization of the eukaryotic and bacterial communities in the model cooling tower

The microbiota of the water changed drastically between inoculation at t = 0 (June 14) and August 9 (t=9 w) but had slight changes afterward, after inoculation with V.v and *Lp* (Figure 2.3 A). This suggests that over the first two months, the microbiota changed reaching equilibrium by or before August 9 (Figure 2.3). *Burkholderiaceae* and *Verrucomicobiceae* were the most predominant bacterial families in the water samples, with relative abundance going from 3.8 % to 25.5 % and 9.5 to 22.29% respectively, excluding the samples taken at inoculation. *Poterioochromonas* was the most predominant eukaryotic genus in water samples with levels going from 16.4 % to 89.4% (Figure 2.3 B). In the biofilm (Figure 2.3 C), *Nitrosomonadaceae* was most predominant bacterial genus in warm pipes (17.1% to 22.5%). In contrast, the levels of *Rhodocyclaceae* (8.2% to 17.1%) and *Burkholderiaceae* (8.9% to 21.7%) were higher in the biofilm formed in the pipes at 22.7°C. The abundance of *Verrucomicrobiaceae* was higher (14.6% to 37.6%) in the biofilm formed on the elastomer surface, especially the one at 30.7°C. *Oligohymenophorea* was the most abundant eukaryotic genus in the biofilm samples (Figure 2.3 C), having a higher abundance in the biofilm formed in the cold pipes (31.7 % to 73.6%). Interestingly the presence of *Oligohymenophorea* in water was only noticed after the inoculation with *Legionella. Vermamoeba* and *Naegleria*, known for being hosts of *Legionella*, were mostly observed in biofilm samples, especially in the biofilm formed in the warm pipes and in the elastomer tubes.


A
Relative abundance (%)
100
80
60
40
20
0
Jun14 a
Jun14 b
Jun14 c
Aug9 a
Aug9 b
Aug9 c
Aug22 b
Aug22 c
Aug24 c
Aug31 a
Aug31 b
Aug31 c
Sep4 a
Sep4 b
Sep4 c
Sep7 a
Sep7 b
Sep7 c
Sep13 a
Sep13 b
Sep13 c
Verrucomicrobiaceae
Sphingomonadaceae
Obscuribacterales_fa
Legionellaceae
Caulobacteraceae
Flavobacteriaceae
Rhodocyclaceae
Burkholderiaceae
Mycobacteriaceae
Chitinophagaceae
Others


B
Relative abundance (%)
100
80
60
40
20
0
Aug9 a
Aug9 b
Aug9 c
Aug22 b
Aug22 c
Sep13 a
Sep13 b
Sep13 c
Vermamoeba
Naegleria
Poterioochromonas
Trypanosomatida
Ochrophyta_unclassified
Oligohymenophorea_unclassified
Metakinetoplastina_unclassified
Others

C


Relative abundance (%)
100
50
0
Cold pipe1 a
Cold pipe1 b
Cold pipe1 c
Cold pipe2 a
Cold pipe2 b
Cold pipe2 c
Cold pipe4 a
Cold pipe4 b
Cold pipe4 c
Cold pipe6 a
Cold pipe6 b
Cold pipe6 c
Cold pipe7 a
Cold pipe7 b
Cold tube
Cold tube
Cold tube
Warm pipe2 a
Warm pipe2 b
Warm pipe2 c
Warm pipe3 a
Warm pipe3 b
Warm pipe3 c
Warm pipe4 a
Warm pipe4 b
Warm pipe4 c
Warm pipe6 a
Warm pipe6 b
Warm pipe6 c
Warm pipe7 a
Warm pipe7 b
Warm tube
Warm tube
Warm tube
Flocs a
Flocs b
Flocs c
Flocs (Sep 13)
Flocs (Sep 13)
Flocs (Sep 13)
PVC pipes at 22.7°C
PVC pipes at 30.7°C
Reyranellaceae
Xanthobacteraceae
Verrucomicrobiaceae
Rhodocyclaceae
Rhodobacteraceae
Nitrosomonadaceae
Burkholderiaceae
Microbacteriaceae
Chitinophagaceae
Microscillaceae
Others

D

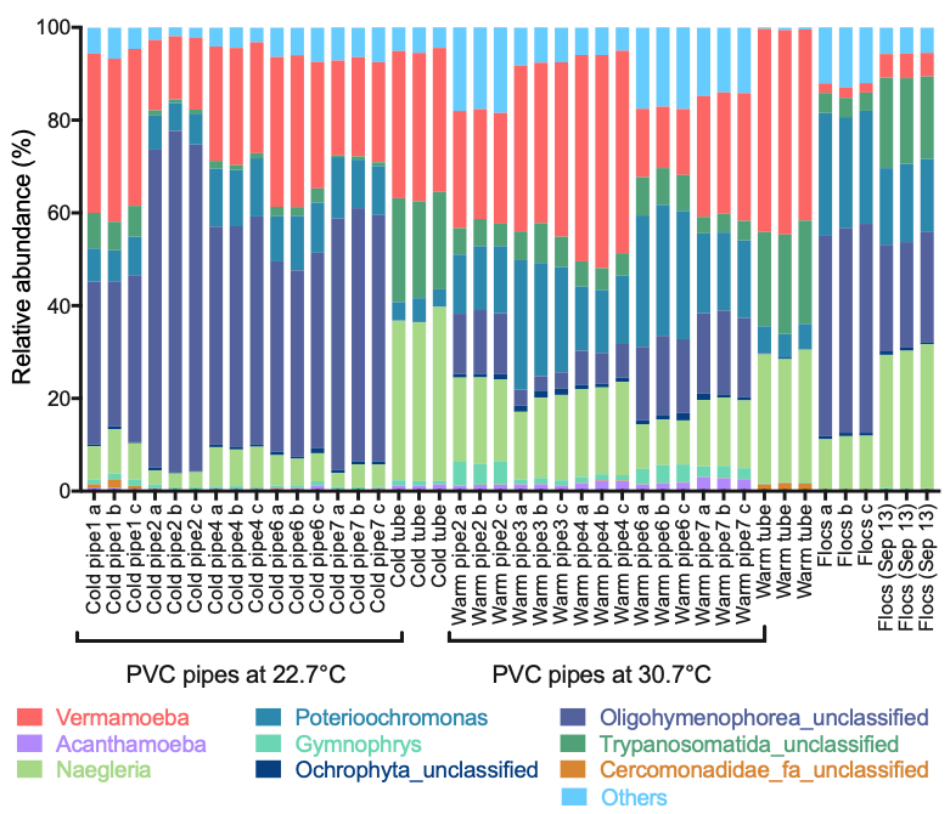

Relative abundance (%)
100
80
60
40
20
0
Cold pipe1 a
Cold pipe1 b
Cold pipe1 c
Cold pipe2 a
Cold pipe2 b
Cold pipe2 c
Cold pipe4 a
Cold pipe4 b
Cold pipe4 c
Cold pipe6 a
Cold pipe6 b
Cold pipe6 c
Cold pipe7 a
Cold pipe7 b
Cold pipe7 c
Cold tube
Cold tube
Cold tube
Warm pipe2 a
Warm pipe2 b
Warm pipe2 c
Warm pipe3 a
Warm pipe3 b
Warm pipe3 c
Warm pipe4 a
Warm pipe4 b
Warm pipe4 c
Warm pipe6 a
Warm pipe6 b
Warm pipe6 c
Warm pipe7 a
Warm pipe7 b
Warm pipe7 c
Warm tube
Warm tube
Warm tube
Flocs a
Flocs b
Flocs c
Flocs (Sep 13)
Flocs (Sep 13)
Flocs (Sep 13)
PVC pipes at 22.7°C
PVC pipes at 30.7°C
Vermamoeba
Acanthamoeba
Naegleria
Poterioochromonas
Gymnophrys
Ochrophyta_unclassified
Oligohymenophorea_unclassified
Trypanosomatida_unclassified
Cercomonadidae_fa_unclassified
Others

Figure 2.3: Microbial community profiling of biofilm and water samples.

Water samples were taken over time to analyze the microbial communities in the water of the model cooling tower: (A) Relative abundance (%) of bacterial OTU in water samples classified at the family level. (B) Relative abundance (%) of the eukaryotic OTU in water samples classified at the genus level; Biofilm was harvested to analyze the microbial communities in the biofilm growing in the PVC pipes and the BTP Pharmed tubes: (C) Relative abundance (%) of the bacterial OTU in biofilm samples classified at the family level and (D) Relative abundance (%) of the eukaryotic OTU in biofilm samples classified at the genus level.

A machine-learning (LEfSe) algorithm was used to identify bacterial families and eukaryotic genera of importance for biofilm and for water (Segata et al. 2011). LEfSe involves both statistical and biological significance analysis and linear discriminant analysis (LDA). The algorithm was able to identify significant taxa for both water and biofilm. Interestingly Legionella was enriched in the water and not in the biofilm. Amoebas such as *Vermamoeba* and *Acanthamoeba* were predicted to be in the biofilm (Figure 2.4).

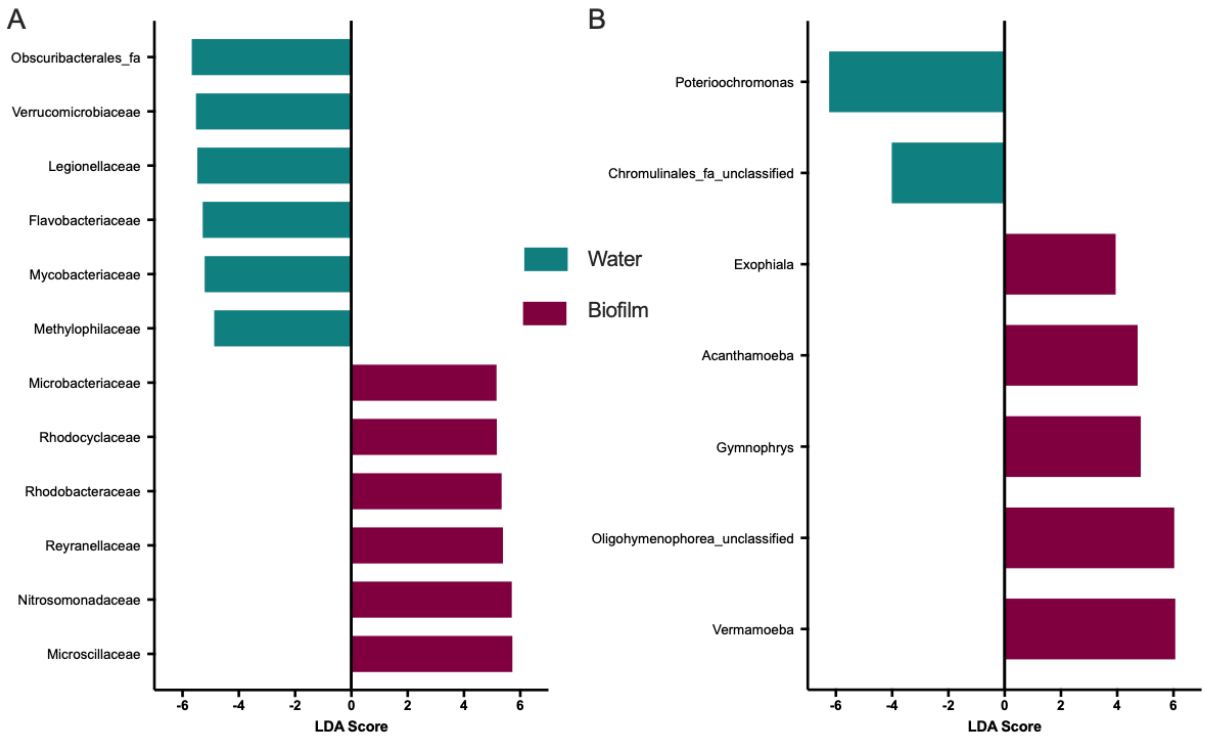
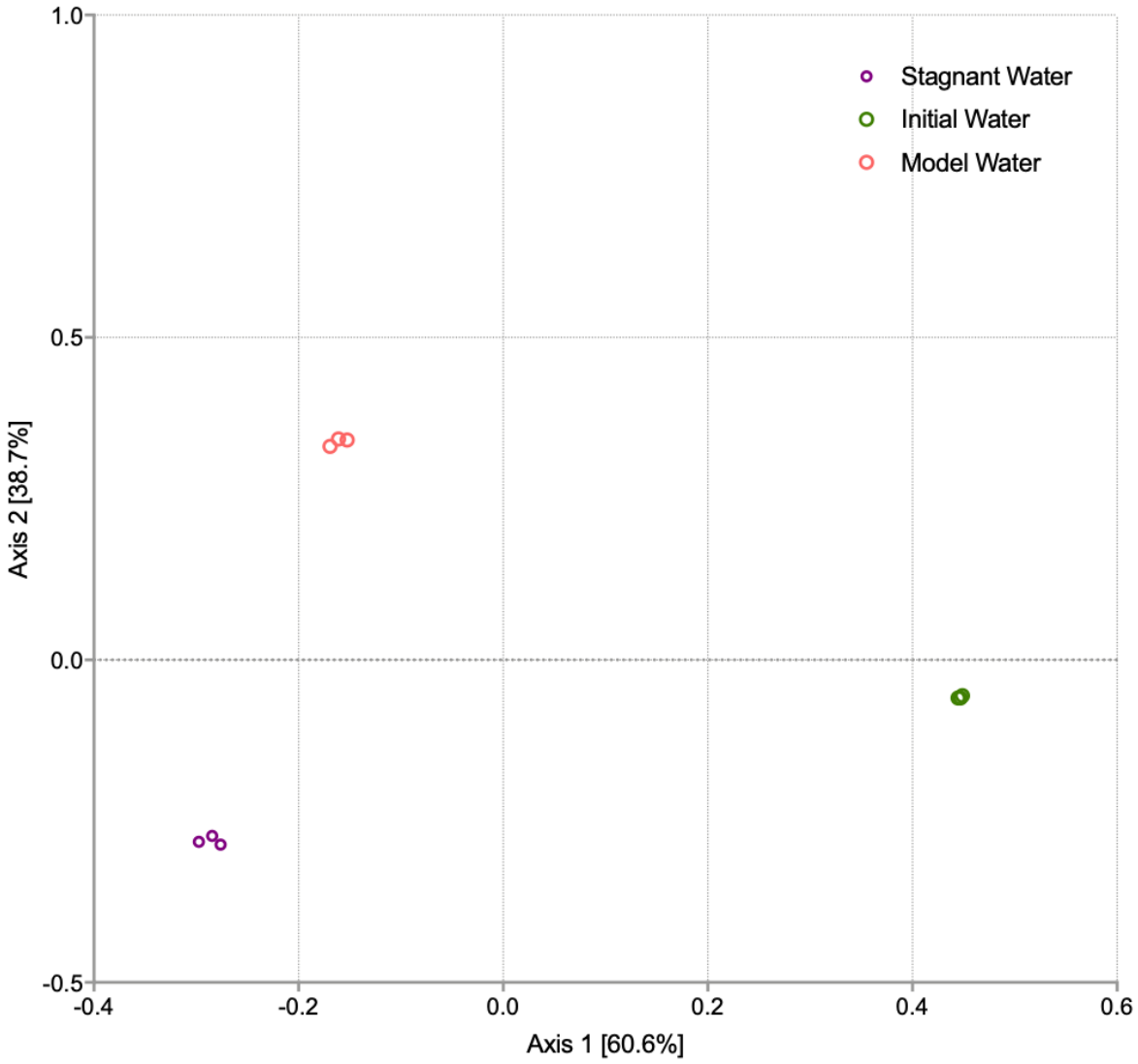


Figure 2.4: LEfSe analysis of biofilm and water samples.

The machine learning algorithm LEfSe was used to identify significant bacterial (A) and eukaryotic (B) taxa associated with biofilm and water. The LDA score is an effect size that measures the importance of the taxa in the condition studied.

Beta diversity analysis was used to confirm that the model had a specific effect on the microbiota (Figure 2.5). Water samples (triplicate) were grouped by type, namely, initial water from the cooling tower (June 14), model water (September 13) and stagnant water (September 13). The triplicates grouped so well it was hard to see all three replicates in the scale of the PCoA. This confirms that the microbiome of the model tower was significantly different than the initial water used to seed the system and shows a significant difference from the microbiome of the stagnant water (Figure 2.5). This result

confirms that circulating the water through our model cooling tower and adding *V. v* and *Lp* affected the microbial composition in a different way than when the same water stagnated in a bottle during the same time. This is also evidenced by the fact that different taxa are enriched in the three different groups (Supplementary Figure 1).



[PERMANOVA] F-value: 269.21 ; R-squared: 0.98901;
p-value < 0.001

Figure 2.5: Beta-diversity of water samples bacterial communities grouped by water type: cooling tower, initial water and stagnant water. Principal coordinates analysis (PCoA) plot of water samples bacterial communities grouped by water type: cooling tower (September 13), initial water (June 14) and stagnant water (September 13). Statistical significance was determined using PERMANOVA.

Presence of *Legionella* in the system

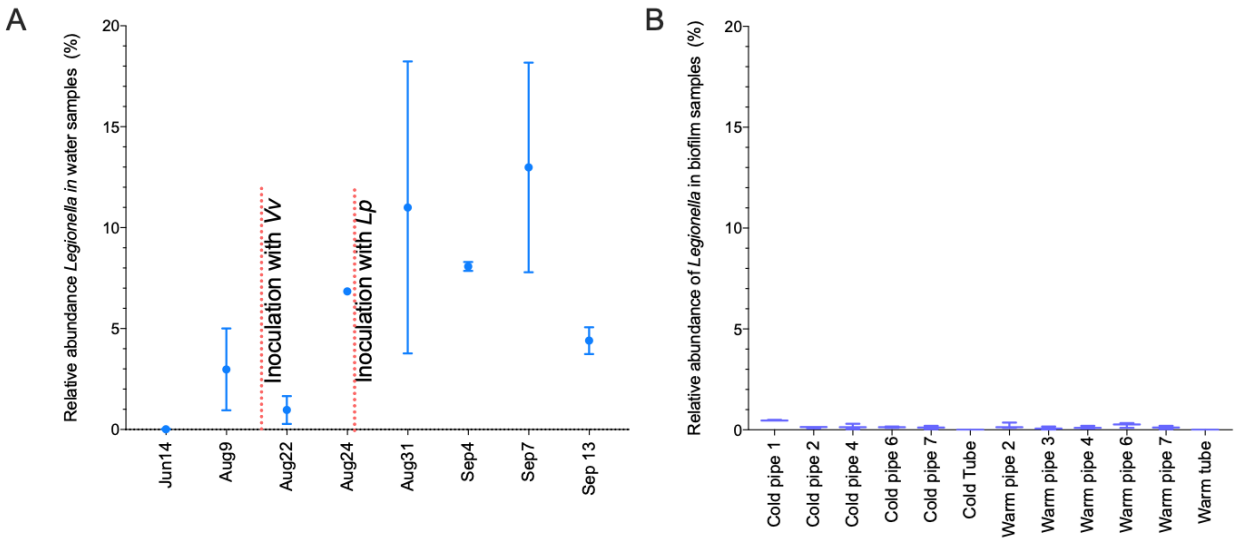
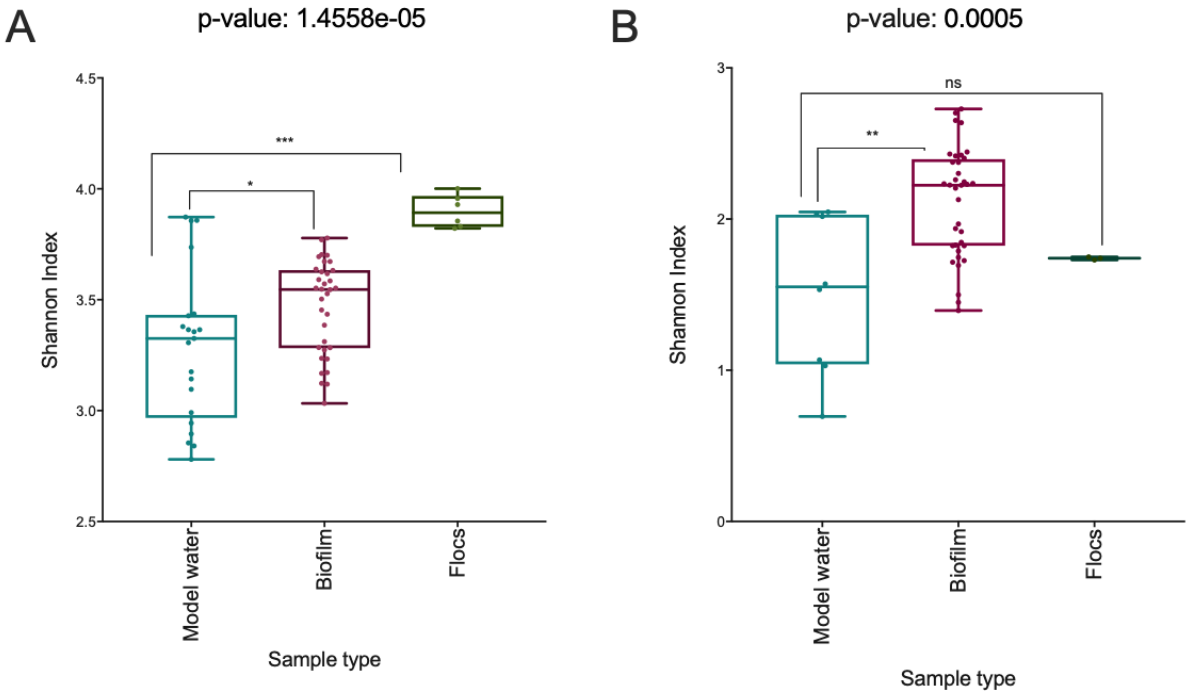


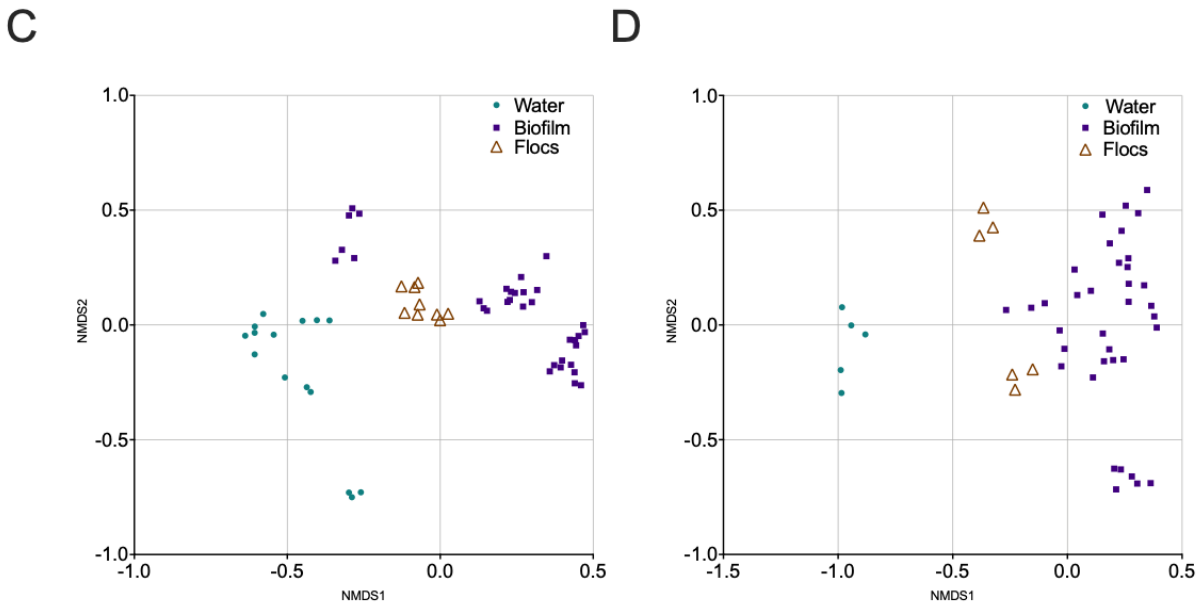
Figure 2.6 Relative abundance of Legionella in the model water (A) and biofilm (B). Data represents the mean of triplicate samples with standard deviation.

The presence of *Legionella* in the system was evaluated using the results of the 16S analysis. The percentage of abundance of the reads of the *Legionellaceae* family was calculated according to the total number of reads for each sample after rarefaction (Figure 2.6). The abundance of *Legionella* at the beginning of the experiment, that is, June 14th, was almost null (0.02%). An increase in the abundance of *Legionella* was observed after inoculation with *Vv* on August 16th (0.46%). A constant increase of the relative abundance of *Legionella* was not observed. The abundance of *Legionella* fluctuated between 12.96 to 4.40 %. *Legionella* in the biofilm samples was lower than in the water samples (less than 0.5 %).

Diversity in the model cooling tower

The Shannon Index was calculated to measure alpha diversity (Figure 2.7 A, B) while beta diversity was analyzed to assess dissimilarities between the communities in the different kinds of samples (Figure 2.7 C, D). There was a significant difference between the alpha diversity in the biofilm samples and in the water samples for both eukaryotic and bacterial communities. Moreover, the difference between the diversity in the model water and in the flocs was significant in the bacterial communities whereas it was not the case for the eukaryotic communities. Interestingly, the communities present in the flocs samples were more similar to the biofilm samples than the water samples (Figure 2.7 C, D).





[PERMANOVA] F-value: 14.68; R-squared: 0.34804; p-value < 0.001
[NMDS] Stress = 0.11458

[PERMANOVA] F-value: 13.859; R-squared: 0.38648;
p-value < 0.001 [NMDS] Stress = 0.1404

Figure 2.7: Alpha-diversity and Beta-diversity of samples from the model, categorized by the type of samples.

Alpha diversity of samples from the model for the bacterial (A) and eukaryotic community (B), categorized by the type of samples: water, biofilm and flocs. Mann-Whitney test was performed to determine the statistical significance. There seems to be more bacterial diversity in the flocs than eukaryotic diversity. Beta diversity of samples from the bacterial (C) and the eukaryotic (D) community categorized by the type of samples.

Influence of the temperature and surface on bacterial and eukaryotic communities in the biofilm

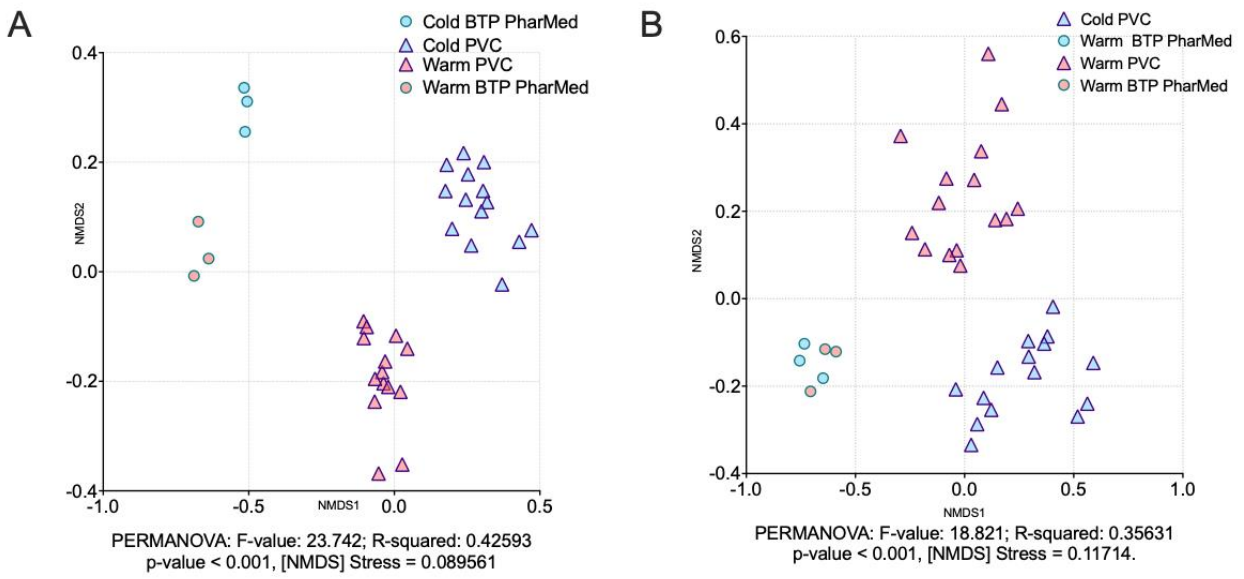


Figure 2.8: Beta-diversity of samples grouped by temperature and material of the surface.

Non-metric dimensional scaling (NMDS) plot of Beta-diversity (Bray Curtis Index) of bacterial (A) and eukaryotic communities of biofilm (B) samples grouped by temperature and material of the pipe.

Beta diversity was calculated to analyze the difference between biofilm samples (Figure 2.8). Biofilm samples were grouped by the temperature of the pipes (22.7 °C and 30.7 °C) as well as the material (PVC and BTP Pharmed). Both the bacterial and eukaryotic communities showed a significant difference ($P < 0.001$) between the biofilm formed at 22.7 °C and at 30°C and the biofilm formed in PVC and BTP (Figure 2.8). However, the temperature did not seem to have a significant effect on the eukaryotic

communities in biofilm growing in the elastomer. Therefore, temperature and material affected the composition of the microbial community of our model system.

Discussion

Cooling towers are an important source of outbreaks of LD. A better understanding between the relationship of the microbiota of cooling towers and the dynamics of *Legionella* colonization is needed to improve management of the systems and evaluation of risk of LD. Several studies have been carried out on model water systems to study the impact of different factors in the dynamics of *Legionella pneumophila* colonization (Farhat et al. 2009; Farhat et al. 2012; Taylor et al. 2013; Zhang et al. 2016; Levorino et al. 2017). To our knowledge, this is the first study using a model system with such complexity. Our model was built using a biospherical approach; we studied an enclosed system where the evolution of the microbiota in the water and in the biofilm would depend on the relatively constant conditions (Rillig and Antonovics 2019). This model reproduced the temperature typically seen in a cooling tower (American Society of Heating Refrigerating and Air-Conditioning Engineers Inc 2008). Operating the model with water from a real cooling tower and using of this water (filtered sterilized) as make up water, led to have nutrient conditions more representative of actual cooling than other studies. However, towers would also receive nutrients from the ambient air that carries pollen, spores, dust and other debris. This study gives us a snapshot of what would happen if a cooling tower was not managed properly for three months.

A unique microbiota developed and evolved with the conditions of the system (Figure 2.5). That is, the microbiota of the water at the end of the experiment was

significantly different from the initial water used to seed the system and the water that was kept in stagnant conditions at 25°C. It has been previously shown that internal factors of water distribution systems such as pH, temperature and water flow play a central role in shaping the microbiome (Douterelo et al. 2017).

It is not possible to compare the microbiome of this model cooling tower with other studies since water source and regional climate shape the microbiome of cooling towers (Llewellyn et al. 2017; Paranjape et al. 2019). However, some similarities were observed between our model cooling tower and other studies. For instance, our results showed that *Burkholdericeae* was abundantly found in the water of the model cooling tower. This is in agreement with other studies studying similar environments (Paranjape et al. 2019; Tsao et al. 2019). *Verrumicrobiaceae* was also an abundant genus in the system, which is consistent with its presence in natural water reservoirs (Zwart et al. 2002; Boucher et al. 2006). Taxa previously studied for their capacity to form biofilms such as *Pirellulaceae, Rhodobacteraceae and Caulobacteraceae,* were identified in the biofilm samples of the model (Entcheva-Dimitrov and Spormann 2004; Elifantz et al. 2013; Miao et al. 2019).

Amoebas were present in biofilm for our model system, as reported previously (Figure 2.4) (Thomas et al. 2004; Thomas et al. 2008; Taravaud et al. 2018). Interestingly, amoebas were mostly found in biofilm from warm pipes (30.7°C) (Figure 2.3). In addition, some organisms seemed to be more affected by temperature and surface material. Our results show that the ciliates *Oligohymenophorea* were more abundant in the biofilm formed at 22.7°C than at 30.7°C (Figure 2.3 D). It is possible that the species of *Oligohymenophorea* present in the system have an optimal growth temperature closer to 20°C. Interestingly, this organism was not detected in BTP biofilm. Amoebas such as

Naegleria, Vermamoeba and *Acanthamoeba* were present in all the biofilm samples. On the opposite, *Naegleria* was more present in biofilm at 30.7 °C and in BTP tubes. When conducting a microbiome analysis in a water distribution system or a cooling tower, it is important to take into account the fact that the microbial communities will be different in different parts of the system due to the effect of the temperature. Taking samples from different components operating at different temperatures gives a more complete picture of the microbiota since the microbial communities may vary within a same system.

The biofilm communities of the pipes were shaped by temperature (Figure 2.8). Significant differences between the cold and warm water pipes were seen for the eukaryotic and bacterial communities. First, the impact of temperature is clear for the bacterial communities growing in PVC and the elastomer; bacterial communities growing at 30.7 °C were significantly different from communities growing at 22.7°C. Temperature also had an effect on the eukaryotic community growing on PVC, displaying a significant difference between communities in cold and warm water pipes. However, the temperature did not have a significant effect on the eukaryotic communities growing on BTP.

In addition to temperature, bacterial communities in the biofilm were also influenced by the material surface. Significant differences were noted among the biofilm samples taken from PVC or elastomer surfaces at a given temperature (Figure 2.8). The impact of the material in the microbiome is well known (Moritz et al. 2010; Pinto et al. 2014; Ji et al. 2015; Ji et al. 2017; Proctor et al. 2017). Different materials release different compounds that might be taken as an energy source by some organisms. For instance, plastic substrates tend to leach biodegradable compounds (Moritz et al. 2010). It has been shown that elastomers (such as BTP) can leach nutrients and an explanation for

the difference in the microbiota in BTP and PVC could be that fastidious organisms might tend to grow biofilms in elastomers instead of PVC due to the difference in the availability of nutrients (Rogers et al. 1994a). Difference in the diameter of the pipes and the tubes might also have an effect on the microbiome. A smaller diameter increases the surface to volume ratio between the water and the surface and might have an impact on biofilm formation.

During the whole experiment, flocs of detached biofilm were observed in the bioreactor. In addition, at the day of the dismantlement, flocs floating in the water inside the pipes and the bioreactor were observed. We analyzed the alpha and beta diversity of these flocs (figure 2.7). Bacterial diversity was significantly higher in the flocs than in the water samples. In addition, the communities were more similar to the ones found in biofilm than the communities present in water. A suitable explanation for this increased diversity is that floc samples had species found in both the water and in the biofilm; the formation of these flocs might be related to the presence of *Proteioohromonas* in the biofilm. As a protective response against grazing by *Proteioochromonas* and other flagellates some bacteria auto aggregate forming flocs in water systems (Blom et al. 2010; Trunk et al. 2018).The presence of species that promote biofilm detachment might represent a risk of dissemination. In addition, detached biofilm represents an additional challenge on the disinfection process since the presence of biofilm in water may augment the chlorine demand and could respond differently to cleaning interventions (Fish and Boxall 2018).

Even though the presence of *Legionella* in biofilms within water distribution systems has been reported in several studies (Armon et al. 1997; van der Kooij et al. 2005; Lau and Ashbolt 2009; Declerck 2010; Moritz et al. 2010; Buse et al. 2012; Abdel-

Nour et al. 2013; Buse et al. 2014; Abu Kweek and Amer 2018), the relative abundance of *Legionella* in the biofilm was extremely low (≤0.5 %) at the end of the present experiment while we were able to detect the presence of *Legionella* in water samples at a higher abundance (up to 11% of reads). Interestingly, *Legionella* was not detected from the elastomer surfaces (Figure 2.6 B). Furthermore, evidence of the ability of *Legionella* to form and integrate biofilm in PVC has previously been reported (Rogers et al. 1994a; Armon et al. 1997). Several factors influence the formation of biofilm by *Legionella* and its ability to integrate biofilms (Rogers et al. 1994a; Piao et al. 2006; Buse et al. 2017; Rhoads et al. 2017). Since the inoculation of the system with *Lp*, a rise in the relative abundance of *Legionella* was observed (Figure 2.6). Two weeks after the inoculation with *Lp*, a drop on the relative abundance of *Legionella* was observed from 11% to 8%. Conversely, we see an augmentation on the relative abundance of *V. vermiformis* (Figure 2.3 B). It has been shown that *Lp* maintains an ecological balance with amoeba within the biofilm environment and that intracellular growth of *Legionella* may be cell concentration dependent (Shaheen et al. 2019). Furthermore, it is unlikely that *Legionella* did not integrate the biofilm at any time during the 12 weeks the model cooling system was operating. The lack of time points in the analysis of the composition of the *Legionella*, prevent us from making any assumptions about the dynamics of the microbiota in the biofilm. The presence of *Naegleria*, *Vermamoeba* and *Acanthamoeba* in the biofilm as well as the temperature being between 22.7 and 30.7 °C in the pipes suggest that the bacterium had favorable conditions for its growth and proliferation (Rowbotham 1980; Moffat and Tompkins 1992; Fields et al. 2002b; Ashbolt 2015). A possible explanation for the presence of *Legionella* in the water and not in the biofilm at the end of the experiment

would be that *Legionella* is released into the water after intracellular replication inside amoeba located more abundantly inside the biofilm. *Legionella* is expelled in the water by the lysis of amoeba before infecting another free-living amoeba (Greub and Raoult 2004; Lau and Ashbolt 2009). The bacterium can also be expelled in cysts from ciliates such as a O*ligohymenophorea* species, *Tetrahymena* as well as amoeba species like *Acanthamoeba* (Bouyer et al. 2007; Berk et al. 2008; Hojo et al. 2012)

Conclusion

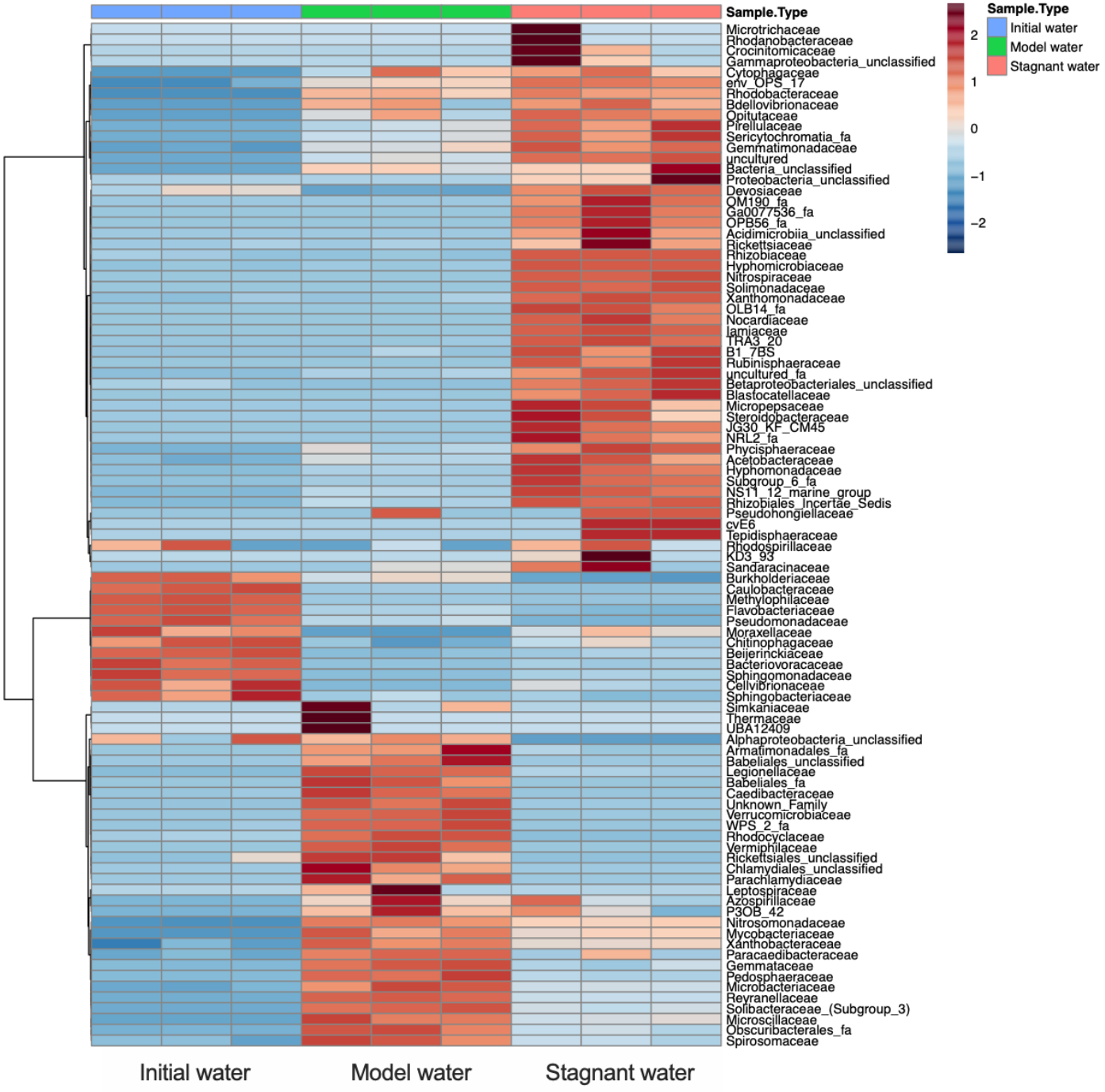
This study illustrates the importance of studying the microbiota in water and in biofilm since communities in the biofilm and the water were different. From our study three main observations emerge. First, in our model, the water temperature and surface material have a great impact in the composition of the microbiota. These factors are not independent of each other and do not affect equally all organisms. Secondly, the host cells were mainly present in the biofilm, while *Legionella* was not present in the biofilm at the time of sampling. This suggests that at different time points in its life cycle, it will be found in different parts of the cooling tower. Thirdly, this study showed that water sampling for detection of *Legionella might be only* adequate at certain points in *Legionella* life cycle. Even though *Legionella pneumophila* might be present in the biofilm, it can be released into the water after growing and reproducing intracellularly inside ciliates and amoeba in unchlorinated or untreated systems. However, during its replication, it might be growing intracellularly inside amoeba in the biofilm. A better understanding of the dynamics of *Legionella* proliferation in amoebae present in biofilms is needed.

Supplementary Material

Supplementary Table 1: Samples taken from the model cooling tower

Sample Name	Sample type	Week of sampling	Additional Information	Temperature	Analyses
Initial Water	Water	0	Water from the actual cooling tower	N/A	HPC/ 16S
Jun 14	Water	0	Day of assembly of the system	N/A	HPC/ 16S
Aug 9	Water	9	Before Inoculation V. vermiformis	N/A	HPC/ 16S/ 18S
Aug 22	Water	10	After Inoculation V. vermiformis	N/A	HPC/ 16S/ 18S
Aug 24	Water	10	Before Inoculation L. pneumophila	N/A	HPC/ 16S
Aug 31	Water	11	After Inoculation L. pneumophila	N/A	HPC/ 16S
Sep 4	Water	12	After Inoculation L. pneumophila	N/A	HPC/ 16S
Sep 7	Water	12	After Inoculation L. pneumophila	N/A	HPC/ 16S
Control water 1	Water	13	Water from actual cooling tower left for 13 weeks in a container at room temperature	Room temperature (22°C)	16S
Control water 2	Water	13	Water from actual cooling tower left for 13 weeks in a container at room temperature	Room temperature (22°C)	16S
Sep 13	Flocs	13	Water from the bioreactor + detached biofilm	N/A	HPC/ 16S/ 18S
Flocs Pipe	Flocs	13	Water from the pipes + detached biofilm	N/A	HPC/16S/ 18S
Cold pipe 1	Biofilm	13	Biofilm grown on PVC pipe	22.7°C	HPC/ 16S/ 18S
Cold pipe 2	Biofilm	13	Biofilm grown on PVC pipe	22.7°C	HPC/ 16S/ 18S
Cold pipe 4	Biofilm	13	Biofilm grown on PVC pipe	22.7°C	HPC/ 16S/ 18S
Cold pipe 6	Biofilm	13	Biofilm grown on PVC pipe	22.7°C	HPC/ 16S/ 18S

Cold pipe 7	Biofilm	13	Biofilm grown on PVC pipe	22.7°C	HPC/ 16S/ 18S
Cold tube	Biofilm	13	Biofilm grown on BTP PharMed tube	22.7°C	16S/ 18S
Warm pipe 2	Biofilm	13	Biofilm grown on PVC pipe	30.7 °C	HPC/ 16S/ 18S
Warm pipe 3	Biofilm	13	Biofilm grown on PVC pipe	30.7 °C	HPC/ 16S/ 18S
Warm pipe 4	Biofilm	13	Biofilm grown on PVC pipe	30.7 °C	HPC/ 16S/ 18S
Warm pipe 6	Biofilm	13	Biofilm grown on PVC pipe	30.7 °C	HPC/ 16S/ 18S
Warm pipe 6	Biofilm	13	Biofilm grown on PVC pipe	30.7 °C	HPC/ 16S/ 18S
Warm pipe 7	Biofilm	13	Biofilm grown on PVC pipe	30.7 °C	HPC/ 16S/ 18S
Warm tube	Biofilm	13	Biofilm grown on BTP PharMed tube	30.7 °C	16S/18S



Supplementary Figure 1: Heatmap representing the predominant taxa in water samples organized by type: Initial water (June 14), model water (September 13) and stagnant water (September 13). The distance measure between data points in the clustering was the Pearson correlation coefficient. Enriched taxa are represented in red.

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General discussion

The objective of this work was to study the dynamics of *Legionella pneumophila* in relationship with the physical conditions in a cooling tower and the resident microbiota. The system was filled with water from a real cooling tower allowing us to have a microbial community that can be found in these systems. The design of this model intended to mimic some of the real features of the cooling towers such as the heat exchanger, the temperature difference between the water at the inlet and the output of the cooling tower and the flow of water circulating outside the tower. However, our design was constrained for biological safety reasons; it was not possible to have an open system harbouring *Legionella pneumophila* and amoeba. This model cooling tower was able to grow a unique microbiota, different from the initial water and the water left stagnant for 3 months (Figure 2.5). This fact suggests that the conditions of the system shaped the microbial community. Our model allowed us to see that different factors shape the microbial communities such as the temperature and the materials. Previous pilots have been developed to assess the effect of these factors (Farhat et al. 2009; Taylor et al. 2013; Levorino et al. 2017). However, generally, these pilots allow the study of either the biofilm formation or the water, but not both. The main factors that we considered to study in this model cooling tower were the temperature, the presence of amoeba and the materials. Our pilot allowed us to study the microbiota in the water and in the biofilm, considering both water and biofilm as different microbiomes that interact between each other. While this model gave us a snapshot of the evolution of the microbiota in a cooling tower, this work can be continued by improving the pilot-scale model.

Air inlet and fill material

The fact that a cooling tower is a system open to the atmosphere affects the nutrient content within it. These heat exchange devices receive inputs of carbon and nutrients (Liu et al 2011) from the air as well as a multitude of organisms that contribute to the diversity. The lack of an important input of air in our model system might constrain the diversity within the resident microbiota. In a cooling tower, the air and the water are in contact in a fill material which can be made out of PVC and ceramic among other materials (American Society of Heating Refrigerating and Air-Conditioning Engineers Inc 2008). It is considered that, because of the big surface that the fill material represents and the contact it has with both the water and the nutrients coming from the air, biofilm can be easily formed in it (Liu et al 2011). The number of studies considering the biofilm formation within the fill material are limited (Liu et al 2009). To improve the model cooling tower, it would be interesting to model the fill material.

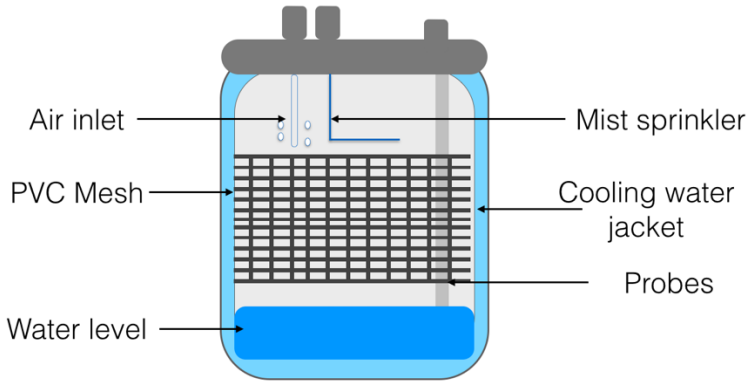

Figure 3.1: Schematic representation of a bioreactor simulating a cooling tower.

A cooling tower model considering the fill material and its exposure to both air and water as well as the exposure to sunlight would be more representative or a real cooling tower. A similar model could be built using a bigger bioreactor, containing fill material made out of PVC, using a sprinkler instead of a tube for the water inlet, decreasing the water level in the bioreactor (Figure 3.1). A higher air flow would enter the bioreactor. It is proposed that the air comes from the exterior of the building to increase the input of environmental organisms and nutrients.

Disinfection procedures

Even though our model allowed us to study what would happen if a cooling tower is not disinfected and gets colonized by *Legionella,* an important factor to consider in future studies would be disinfection. It has been shown that the use of disinfectants affects both the diversity of the microbiota and the presence of *Legionella* in a cooling tower (Hwang et al. 2012; Paranjape et al. 2019). The presence of *Legionella* in the biofilm was not detected at the end of the experiment. This might be related to the absence of disinfectant of the water. The relationship between biofilm formation or surface adhesion by *Legionella* and chlorine residual has not been proven. However, a relationship between chlorine stress and biofilm formation by *Pseudomonas* aeruginosa has already been described (Kekeç et al. 2016). Furthermore, in a future study it would be important to consider a disinfection regime to simulate better the conditions of a cooling tower. It may be possible that integration of *Lp* in biofilm is triggered by environmental stresses. In this study, *Lp* was not exposed to stress due to the lack of nutrients since there was a

population of host cells and was not exposed to oxidative or thermal stress due to the absence of disinfection procedures.

Other materials

Another characteristic of a cooling tower that could be better simulated in further research is the material of the pipes. Generally, cooling towers employ steel, brass, copper, stainless steel and other metals and it is clear that the materials affect microbial diversity and biofilm development (Rogers et al. 1994a; Rogers et al. 1994b; American Society of Heating Refrigerating and Air-Conditioning Engineers Inc 2008; Liu et al. 2011; Proctor et al. 2017). Nowadays, polymer pipes, such as cross-linked polyethylene (PEX), polyethylene (PE), and polyvinyl chloride (PVC), are increasingly used in water supply systems because their low cost and ease of installation (Lee 2013). Our study revealed that PVC can support the growth of a multitude of *Legionella* hosts cells such as *Oligohymenophorea, Naegleria, Vermamoeba* and *Acanthamoeba*. The presence of organisms that promote Legionella proliferation in cooling towers represents a risk factor for a LD outbreak. Thus, replacing metallic materials that can have an antimicrobial effect on the microbial growth with polymer pipes might lead to an increased colonization of these systems.

Biofilm sampling

As said previously, our system allowed us to determine that host cells were mainly present in the biofilm. It would be extremely interesting to study the evolution of the biofilm bacterial and eukaryotic communities through time and be able to see if *Legionella* was

present in the biofilm at some point. We hypothesised that *Legionella* was not in the biofilm at the end of the experiment because it was released back into the water after replication inside amoeba and ciliates (Bouyer et al. 2007; Berk et al. 2008; Hojo et al. 2012). Another fact that led us to believe that this could have happened is an augmentation of the relative abundance of host cells two weeks after the inoculation with *Legionella*. The presence of *Oligohymenophorea* in water was only observed after the inoculation with *Legionella* (Figure 2.3). This presence of *Oligohymenophorea* DNA in the water samples might have been caused by the lysis of the cells. To have more biofilm samples will allow us to have a better idea of what are the dynamics of both host and *Legionell*a in the biofilm. To be able to sample at different times, a good strategy to adopt would be to add coupons to some of the pipes. Sampling different coupons at different times would allow to have a better understanding on the evolution of the microbiota in the biofilm during *Lp* colonization.

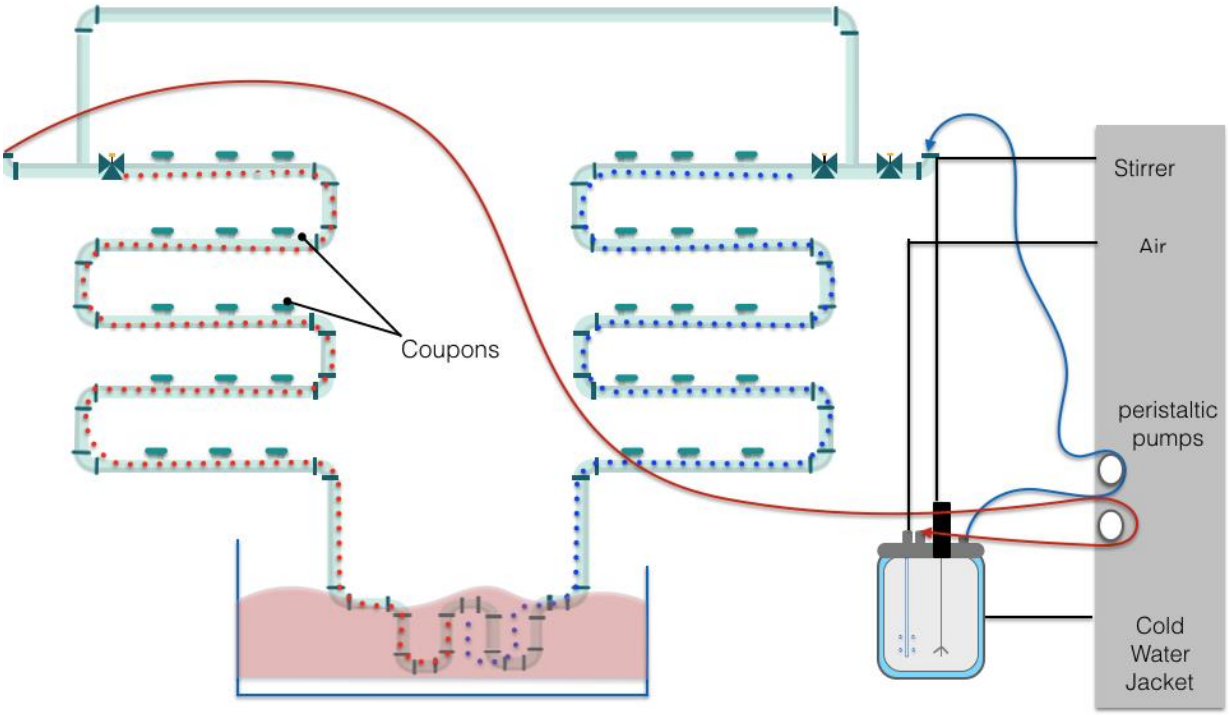

Figure 3.2 Schematic representation of a new model cooling tower with coupons.

Coupons could be added at the top of the pipes in a new design. This would allow an easy sampling. The fact that the coupons will we horizontally placed will avoid that hydrodynamic conditions of the system are altered at some points. A Bypass would be added to redirect the flow of the system. The pipes would be emptied before the sampling and the flow in the pipe network would be stopped with a set of valves.

Hydrodynamic Conditions

Finally, the hydrodynamic conditions of the systems affect the biofilm development. (Vieira et al. 1993; Douterelo et al. 2017; Ling et al. 2018). Biofilm development is different

in systems subject to laminar and turbulent flow. Manuel et al. (2010) conducted a study to analyse the effects of water stagnation and flushing on the microbiota of drinking water promoting the formation of biofilms under turbulent or laminar flow and stagnation conditions. Their results showed that under laminar flow, an increase of cells in sessile state was observed while under turbulent flow there was an increase on the planktonic cells. That seemed to indicate that growth and detachment of biofilm is affected by the regime of the flow. Moreover, they hypothesized that a change in the shear stress of the water (turbulent flow to stagnant conditions) could promote the formation of biofilm. Our system having a flow rate of 1L/ h and a diameter of 0.5 inch, had a laminar flow. However, in cooling tower there is not only one regime. The system can have parts where conditions promote a turbulent flow while in some other parts the system would be laminar. A model cooling tower that promotes shifts in shear stress on the biofilm might allow a better development of biofilm. This could be done by increasing and decreasing the flow at some time points.

Final conclusion

A model cooling tower was developed to study the abiotic and biotic factors that affect Legionella colonization in cooling tower. This model allowed us to have a better understanding of the microbiota inside the system. Having water and biofilm samples gave us a snapshot of the different communities that can exist in a cooling tower. Our results indicated that was a greater microbial diversity in the biofilm than in the water and that the community was shaped by the temperature and the material of the surface where biofilm grew. Furthermore, the study of the microbial community in the biofilm allowed us to see that the hosts cells were found attached to the surface and not in the water. This raised questions since *Legionella* was not found associated with these hosts cells in the biofilm. Hence, it is important to be able to have different samples of biofilm at different time points to have a good understanding in the dynamics of *Lp* in a cooling tower since the bacterium may change from sessile to planktonic state during the colonization of the system.

A cooling tower is a complex environment, due to the differences of temperature and material in different parts of the system. To be able to effectively control *Legionella* propagation, it is fundamental to understand the different factors that might have an impact in the microbiota. Abiotic factors inherent of the design of the cooling tower have a substantial impact shaping the microbiota. These factors do not affect each organism equally and might be related to the interaction of different microbes. It is also important to understand that a cooling tower is not a closed system and that its relationship with its surrounding environment might affect the microbiota. Thus, a better understanding on how the air inlet affects the microbiota is needed since important part of the biomass of a

cooling tower is obtained through the air. Furthermore, this study showed that water sampling for detection of *Legionella* can be adequate in certain conditions. Even though *Legionella pneumophila* might be present in the biofilm of some cooling towers, it seems to be released back into the water after growing and reproducing intracellularly inside ciliates and amoeba, making water samples valid for detection of *Lp*. Having said that, microbiome analyses are a powerful tool for preventing outbreaks. For instance, knowing that there is a host population in the biofilm may allow to carry out corrective measures to avoid an outbreak. Having analyses on the microbiota in the water as well as in the biofilm in different parts of the tower can be helpful when identifying susceptible parts of the system that could be eventually colonized by *Legionella pneumophila.*

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