Legionella and free-living amoebae interactions: an ecological perspective of drinking water safety and controlling legionellosis

by

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ABSTRACT

Legionnaires' disease (LD) outbreaks have been a growing concern for public health as well as for the water industry and building management. The number of outbreaks has significantly increased over the last decades worldwide, especially in the United States and Europe. This disease is mainly caused by a Gram-negative opportunistic water-based pathogen, *Legionella pneumophila.* The primary sources of human exposure to this bacterium are the aerosols from engineered water systems and drinking water premise plumbing (PP) where the pathogenic *Legionella* spp. regrow. *Legionella* spp. colonize the water-biofilms and grow intracellularly in the free-living amoebae (FLA) and other microeukaryotes that are present in built aquatic environment and even in high-quality drinking water. Therefore, the relationships of *L. pneumophila* with FLA and other microorganisms within the drinking water-biofilms under premise plumbing conditions were examined to aid developing safe water management strategies and better public health protection from this pathogen. This study identified a novel risk of *Legionella*-FLA interactions in drinking water by demonstrating that the *Legionella*-containing vesicles of respirable size may contain 23-873 *Legionella* cells per vesicle, thus could potentially serve as a single dose for human infection. Moreover, this study demonstrated that FLA-bacteria interactions may result in a natural selection of *L. pneumophila* within water-biofilms due to the preferential feeding behavior of FLA. These interactions also help *L. pneumophila* to persist within the water-biofilms for years. The feeding behavior of FLA and their reluctance to feed on *L. pneumophila* was later confirmed by real-time monitoring of known bacterial species. Overall, these observations led to a hypothesized growth model for pathogenic *Legionella* spp. in PP by suggesting uptake of *Legionella* spp. by FLA only under certain conditions (such as during food

scarcity), followed by intracellular growth of the bacterium, lysis of the FLA host, release of the newly replicated bacterial cells to the water and re-initiating the cycle if conditions permit. Difference in water-biofilm bacterial community compositions (based on metagenomics analysis) on Cu and PVC pipe material at room temperature (RT) and 40 °C indicated that the temperature was a stronger determinant of water-biofilm bacterial community compositions than pipe material. Nonetheless, Cu pipe material supported higher colonization of *Legionella*, whereas PVC supported more mycobacteria, both of which are opportunistic water-based pathogens. Finally, a probiotic, biological control option for *Legionella* was tested and found promising. The probiome selector (naturally-developed water biofilms on virgin PVC granules) affected the downstream biofilm bacterial community and reduced the colonization of *Legionella* in Cu biofilms. Further optimization of the probiome (identified through vigorous microbiome screening and process optimization) is still required, but has the potential to be an alternative sustainable strategy to control *Legionella* spp. (instead of high-temperature or heavy chemical disinfection) in high *Legionella*-risk built aquatic environments. Moreover, the water-biofilm community profiles indicated that microbial community mapping could be useful to identify the vulnerable or compromised sites of a PP, and in combination with risk assessment and Hazard Analysis Critical Control Point (HACCP)-based water management approaches, it could ensure better water and public health safety.

PREFACE

This thesis is an original work by Md Shaheen. The modified versions of Chapter 2, 3 and 4 of this thesis have already been published in scientific journals. The published articles are listed below. Chapters 5 has also been prepared as manuscript and is ready for submission to peer-reviewed journal.

1. **Shaheen, M.** and Ashbolt, N.J. (2018) Free-living amoebae supporting intracellular growth may produce vesicle-bound respirable doses of *Legionella* within drinking water systems. *Exposure and Health* 10(3), 201-209.

M. Shaheen was responsible for the experimental work, data analysis and manuscript composition. N.J. Ashbolt was the supervisory author and reviewed the manuscript.

2. **Shaheen, M.**, Scott, C., & Ashbolt, N. J. (2019). Long-term persistence of infectious *Legionella* with free-living amoebae in drinking water biofilms. *International Journal of Hygiene and Environmental Health*, 222(4), 678-686.

M. Shaheen was responsible for the experimental work, data analysis and the manuscript composition. C. Scott performed the qPCR experiment and reviewed the manuscript and N.J. Ashbolt was the supervisory author and reviewed the manuscript.

3. **Shaheen, M.**, & Ashbolt, N.J. (2021). Differential bacterial predation by free-living amoebae may result in blooms of *Legionella* in drinking water systems. *Microorganisms* 9(174). https://doi.org/10.3390/ microorganisms9010174

M. Shaheen was responsible for the experimental work, data analysis and the manuscript composition and N.J. Ashbolt was the supervisory author and reviewed the manuscript.

Other scholarly contributions during the PhD study period:

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Both L. Palli and M. Shaheen contributed equally to the manuscript composition as an editorial for the special edition of the journal.

- Setty, K., Loret, J.F., Courtois, S., Hammer, C.C., Hartemann, P., Lafforgue, M., Litrico, X., Manasfi, T., Medema, G., **Shaheen, M.** and Tesson, V. (2019). Faster and safer: Research priorities in water and health. *International Journal of Hygiene and Environmental Health*, 222(4), 593-606.

M. Shaheen was responsible for the composition of chapter (Water microbiology) and reviewing the manuscript. K. Setty coordinated the manuscript composition, compiled and reviewed the manuscript. All other authors including K. Setty contributed to specific chapters and reviewed the manuscript.

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List of Abbreviations

AR	Annular Reactor
ARB	Amoeba resistant bacteria
ARM	Amoeba resistant microorganisms
ASHRAE	American Society of Heating, Refrigerating and Air-Conditioning Engineers
ASV	Amplicon Sequence Variant
ATCC	American Type Culture Collection
BCC	Bacterial Community Compositions
BCYE	Buffered Charcoal Yeast Extract agar medium
BCYE-PCV	BCYE agar with Polymyxin B, Cycloheximide and Vancomycin
BFP	Blue fluorescent protein
CDC	The United States Centers for Disease Control and Prevention
CFU	Colony forming units
Cu	Copper
DWDS	Drinking water distribution systems
DWS	Drinking water systems
EPA	The United States Environmental Protection Agency
EWGLI	European Working Group for *Legionella* Infections
EWS	Engineered water systems
FLA	Free-living amoebae
GFP	Green fluorescent protein
GU	Genomic Units
HACCP	Hazard Analysis Critical Control Point
HDPE	High density polyethylene
HPC	Heterotrophic Plate Counts
HT	High temperature (40 °C)
Dot/Icm	Defect in organelle trafficking/intracellular multiplication
ISO	International Organization for Standardization
LB	Luria Bertani agar medium
LCV	*Legionella* Containing Vacuoles
LD	Legionnaires' Disease

LEfSe	Linear discriminant analysis Effect Size
LOD	Limit of Detection
Mip	Macrophage infectivity potentiator
NCBI	National Center for Biotechnology Information
NNA	Non-nutrient Agar
NNDSS	National Notifiable Diseases Surveillance System
NTM	Nontuberculous Mycobacteria
OPPPs	Opportunistic premise plumbing pathogens
OSHA	The United State Occupational Safety and Health Administration
OTU	Operational Taxonomic Unit
PAGE's saline	A salt solution for amoeba developed by F. C. Page
PBS	Phosphate Buffered Saline
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational multivariate analysis of variance
PEX	Crosslinked Polyethylene
PHAC	Public Health Agency of Canada
PP	Premise plumbing
PVC	Polyvinyl Chloride
PYG	Peptone Yeast Extract Glucose medium (liquid)
QMRA	Quantitative Microbial Risk Assessment
qPCR	Quantitative Polymerase Chain Reaction
RA	Relative abundance
RFP	Red fluorescent protein
RT	Room temperature/ 22 °C (±1 °C)
SCGYEM	Serum-casein-glucose-yeast extract medium
SSU	Small sub unit
UV	Ultra violet
VBNC	Viable but non-culturable
VHA	Veterans health administration
WHO	World Health Organization
WSP	Water safety plan

Chapter 1

Chapter 1: Background and literature review

1.1 *Legionella* spp. and legionellosis

Legionella spp., especially the species *L. pneumophila* has become a major public health concern in developed countries due to the severity of impact and continued increase in the incidence rate of Legionnaires' disease (LD). This is an acute and often fatal pneumonia-like infection in humans. Pathogenic legionellae also cause Pontiac fever, a mild flu-like self-limiting infection (Glick et al. 1978, Rowbotham 1980). Pontiac fever, in most cases, resolves on its own, and therefore, prevalence data are largely unknown for this disease. Both of these infections are commonly termed as legionellosis. *Legionella pneumophila* has been reported to be responsible for 91.5% of the total legionellosis cases, especially serogroup 1, which is responsible for 84.2% of the reported cases worldwide. The other two major species, *L. longbeachae* and *L. bozemanii* are responsible for 3.9% and 2.4% of the total legionellosis cases, respectively (Yu et al. 2002). Although *Legionella* spp. are respiratory pathogens, no human-to-human transmission has been reported except one speculated case (Borges et al. 2016, Correia et al. 2016).

Legionella spp. are the number one cause of drinking water-related illness and death in the United States (US) and ranked fourth by the health burden amongst all infectious diseases in Europe (Cassini et al. 2018). In 2010-2013, two-thirds of the drinking water-related illness in the US was due to *Legionella* spp. (Beer et al. 2015, Gargano et al. 2017). Legionellosis has been a notifiable disease in the US and recorded through the National Notifiable Diseases Surveillance System (NNDSS); however, the passive monitoring of legionellosis as a water-borne disease began in 2001. In Canada, legionellosis has also been a notifiable disease since 1986 (PHAC 2020). The reported cases of LD are ever-increasing and grew by a factor of 5.5 from 2000 to 2017 (CDC 2018, NASEM 2020) (Figure 1.1).

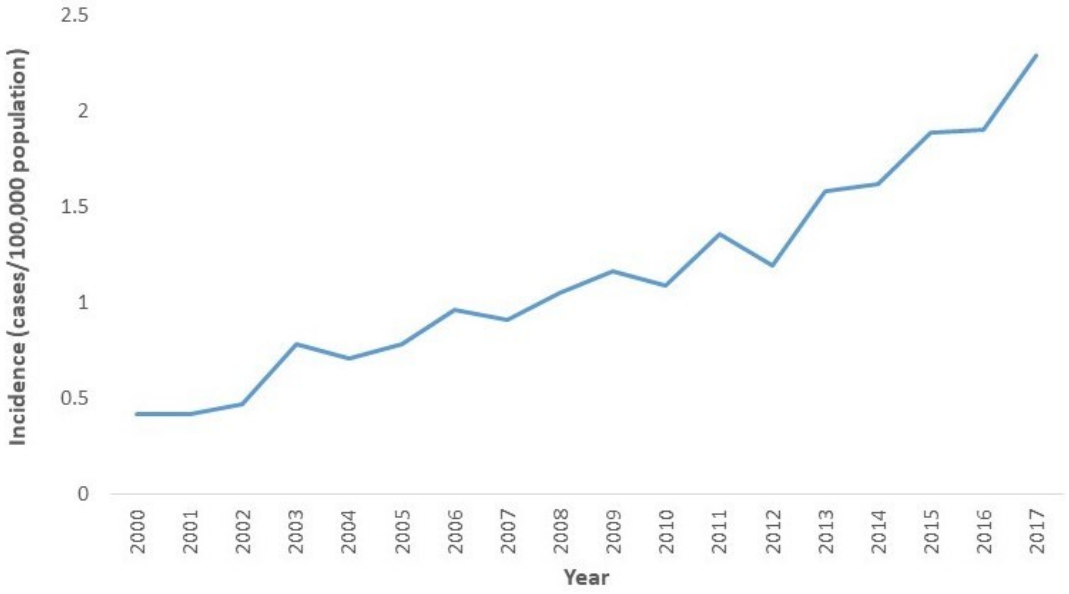

Figure 1.1: The reported cases of Legionnaires' disease in the US shows a continuously increasing occurrence of LD (CDC 2018, NASEM 2020).

The increased incidence of LD could be partly due to the implementation of a better monitoring system, aging water systems, an increase in high-risk populations like the elderly, immunosuppressed and smokers, and also due to climate change. Therefore, it is a great challenge for the water industry and public health authorities to ensure water safety by controlling human exposure to *Legionella* spp. and other water-based opportunistic premise plumbing pathogens (OPPPs) (i.e., nontuberculous *mycobacteria* (NTM) and *Pseudomonas aeruginosa*). In the US, an average of 13,000 hospitalization per year (range 8000-18000/year) occurs exclusively due to LD and the associated total hospitalization cost is about $434 million/year (Collier et al. 2012). However, the total number of cases could be 10 times higher than the reported number since sporadic cases are often not diagnosed and thus are not notified (NASEM 2020). Moreover, the self-limiting nature of Pontiac fever and overlapping symptoms with the common cold means there is a large under-reporting of the true disease burden from

Legionella infections (Jones et al. 2003). LD outbreaks are not very common in Canada, but several reported outbreaks have been associated with relatively high mortality. The LD outbreak in Quebec in 2012 was one of the most devastating outbreaks in Canada and was responsible for 182 confirmed cases with 13 fatalities (Lévesque et al. 2014).

Inhalation of pathogenic *Legionella*-containing water aerosols is the main route of exposure to *Legionella spp.* for humans (Hines et al. 2014, Walser et al. 2014). In urban settings, water aerosols can be generated during common water uses (showers/taps) and in mass-scale from building cooling-towers, decorative fountains, humidifiers, irrigators and hot tubs. Therefore, developing a monitoring system for pathogenic *Legionella* spp. and identifying conditions favorable for their persistence and growth to critical concentrations within these engineered water systems (EWS) are crucial for ensuring water and public health safety.

1.2 *Legionella pneumophila*

Legionella spp. are Gram-negative non-spore-forming, aerobic, facultative intracellular rod-shaped bacteria. They are non-encapsulated and usually have one or more polar or sub-polar flagella (limited motility). In contrast to most other Gram-negative bacteria, *Legionella* spp. have high amount of branched-chain fatty acid in the cell wall. *Legionella* spp. are inherently resistant to many antibiotics. These bacteria are also able to sustain a wide pH range and very high temperature and may grow at 20 to 50 °C at pH 5.5 to 9.2 (Plouffe et al. 1983, Rogers et al. 1994b, Wadowsky et al. 1985). *Legionella pneumophila* came into attention after discovering it as the causative agent of a mysterious acute respiratory illness in 1976 when 182 members of the Pennsylvania American Legion developed pneumonia (29 individuals died) after returning from a convention in Philadelphia (Fraser et al. 1977). However, members of the legionellacease

family had been isolated from clinical samples as early as 1943 and retrospective analysis described it as the etiological agent for unsolved epidemics of acute respiratory disease in 1950 (Winn 1988). There are over 65 species with 70 serogroups, but only a few species are responsible for causing legionellosis and *L. pneumophila* is the most dominant one (Burillo et al. 2017, Mondino et al. 2020). The type IV secretion system (dot/icm) is considered as the main virulent factor for pathogenic *Legionella* spp. There are about 1800 effector proteins that can be transported to the host cells through this IV secretion system. The macrophage infectivity potentiator protein (Mip), one of the main effector proteins of pathogenic *Legionella* spp. is a 24 kDa surface-exposed protein and is necessary for intracellular survival and growth in human macrophages or FLA trophozoites (Cianciotto et al. 1989, Miyamoto et al. 1993). Pathogenic *Legionella* spp. are considered to have co-evolved with fresh water protozoa (mainly free-living amoeba and ciliates) by acquiring mechanisms to withstands the intracellular conditions of the food vacuoles of protozoa and to use the intracellular nutrients for multiplying within. Once phagocytosed by FLA, the infectious form of the *L. pneumophila* cells quickly adapt to the intracellular environment of the food vacuoles and undergo intracellular differentiation by changing its morphological and physiological features and also interfere with the host defense system (Robertson et al. 2014). At this stage the food vacuoles are referred to as *Legionella*-containing vacuoles (LCV). Accumulation of nutrients to a threshold amount (*L. pneumophila* induced transport or inherent characteristics of FLA) in the LCV from host cell cytoplasm triggers the intracellular multiplication of the *L. pneumophila* cells. A life cycle of *L. pneumophila* in water environment and FLA and ciliates has been described with different developmental states of cells in the following table (Table 1.1) and in Figure 1.2.

Table 1.1: The developmental states of *L. pneumophila* (Robertson et al. 2014)

Name used (abbreviation)	Main characteristics	Primary references
Exponential phase form (EPF)	Grown extracellularly, non-infectious to host cells, sensitive to stress, replicates actively	(Byrne and Swanson 1998)
Stationary phase form (SPF)	Grown extracellularly, infectious to host cells, resistant to stress	(Byrne and Swanson 1998)
Filamentous form (FF)	Grown extra- and intra-cellularly, infectious to host cells, forms dense biofilms	(Piao et al. 2006, Rodgers et al. 1978)
Mature infectious form (MIF)	Grown intracellularly, infectious to host cells, resistant to stress	(Garduño et al. 2002)
Immature intracellular form (IIF)	Grown in cultured macrophages, morphologically undifferentiated, less infectious and less resistant to stress than MIFs, elongated	(Abdelhady and Garduño 2013)
Replicative phase form (RPF)	Grown intracellularly, replicates actively	(Faulkner and Garduño 2002)
MIF-EPF intermediate	Grown extracellularly upon reviving from mature infectious forms in culture media, shows intra-periplasmic vesicles	(Faulkner and Garduño 2002)
MIF-RPF intermediate	Grown intracellularly in response to the presence of amino acids, a precursor to the initiation of replication in the LCV	(Sauer et al. 2005)
RPF-MIF intermediates	Grown intracellularly in the late stages of the infection cycle, display unique envelope profiles. Might be similar to IIFs	(Faulkner and Garduño 2002)
VBNC derived from a SPF	Grown extracellularly in response to sustained stress, resuscitates in the presence of amoebae	(Al-Bana et al. 2014, Steinert et al. 1997)
VBNC derived from a MIF	Grown extracellularly in response to stress, shows an intact cell ultrastructure, does not resuscitate in amoebae	(Al-Bana et al. 2014)
VBNC derived from an EPF	Apparently more fragile than the other VBNC mentioned above	(Ohno et al. 2003)
Pelleted MIF	Grown within ciliates and amoebae, show unique developmental traits	(Berk et al. 2008, Berk et al. 1998)
Pelleted VBNC	Grown within ciliates, may show unique developmental traits	(Al-Bana et al. 2014)

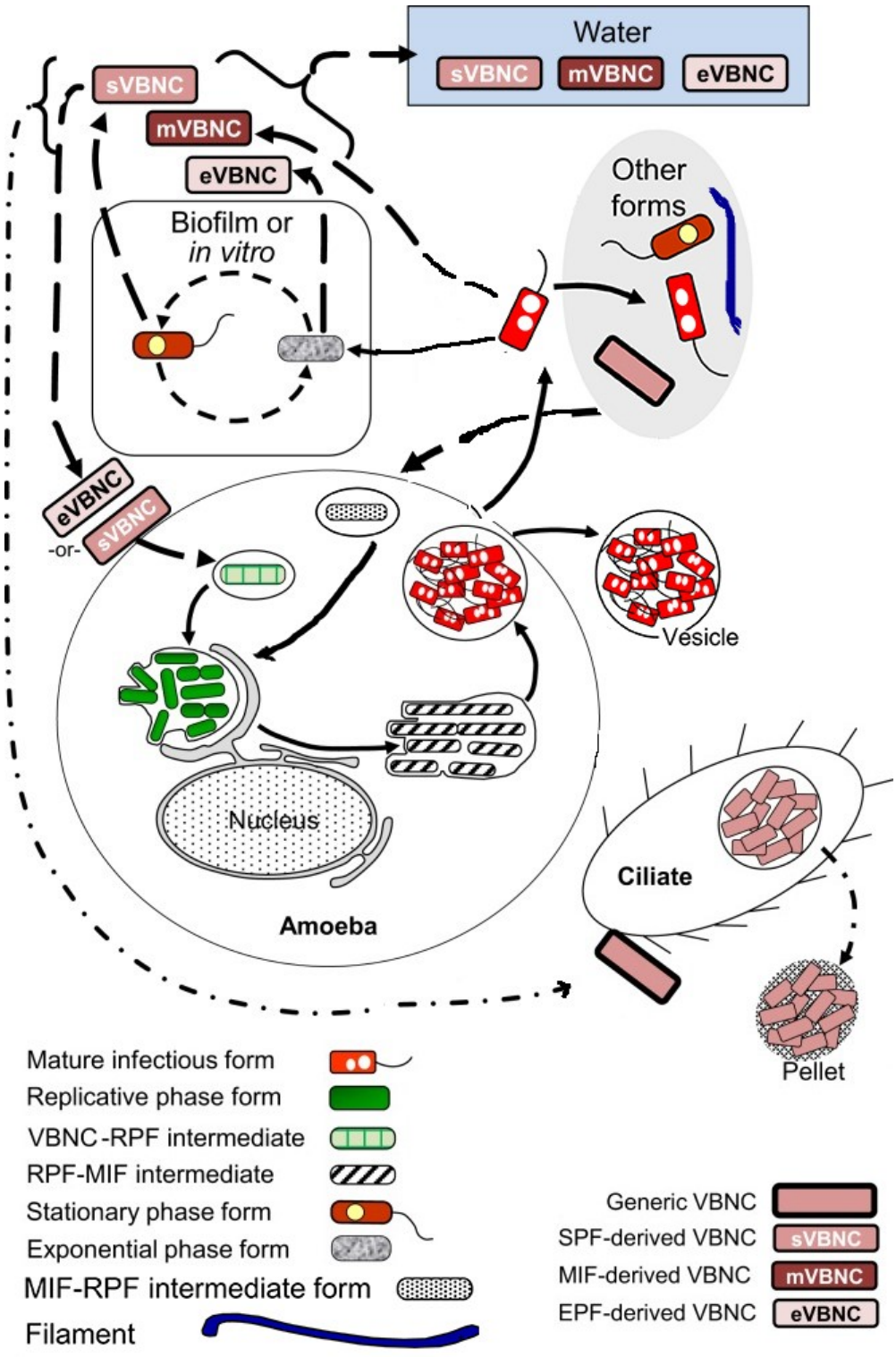

Figure 1.2: Schematic presentation of *L. pneumophila*'s life cycle in natural water environment (Intracellular in different protozoa and planktonic in water). The bacterium shows different developmental forms in protozoa and may present as cells with various physiological and morphological states in water (Robertson et al. 2014).

1.3 Free-living amoebae

FLA have two main forms of life: an active trophozoite form which is able to move, divide and feed (e.g., phagocytosis) and a dormant cyst form which is a strategic form of survival during unfavorable environmental conditions (nutrient limitation, osmotic or temperature stress). Many FLA like *N. fowleri* and *Willaertia magna* (Robinson et al. 1989) also have a flagellated form which can be transformed from trophozoite state (Figure 1.4) (Mehlhorn 2008, Silva and Moser 2021).

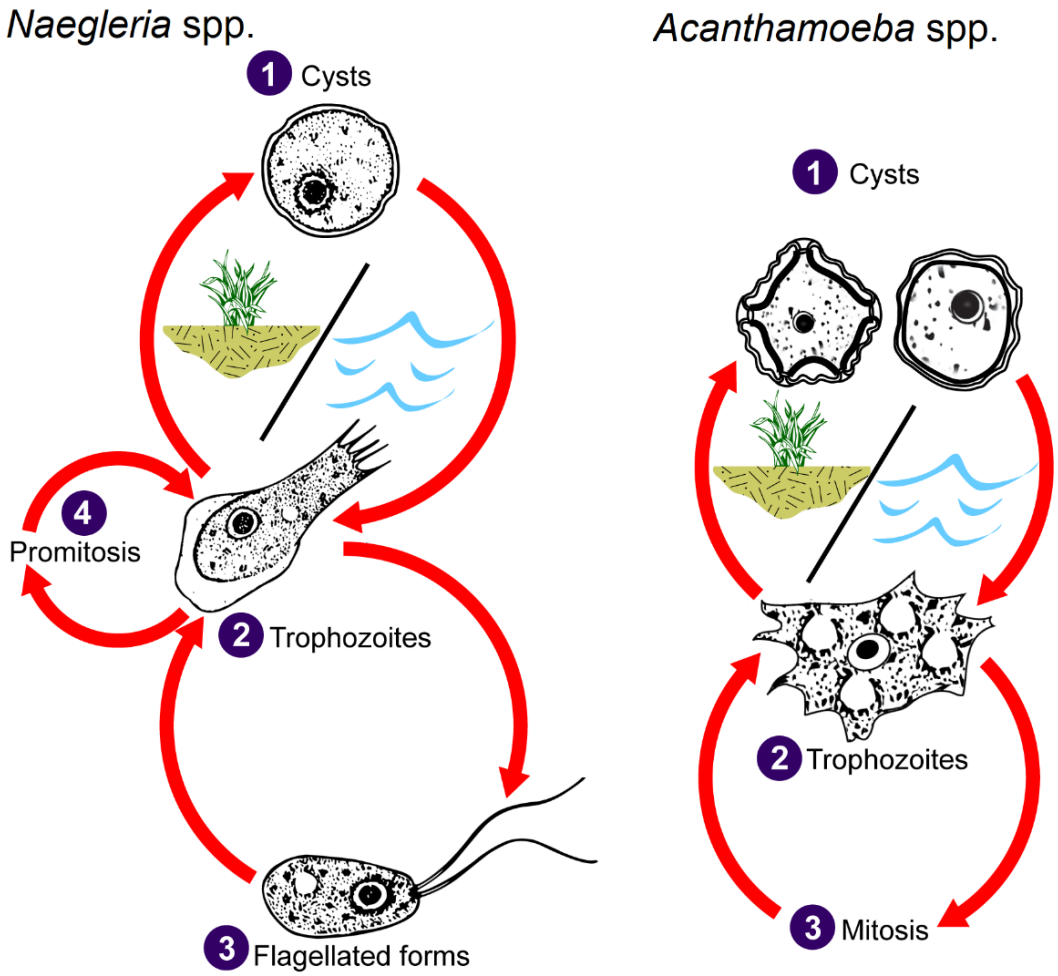

Figure1.3: Life cycle of free-living amoebae (FLA). FLA has mainly two developmental forms: cysts and trophozoites. Many FLA like Naegleria spp. has a flagellated form (Mehlhorn 2008).

In EWS, FLA are potential reservoirs for many pathogenic bacteria such as *Legionella* spp., nontuberculous mycobacteria. Fifteen amoebae species have been reported to support intracellular growth of *L. pneumophila* (Hägele et al. 2000, Robertson et al. 2014). Many FLA are also pathogenic including *N. fowleri* which causes fatal meningoencephalitis (known as brain-eating amoeba), *Acanthamoeba* spp. which causes keratitis (corneal inflammation in humans). Amoebae are considered as training ground for *L. pneumophila* since it uses the same mechanisms to infect both amoebae and human macrophages (Molmeret et al. 2005). Intracellularly grown *L. pneumophila* cells are more virulent than microbiological media-grown cells due to having increased expression levels of virulence genes (Barker et al. 1995, Cirillo et al. 1994).

1.4 Ecology of *Legionella* spp. within engineered aquatic systems

Legionella spp. are very common in any aquatic environment, however, engineered aquatic environments like building cooling towers, hot tubs, and premise plumbing (PP), shower-heads, whirlpools/hot tubs have been reported to be associated with major LD outbreaks (Ashbolt 2015, Craun et al. 2010, Fields et al. 2002). Since *Legionella* spp. are part of the native water microbiota, their presence and colonization in the drinking water distribution systems (DWDS), PP or any EWS are not necessarily an indication of poor performance of the water treatment process or inadequate maintenance of the water distribution systems. However, what makes *Legionella* spp. a problematic pathogen is its ability to rapidly grow to critical concentrations within the water systems under certain environmental conditions (e.g., temperature, water stagnation, disinfectant residual, etc.). Moreover, corrosion products like iron (an essential nutrient for *Legionella* spp.) released from plumbing also influence growth of *Legionella* spp. in DWDS (NASEM 2020, Reeves et al. 1981, Rhoads et al. 2017, Ristroph et al.

1981). Exposure to high concentrations of *Legionella* spp. via aerosols initiates infections in human lungs (Hamilton et al. 2019, Schoen and Ashbolt 2011). *Legionella* spp. have been reported to be present at typically low concentrations in water-biofilm environments, however limited information is available on how the water environment supports rapid growth (i.e. planktonic or biofilm-associated growth) – as recently reviewed by the U.S. National Academies of Science, Engineering and Medicine (CDC 2018, NASEM 2020).

Water in EWS is not sterile and over time the native microbiota of water develops biofilms on pipe surfaces, fixtures, or other contact points. Different *Legionella* spp. may have different preferred ecological niches but biofilms within PP and warm aquatic environments (cooling towers, hot tubs) are considered central to the ecology of pathogenic *Legionella* spp. The structure and physio-chemical properties of the biofilms affect the colonization and release of *Legionella* spp. Disinfectants have been reported to have effects on biofilm structures, as well as release and inactivation of *L. pneumophila*, however, they cannot completely remove biofilms (Shen et al. 2016, Shen et al. 2015). Biofilms protect *L. pneumophila* and other pathogens from disinfection by acting as a physicochemical barrier (Gião et al. 2009, Kim et al. 2002, Lau and Ashbolt 2009). It is generally understood that the released *L. pneumophila* from biofilms would recolonize downstream within DWDS or in PP, to which subsequent growth leads to problematic concentrations of *L. pneumophila* for human exposures. Water-biofilms are growth supportive environments where pathogenic *Legionella* spp. interacts with other bacteria, viruses and eukaryotic microorganisms like free-living amoebae (FLA), ciliates and nematodes (Keevil et al. 1993). Free-living amoebae are native members of the water-biofilm microbial community and are frequently present in EWSs, including drinking water systems and are particularly important for the persistence and growth of *Legionella* spp. (Buse et al. 2013b, Delafont et al. 2013,

NASEM 2020, Thomas et al. 2014). Pathogenic *Legionella* spp. are able to resist the host immune responses of FLA, multiply intracellularly and lyse the host cells within EWS. In aquatic environments, FLA live by grazing on bacterial biofilms which may naturally contain *Legionella* spp. (Lau and Ashbolt 2009) and FLA grazing is crucial for the rapid growth and pathogenicity of these bacteria (Buse and Ashbolt 2011, Thomas et al. 2011, Thomas et al. 2014). *Acanthamoeba* spp.*, Vermamoeba (Hartmannella)* spp. and *Naegleria* spp. are the most reported amoeba genera in treated water. A typical drinking water system may contain 0-100 amoebae/L of water and 70-2900 amoeba-ciliates/cm^2 of biofilms (Hoffmann and Michel 2001, Långmark et al. 2007).

1.5 Energy conservation, *Legionella* spp. and novel control approach

The recent move to support water conservation through implementation of green-building codes (energy-efficient buildings) impacts the physicochemical conditions of the building water (e.g., temperature, hydraulic retention time and residual disinfectant concentration) and thus affects biofilm formation and microbial community compositions of the water-biofilms. Low-flow fixtures lead to more prolonged stagnation of water and may cause loss of residual disinfectant, up to 144 times faster in energy-efficient buildings than in DWDS (Rhoads et al. 2016). Moreover, elevated levels of OPPP gene markers were also reported in energy-efficient building water. Pipe materials also influence the colonization of *L. pneumophila* (Buse et al. 2014a, Giao et al. 2015, Lu et al. 2014, Rozej et al. 2015, Wang et al. 2014). Within well-managed systems, the disinfection process or agents remain the same for years and use of the same disinfection procedure could cause a selection of a microbial community that favors the colonization and growth of *Legionella* spp. and similar opportunistic respiratory pathogens like

NTM. Moreover, the selection of beneficial FLA host or similar bacteria-eating eukaryotes could also help the persistence of *Legionella* spp. Chloramine has been reported to be effective in controlling *Legionella* spp. but also helps NTM persist in the bulk water as well as biofilms (Rhoads et al. 2017). The use of chlorine to make water potable was reported as early as in 1835 but to disinfect drinking water was started in 1890 (Akin et al. 1982, Nozaic 2004, White 1972). However, chlorine or other disinfectants like monochloramine may quickly dissipate in PP (Nguyen et al. 2011, Zhang and Edwards 2009). Chlorine is a strong oxidant and can react with common pipe materials like copper, PEX (cross-linked polyethylene), lead etc. (Edwards and Dudi 2004, Sarver et al. 2011, Whelton et al. 2011). The corrosion products such as CuO (produced in Cu pipes by reacting with chlorine) cause loss of residual disinfectants (Edwards et al. 1996, Hidmi and Edwards 1999, Lagos et al. 2001). Warm water (30-42 °C) along with localized corrosion products that minimize the disinfectant residuals (Rhoads et al. 2020) together may also favor FLA-*Legionella* interactions and intracellular growth of *Legionella* spp. One recent example was the water crisis in Flint, Michigan where the corrosive Flint River water dislodged pipe deposits, damaged the iron pipe that consumed residual chlorine and caused high concentrations of *L. pneumophila* in building water (Masten et al. 2016, Pieper et al. 2017). However, the biology of pathogenic *Legionella* spp. from microbial community perspectives within the biofilms/water environment is not well studied. Biofilm microbial diversity dynamics could be critical to understand the growth and interactions of *Legionella* spp. with other microorganisms (prokaryotes and eukaryotes) in EWS. A more fundamental understanding of the ecological interactions of pathogenic *Legionella* spp. would aid in identifying more sustainable and targeted monitoring and control strategies than are available today.

1.6 Current *Legionella* management and regulatory requirements:

Since water aerosols are the primary infection source causing legionellosis, controlling the dissemination and transmission of the causative bacteria in water is the key to prevent legionellosis cases. The current regulatory guidelines are mainly focused on the management of EWS using generic treatment options (high heat, a heavy dose of chemical disinfectants) to kill *Legionella* spp. or prevent its growth within these systems (ASHRAE 2015, WHO 2007). The focus of almost all guidelines is recommending actions to be taken after an outbreak occurs. Given the predominance of sporadic cases (>70%) (NASEM 2020), this reactive approach is unlikely to be fully successful. The risk of legionellosis broadly depends on three factors: ecological conditions for growth of pathogenic *Legionella* spp. in water systems, transmission of the bacteria within aerosols from the aquatic environment to humans, and an individuals' susceptibility. Therefore, to take effective measures to prevent the legionellosis, controls can be taken to minimize the impact of each factors (Figure 1.4).

Legionellosis is of serious concern in developed countries but is probably a global health concern, not yet fully recognized. However, the geographical distribution of legionellosis incidence is unknown due to the lack of adequate surveillance and the differences in priorities and knowledge levels for water-borne diseases in different countries. Therefore, the global disease burden associated with *Legionella* spp. is unknown. The current guidelines for monitoring water systems to control legionellosis are generally governed by public health regulatory authorities and also by some utility and professional associations. However, the integration or harmonization of the recommendations by different authorities is absent. The regulatory frameworks are also different from country to country and even within the country (NASEM 2020, Van Kenhove et al. 2019). The most common disagreements among the different

guidelines are the target concentrations of pathogenic *Legionella* spp. in water for different action plans and identification/enumeration methods, since the detection of *Legionella* spp. is not predictive of a disease outbreak and the relationships between the *Legionella* spp. concentrations in water and disease incidences has not been characterized (Stout et al. 2007). However, all the guidelines recommend some common control strategies like avoiding water stagnation, maintaining hot and cold-water systems at recommended temperature settings at all points and ensuring appropriate residual disinfectant levels (except the Netherlands, part of the Germany, Denmark, Switzerland and Austria where a residual disinfection level is not used) (Medema et al. 2013). In such cases, engineering controls are used to minimize growth of *Legionella* spp., but without aiming to achieve any target concentration. The control strategies for preventing transmission of the bacteria and awareness programs for vulnerable groups and service management (building management, assisted living services management and water utility management) about the disease have usually not been given sufficient priority and therefore have contributions in increased legionellosis cases.

The control of *Legionella* spp. and legionellosis in the US is of concern by four different public health organizations, the Center for Disease Control and Prevention (CDC), the Environmental Protection Agency (EPA), the Veterans Health Administration (VHA), and the Occupational Safety and Health Administration (OSHA). Different states, local government and ASHRAE (The American Society of Heating, Refrigerating and Air-Conditioning Engineers) also have their own recommendations for controlling legionellosis (Parr et al. 2015). Moreover, the ASHRAE Standard 188 is the first legally enforceable standard that requires building owners or operators to have preventive and reactive measures in place by addressing all aspects of controlling legionellosis outbreaks through a Hazard Analysis Critical Control Point (HACCP)

plan (ASHRAE 2015). The European Working Group for *Legionella* Infections (EWGLI) advises monitoring cooling-tower water systems for *legionellae*, with action limits (action levels of 10^3 to $>10^4$ cfu/L) for particular preventive maintenance and mitigation plan (EWGLI 2019). *Legionella*-specific water safety plans for drinking water distribution systems (Cunliffe et al. 2014) and buildings (WHO 2007) provide comprehensive advice on *Legionella* preventive maintenance and mitigation plan. Specific advice on *Legionella* spp. targets for drinking water have been adopted by Germany (10^4 cfu/100 mL), France (partial) (250-10^4 cfu/L) and the Netherlands (10^2 cfu/L) based on recommendations by EWGLI (Van Kenhove et al. 2019).

The water safety plan (WSP) approach would be the best management practice for controlling *Legionella* spp. in water systems since it aims to ensure water safety from the source to the point of use. There is scope for adopting quantitative microbial risk assessment (QMRA) targets for different water uses (Hamilton et al. 2019), but this approach has not been widely implemented within WSPs or other management plans. QMRA drinking water models primarily focus on enteric pathogens, without considering the regrowth potential of OPPPs and *Legionella*-FLA interactions in water systems. Current guidelines discuss FLA as an intracellular growth host for pathogenic *Legionella* spp. but none of them specify any monitoring guidelines or target concentrations for FLA.

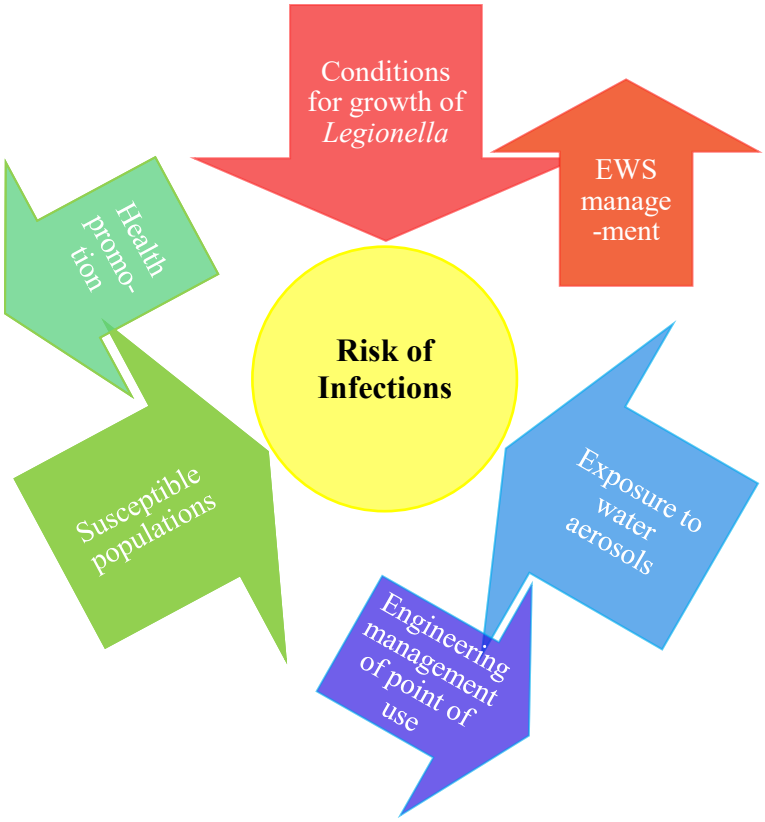

Figure 1.4: A schematic diagram of factors influencing the risk of getting an infection by *Legionella* spp. and approaches for controlling legionellosis in humans. Regrowth of pathogenic *Legionella* spp. is responsible for their high concentrations (respirable infection dose) in water; therefore, maintaining water systems unfavorable for the growth of *Legionella* spp. reduces the risk. Exposure through inhalation of *Legionella*-containing aerosols causes infection, thus reducing aerosol generation would also reduce the risk. Finally, empowering different stakeholders through awareness about the factors affecting exposure to *Legionella* spp. may also contribute to minimizing LD risk.

1.7 Knowledge gaps

Unlike enteric pathogens, regrowth of pathogenic *Legionella* spp. within EWS rather than contamination from external sources is the main cause of the high presence of these bacteria in water. *Legionella* spp. is considered to be a fastidious and slow-growing organism with specific nutritional requirements (iron and cysteine) and treated water is not a nutrient-rich environment;

therefore, it is unlikely that a rapid increase of *Legionella* spp. concentrations through planktonic growth within the DWDS and PP occurs. The intracellular growth within susceptible amoeba trophozoites is the most likely mode of replication that could support high concentrations of *Legionella* spp. in a relatively short period within a water system. However, the information regarding the events of interactions between pathogenic *Legionella* spp. and FLA within a multispecies water-biofilm environment is very limited. *Legionellae* spp. are also known to form viable but non-culturable (VBNC) cells and environmental stressors like drinking water disinfectants could induce their VBNC state (Alleron et al. 2008, Buse et al. 2013a, Vatansever and Türetgen 2015). VBNC *Legionella* cells are able to multiply intracellularly in specific host cells and have the potential to cause infection in humans (Steinert et al. 1997). The FLA-*Legionella* ratio should also be considered for initiating intracellular growth within FLA from a dose-response point of view. Therefore, the population dynamics of FLA and *Legionella* spp. are very important to determine the growth potentials of these bacteria. Different species of FLA have been isolated containing *Legionella* spp. or other bacteria from natural and EWS; however, the details of these relationships are still unknown. For instance, being a very small fraction of the bacterial biofilm communities, how likely a *Legionella* cell would be picked up by the FLA in the water-biofilms environment. Likewise, whether FLA have specific preference (inherent or *Legionella*-mediated) for any *Legionella* spp.

Therefore, studying the interactions of *L. pneumophila* with FLA and other water-biofilm bacteria would provide valuable information for developing sustainable and specific control strategies to prevent the growth of this bacterium in the aquatic environment and to ensure safe water within EWS. The effect of high concentrations of *L. pneumophila* (theoretical outbreak scenario) on the bacterial community of water biofilms will increase our knowledge about the

ecology of treated water systems, aid in developing a predictive model for possible outbreaks/sporadic cases (critical concentration of *Legionella* spp.) and may help us to find specific monitoring/control strategies to improve water system management.

1.8 Research questions, objectives and research justification

A comprehensive literature review and my initial research findings suggest several key questions that are needed to be answered to understand the growth and infection potentials of pathogenic *Legionella* spp. within the water systems in the built environment. *L. pneumophila* may only be present naturally in very low numbers in treated water and therefore, the ecological interactions (interactions with biotic and abiotic factors) toward reaching a high concentration of Legionella spp. would be important for its persistence and growth within PP and built aquatic environments and its infection potential. It is hypothesized that natural biofilms developed on different piping material act as suitable environments for FLA and L. pneumophila, but that these ecological biomes affect interactions between the FLA and L. pneumophila and for which certain conditions lead to rapid growth of Legionella spp. and associated shifts in the microbial population dynamics of water-biofilms that are problematic for human health. Since intracellular growth within susceptible FLA hosts appears the most likely mode for rapid multiplication, studying *Legionella* spp. and FLA together *in vivo* within water-biofilms is critical to understand the different stages of their interactions. Also, studying the effect of abiotic factors like the pipe materials, residual concentrations of disinfectants, water temperature, and flow dynamics on *Legionella* spp. and other microbial biofilm community members would help understand the ecological complexity of PP. Most of the current studies on FLA-*Legionella* interactions have been carried out with pure cultures in rich microbiological media without considering the real-

life conditions of DWDS or PP. Therefore, an in-depth study of the growth dynamics of *L. pneumophila* in simulated drinking water biofilm environments should provide fundamental insight to their functions in premise plumbing and could lead to the development of appropriate biological and engineering control for *L. pneumophila* and other similar water-based saprozoic opportunistic pathogens.

Therefore, the research objectives were to:

1. Evaluate the intracellular growth of *L. pneumophila* within amoeba trophozoites at different temperatures and determine the vesicle-bound *Legionella* cell concentrations for infection-dose potentials;

Different FLA have been reported to provide variable support for the intracellular growth of *L. pneumophila* at different temperatures (Buse and Ashbolt 2011) which could lead to problematic concentrations of the bacteria in PP (Schoen and Ashbolt 2011). FLA trophozoites produce vesicles to release unwanted materials or waste from cells and often these vesicles contain undigested/intracellularly grown bacteria. The aim is to study the vesicle formations by FLA during the intracellular growth of *L. pneumophila* in tap water at different temperatures and determine the public health significance of these vesicles from an infection dose potential of *L. pneumophila*.

2. Characterize the attachment, colonization of *L. pneumophila* and its interactions with different FLA strains within predeveloped natural tap water-biofilms;

Water-biofilms are the locations for most microbial activity in the drinking water systems (Prest et al. 2016), yet specific interactions of *L. pneumophila* and FLA within real-life drinking water

biofilms is poorly understood. If a low number of *Legionella* and FLA cells can be present in a water system, it is important to understand how the specific interactions occur between *Legionella* spp. and FLA in the presence of other water-biofilm bacteria to initiate the intracellular growth of *L. pneumophila* and how a sustainable and balanced ecosystem is maintained within the water systems. Preferential feeding (chemotactic attraction to food) of protozoa has been described (Dopheide et al. 2011), however, it is important to understand how FLA would behave in natural biofilm environments with *L. pneumophila* and which organism plays the major role in the internalization of *Legionella* cells within FLA trophozoites to initiate intracellular growth.

3. Characterization of the *L. pneumophila*- FLA interactions in the presence of other bacteria (known multispecies environment) in tap water;

A real-time observation of the interaction of *L. pneumophila* with FLA in the presence of known bacteria would provide detailed information on the preferential feeding behavior of FLA and the impact of the intracellular presence of *L. pneumophila* on other coexisting (within the same trophozoites or food vacuoles) bacteria.

4. Compare the microbial community compositions of tap water-biofilms grown on different pipe materials (Cu and PVC) at two different temperatures (simulating hot and cold-water pipe conditions during water stagnation) and determine the effect of high concentration of *L. pneumophila* on the microbial community;

Pipe materials have been found to affect biofilm formation, water-biofilm microbial community and pathogenic *Legionella* spp. colonization. High concentrations of *Legionella* spp. have been

usually found in PP associated with LD outbreaks. Therefore, determining the water-biofilm microbial community compositions and possible community shifts in response to a high concentration of *L. pneumophila* may lead to the development of alternative monitoring strategies or predictive models for vulnerable PP.

5. Determine the shifts in water-biofilm microbial population compositions and *L. pneumophila* colonization in response to upstream predeveloped natural water biofilms (sloughed-off from upstream pre-developed biofilms mimicking an introduction of biofilms from upstream to downstream in DWDS as a probiotic approach to control *Legionella* spp.).

All the current approaches for controlling *Legionella* spp. are general maintenance (not specific to *Legionella* spp.) of the water systems by flushing the system with either hot water or high doses of disinfectants in response to certain concentrations of *Legionella* spp. (generally after an outbreak or routine treatment of building with a history of high concentration of *Legionella* spp.). A proactive control strategy would be better for minimizing the legionellosis outbreaks and also could be more sustainable by limiting the unnecessary maintenance or use of chemical disinfectants.

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Chapter 2

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Chapter 2: Free-living amoebae supporting intracellular growth may produce vesicle-bound respirable doses of *Legionella* within drinking water systems

2.1 Abstract

Legionella pneumophila has recently been described as a major cause of water-related outbreaks in developed countries. In drinking water distribution and premise plumbing systems *Legionella* spp. grow within free-living amoeba hosts in biofilm. Exposure to *Legionella*-containing water aerosols generated during common water usage and in built-environments are responsible for causing legionellosis in humans. The study reported in this chapter demonstrated that the amoeba, *Willaertia magna* phagocytosed *L. pneumophila* in drinking water, supported intracellular growth and released *L. pneumophila* in vesicles of variable sizes before the amoeba trophozoites were completely lysed to release the *L. pneumophila* cells in the water environment. The vesicles produced in water at temperatures 22-40 °C varied in number and ranged from 3 to 20 µm in diameter, mostly falling into the respirable size. The respirable-size vesicles that have a diameter of 3 to 10 µm may contain between 23 to 873 *L. pneumophila* cells which could serve as a single human dose to initiate infection according to the current human dose-response information. This study suggested that current culture-based standard monitoring of drinking water for *L. pneumophila* would underestimate the true human health risk not only due to the inability to resolve viable but non-culturable bacterial cells but also due to underestimating the health impact of vesicle-bound *Legionella* cells. Thus, the regulatory guidelines for monitoring and controlling *L. pneumophila* should include amoebae in the surveillance of drinking water and introduce evidence-based strategies to control amoebae as an indirect and effective way of controlling legionellosis.

Keywords: Legionnaires' disease, *Legionella pneumophila*, free-living amoebae, drinking water systems, quantitative microbial risk assessment, and respirable infectious dose.

2.2 Introduction

Legionella spp. are Gram-negative bacteria that are generally present in natural and anthropogenic water environments (Atlas 1999, Fliermans et al. 1981). Recently the species, *L. pneumophila* has been brought to attention due to the high incidence of Legionnaires' disease (LD) outbreaks especially in developed countries, where drinking water systems are mostly well maintained and have good control over enteric pathogens (Beaute et al. 2013, Beer et al. 2015, Gargano et al. 2017). Legionnaires' disease is an acute pneumonia-like infection in humans with a high case-fatality rate, and caused by various *Legionella* spp. *Legionella pneumophila* is reported to be responsible for 80-85 % of the cases and two-thirds of these cases are caused by serogroups 1 and 6 (Yu et al. 2002). *Legionella* spp. also cause Pontiac fever, a self-limiting mild form of a flu-like infection in human, assumed to involve an endotoxin reaction (Castor et al. 2005, Neumeister et al. 1998). Exposures to *L. pneumophila*-containing water aerosols generated from common water uses, from building cooling-towers, decorative fountains and humidifiers are considered to be essential for initiating *L. pneumophila* infections in human lungs (Hines et al. 2014, Walser et al. 2014). Although *Legionella* spp. are common in natural and built environments, *L. pneumophila* in built environments are specifically identified with disease outbreaks (Ashbolt 2015, Craun et al. 2010, Fields et al. 2002). Colonization of environmental biofilms and intracellular growth in free-living amoebae (FLA) are considered to be the key factors for rapid growth of *L. pneumophila* to problematic concentrations in drinking water systems (Declerck 2010, Lau and Ashbolt 2009, Murga et al. 2001, Schoen and Ashbolt 2011).

No human dose-response information is available for *L. pneumophila*. However, using an aerosol infection model, the median infection dose of *L. pneumophila* for guinea pigs has been reported to be less than 129 bacterial cells (Armstrong and Haas 2007a, Berendt et al. 1980).

Guinea pig dose-response data has been used widely for quantitative microbial risk assessment (QMRA) modeling for *Legionella* spp. (Armstrong and Haas 2007b, Bouwknegt et al. 2013, Hamilton and Haas 2016). In the absence of specific dose-response data for humans, studies have considered 1-100 *L. pneumophila* cells deposited within the alveoli as being a likely dose to initiate infection (Armstrong and Haas 2008, Schoen and Ashbolt 2011). A reverse QMRA model analysis using 1 - 100 cells as an alveoli dose estimated that 3.5×10^{6} - 3.5×10^{8} *L. pneumophila* cells per litre of water may be required to cause infection during a typical showering event (Schoen and Ashbolt 2011). Retrospective investigations of many LD outbreaks reported a similar concentration of *L. pneumophila* in treated drinking water or cooling-tower water, along with occurrence in domestic showers (Collins et al. 2016), supporting the fact that a very high concentration of *L. pneumophila* in water maybe necessary for disease outbreaks.

Free-living amoebae are commonly reported in drinking water distribution systems and in premise plumbing worldwide (Delafont et al. 2013, Ovrutsky et al. 2013, Thomas et al. 2008), mostly identified as members of the genera *Acanthamoeba, Vermamoeba (Hartmannella), Naegleria* and *Balamuthia* (Coşkun et al. 2013, Delafont et al. 2013). Microbial community analysis of drinking water systems has also revealed the presence of intracellular bacterial species including pathogenic *L. pneumophila* within these amoeba hosts (Lienard et al. 2017). In addition to laboratory-grown amoeba strains, environmental isolates of amoeba from different water systems and natural hot springs have been found to support the intracellular growth of *L. pneumophila* (Amarouche-Yala et al. 2015, Cateau et al. 2014, Dupuy et al. 2016, Rasch et al. 2016). A theoretical calculation confirmed by flow-cytometric isolation of amoeba trophozoites, and culture-based enumeration of *L. pneumophila* indicated that a maximum of 1348 and 385 *L. pneumophila* cells could be produced within a trophozoite of *A. polyphaga* and *N. fowleri*

respectively, through intracellular growth (Buse and Ashbolt 2012). However, FLA and some other phagotrophic protists like ciliates produce and expel vesicles to release undigested food or toxic foreign particles to the surrounding environment (Hohl 1965). Bacteria like *L. pneumophila* that are able to grow intracellularly or able to at least resist digestion in amoeba trophozoites may also be released in vesicles (Berk et al. 1998). Hence, not only the planktonic *L. pneumophila* concentrations in water, its ratio to amoebae, viability states and intracellular and vesicle-bound concentrations in engineered water should be the necessary considerations when assessing potential human health risk.

Though FLA are ubiquitous within drinking water systems, most water and health regulatory authorities have not mentioned their impact on water quality, except for sporadic concerns over *N. fowleri* for causing brain infection (fatal brain-eating amoeba infection) (Cope et al. 2015, Morgan et al. 2016, Parr et al. 2015) and *Acanthamoeba* spp. for eye infections of contact lens wearers (Walochnik et al. 2015). Among the protozoa, only removal of oo/cysts of the parasitic members *Cryptosporidium* spp. and *Giardia* spp. are required by the most water treatment regulations e.g. in the USA (McTigue and Cornwell 2013). Recently, numerous organizations for health and environment are taking the rise of legionellosis outbreaks/cases very seriously and have developed control strategies for *L. pneumophila* in different built environment settings like healthcare facilities, and large building water supplies (Lindahl and Pearson 2015). Most of the regulatory documents emphasize the need for routine maintenance of water systems and the minimization of water stagnation within the critical growth temperature window (25-45 °C) (Boppe et al. 2016), with few recommending a limit for *L. pneumophila* in pipe water (ASHRAE 2015, WHO 2007). However, the standard method for enumeration of *Legionella* spp. is culture-based which is unable to resolve certain forms of these bacteria referred to as viable

but non-culturable states (Meier and Bendinger 2016). Although amoebae play a major role in *Legionella* spp. growth, almost all of the regulatory authorities have not included control strategies for amoebae. Only the *Guidelines for Managing Microbial Water Quality in Healthcare Facilities, Queensland, Australia* mentioned the need to control *A. polyphaga* and *N. fowleri* as human pathogens and to a lesser extent as a means of controlling *L. pneumophila* growth in drinking water (Cumming et al. 2013).

Therefore, it is critical to understand the impact of FLA on the growth of opportunistic pathogens like *L. pneumophila* in drinking water pipe environments. Here the vesicle formation during intracellular growth of a pathogenic *L. pneumophila* in *W. magna* was characterized by direct microscopy and image analysis, so as to illustrate how vesicle-bound *L. pneumophila* in drinking water could maximize the exposure to infectious *Legionella* cells if aerosolized. *W. magna* as a model FLA was chosen due to its close morphological and genetic homology to the more commonly identified *Naegleria* spp. (Robinson et al. 1989). Specifically, the size distribution of vesicles containing packed *L. pneumophila* cells released from trophozoites emphasized their potential public health impact from drinking water aerosol exposures.

2.3 Materials and methods

2.3.1 *L. pneumophila* culture

L. pneumophila Lp02 (ATCC®33152) harboring a plasmid containing Green fluorescent protein gene (Plasmid GFP) (from Ann Karen Brassinga, University of Manitoba, Canada) was grown on BCYE (Buffered Charcoal Yeast Extract) agar plates without antibiotics at 37 °C for four days. The cells were suspended in filtered-sterile tap water and washed with the same medium for three times. The number of *L. pneumophila* cells in tap-water suspension was

determined by culture on BCYE plates and cross-checked by comparing the number with the direct count of fluorescent cells obtained by microscopy with the aid of a hemocytometer.

2.3.2 *W. magna* culture

W. magna Z503 (ATCC®50035) was purchased from American Type Culture Collection (ATCC) and routinely grown in Serum Casein Glucose Yeast Extract Medium (SCGYEM) at 30 °C for 72 h in 25-cm^2 cell-culture flasks to obtain trophozoites. The trophozoites were washed three times with filtered-sterile tap water and re-suspended in sterile tap water to a concentration of approximately 1×10^5 trophozoites mL^{-1}.

2.3.3 *L. pneumophila* and *W. magna* co-culture

L. pneumophila cells and *W. magna* trophozoites were mixed at a ratio of 100:1 in filtered-sterile tap water. Six millilitres of the suspension were dispensed in each of nine 25-cm^2 cell-culture flasks (about 5.0×10^5 trophozoites per flask). Flasks were incubated in triplicate at room temperature (22±1 °C), 30 °C and 40 °C. The experiments were undertaken three times.

2.3.4 Fluorescence microscopy

L. pneumophila and *W. magna* co-cultures were observed at multiple time points (at 24, 48, 72 and 96 h of incubation) to check the intracellular growth of *L. pneumophila* in *W. magna* using the EVOS Cell Imaging Systems (ThermoFisher Scientific). Three random bright field and fluorescent images were taken from each 25-cm^2 cell-culture flasks of each temperature setting to determine the size distribution of the produced vesicles.

2.3.5 Image analysis and data processing

A total of 81 field-of-view images were processed using ImageJ software (version 1.50). The area of the *L. pneumophila* -containing food-vacuoles when appropriate and vesicles (fluorescent green) were calculated from each image by applying an appropriate threshold setup and filter. The area demarcation of the random vesicles and food-vacuoles by the software were cross-checked visually with the original images and appropriate settings were chosen to obtain the closest approximate calculated area for each green fluorescent vesicle. Assuming spherical shaped vesicles, their diameters were calculated from the corresponding areas obtained by processing the images with the ImageJ software.

2.3.6 Calculating the number of *L. pneumophila* in vesicles

Legionella spp. are rod-shaped bacteria and are reported to have a length of 1 to 2 μm (average 1.5 μm) and diameter of 0.3 to 0.9 μm (average 0.6 μm) (Diederen 2008). The estimated volume of each *Legionella* cell assuming cylindrical-shaped is 0.42 μm^3. The maximum random close packing density of spherocylindrical objects within a closed three-dimensional shape is about 0.70 (Williams and Philipse 2003). The random close-packing density provides a mathematical expression about how many solid objects of a particular size and shape could be accommodated in an empty closed boundary of another three-dimensional object. The number of *L. pneumophila* cells in each vesicle was estimated using the following formula:

$$\text{No. of } \textit{L. pneumophila} \text{ cell} = \frac{\text{Vesicle volume} \times \text{Random close-packing density}}{\textit{L. pneumophila} \text{ cell volume}}$$

2.4 Results

W. magna supported intracellular growth of pathogenic *L. pneumophila* Lp02 strain (with plasmid GFP) under the studied conditions. The trophozoites internalized *L. pneumophila* in drinking water at each temperature examined (22±1 °C, 30 °C and 40 °C) within 24 h of co-culture. *L. pneumophila* exhibited resistance to the host digestion and variable intracellular growth within the food-vacuoles at the studied temperatures. The newly replicated bacteria were released from the amoeba trophozoites either as free planktonic cells or in a package of variable sizes vesicles (Figure 2.1).

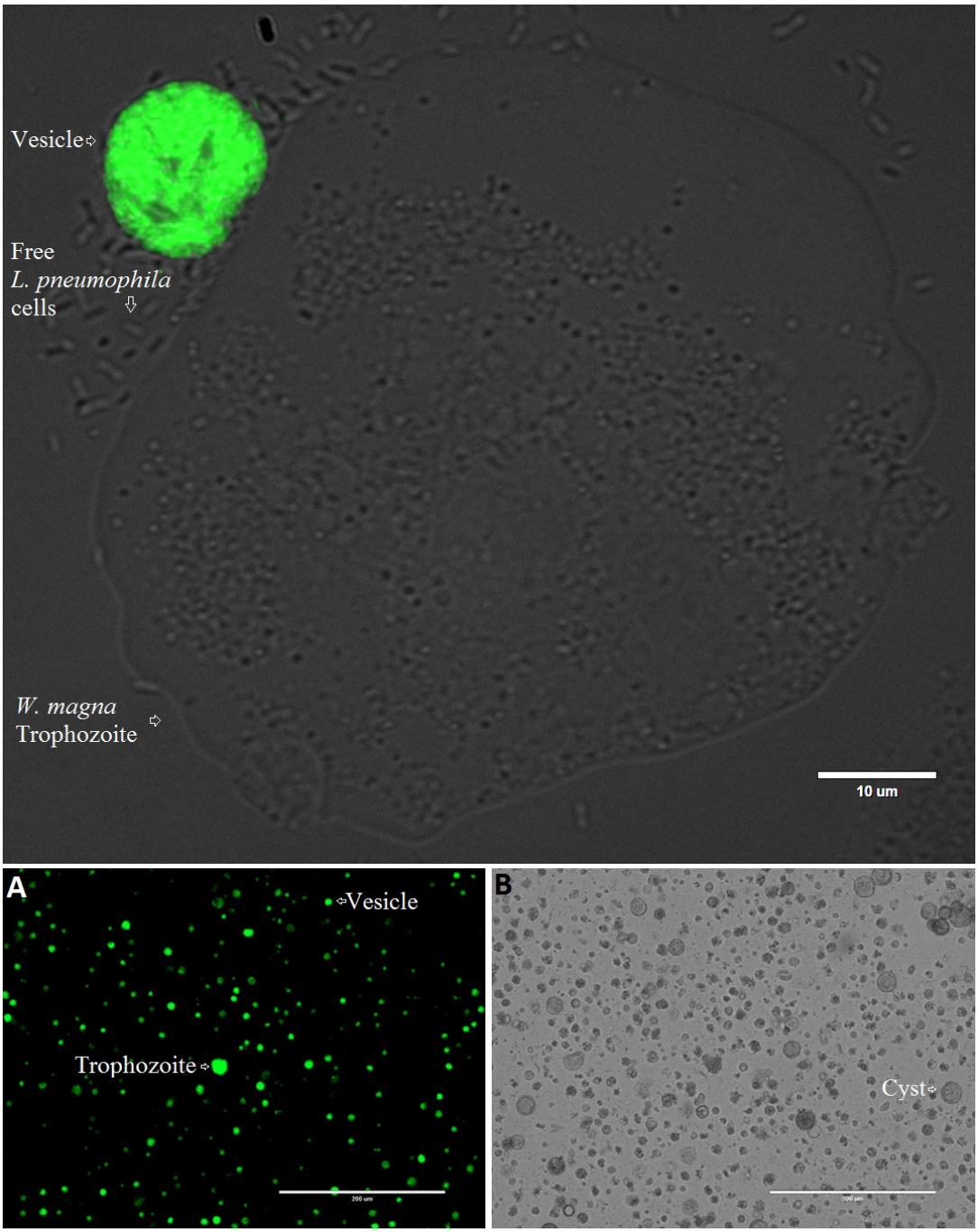

Figure 2.1: *L. pneumophila*-containing vesicle (green) releasing from a *W. magna* trophozoite (Scale bar 10 µm) (Top). Free *L. pneumophila* cells were also fluorescent but not visible because of weak fluorescence intensity. Images showing vesicles and trophozoites containing *L. pneumophila* (Scale bar 200 µm) (Bottom) (A. Green-Fluorescent, B. Bright-field image of the same frame).

Planktonic *L. pneumophila* cells were also fluorescent but not visible in the image because the intensity is much weaker compared to the cumulative fluorescence of the vesicle-bound cells. *Legionella* cells were observed to be mostly in pairs when they were released from the vesicles. Careful observation of the bright-field and epifluorescent microscopic images revealed that the *L. pneumophila* cells in vesicles and in vacuoles were tightly packed and were also in pairs or in very short chains (Figure 2.2). More vesicles were generated at 30 °C than at room temperature 22 °C (±1 °C) ($p = 0.02$) or 40 °C ($p = 0.001$). The vesicles containing *Legionella* were observed as early as 48 h after inoculation and at 96 h the co-cultures were found to have mostly *L. pneumophila*-containing vesicles with hardly any live trophozoites, at all the temperature settings. Initiation of the infection began with arresting the movement of amoeba trophozoites, with the physiological states of both the bacteria and the amoeba trophozoites appearing to influence the infection process.

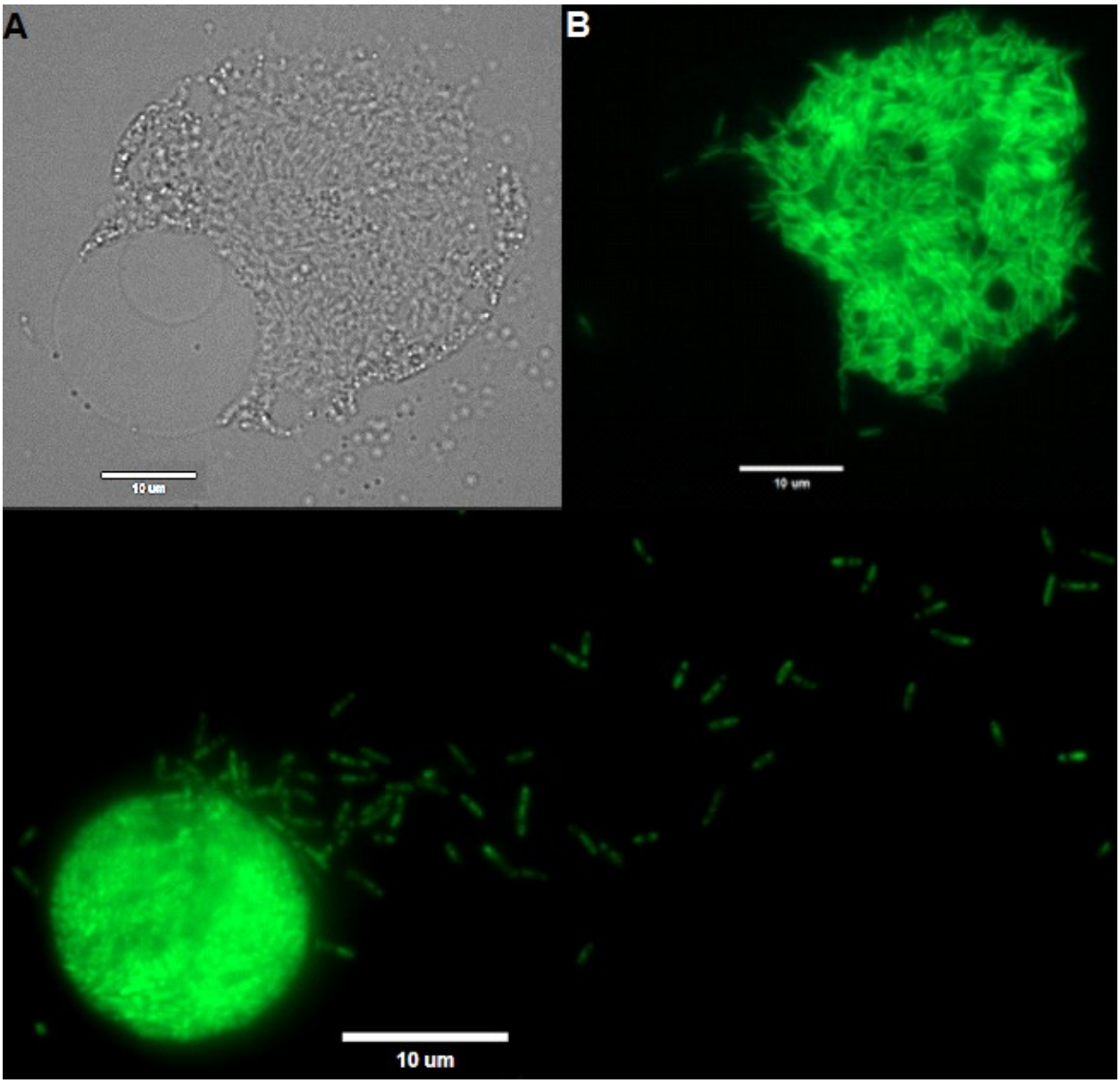

Figure 2.2: *Intracellular growth of* L. pneumophila *within a* W. magna *trophozoite. A. Bright-field image and B. Green-fluorescent image showing tightly packed* L. pneumophila *cells within an amoeba trophozoite.* L. pneumophila *cells are releasing from a free vesicle (Scale bar 10 µm) (Bottom).*

At 40 °C trophozoites appeared stressed and poorly supported intracellular growth of the bacteria. The average total number of vesicles calculated from nine random images of each

experimental trial, taken at 96 h of co-culture at room temperature, 30 °C and 40 °C were 973±177, 1288±113 and 297±185 respectively (Figure 2.3).

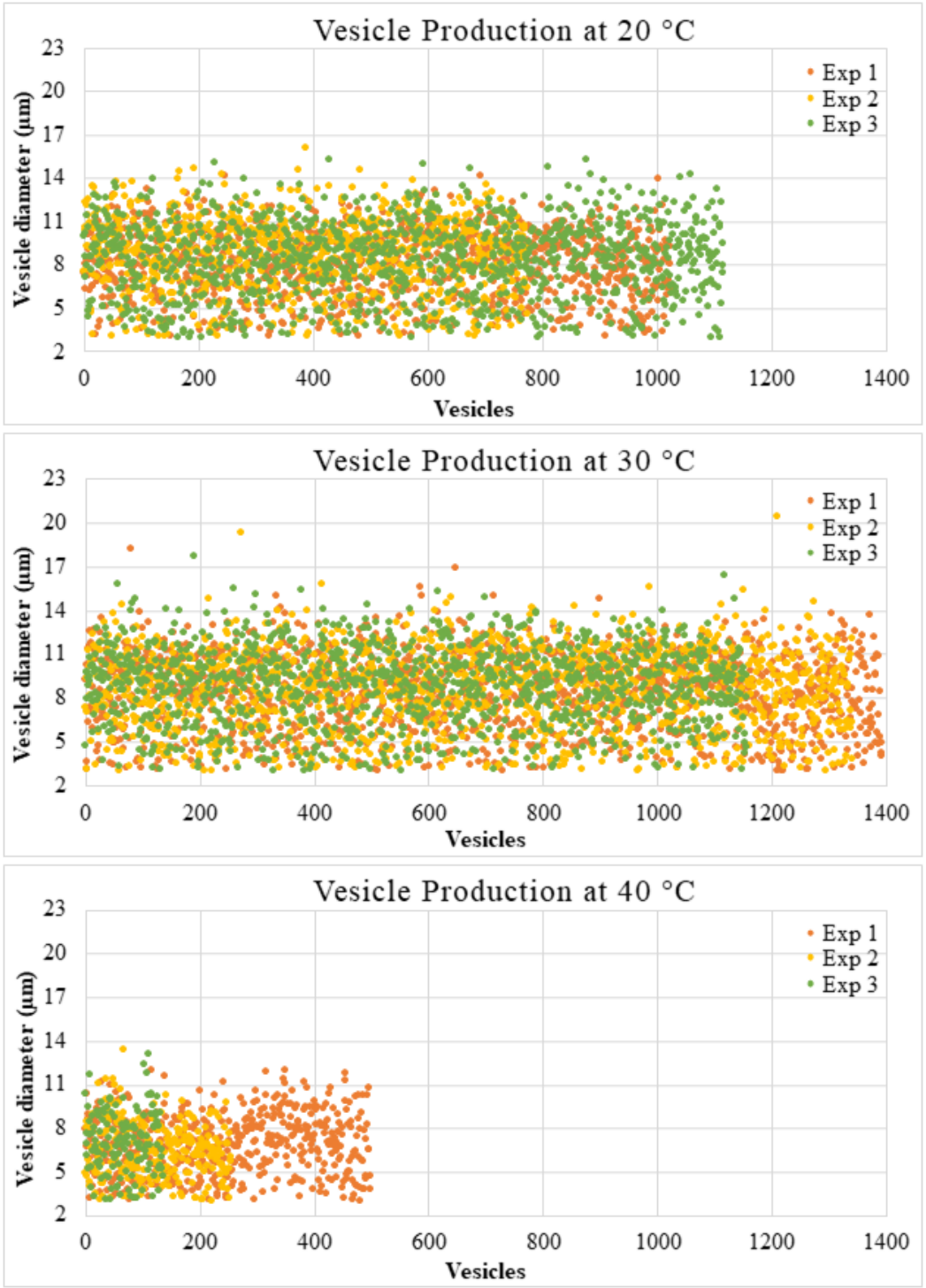

Figure 2.3: The number and size distribution of vesicles formed at different incubation temperatures. Three colors represent three trials of the experiment and each dot represents a vesicle. The number of vesicles was determined from nine random field-of-view images of each experimental replicate at each incubation temperature. The number of vesicles varies at different temperature but the size distribution was very much the same.

However, the vesicles formed at all three incubation temperatures ranged from 3 to 20 µm in diameter and mostly fell into the respirable-size (< 10 µm) range. The *L. pneumophila* containing vesicles formed at 40 °C were generally smaller than those formed at relatively lower temperatures. The average percentage of respirable-size vesicles at room temperature, 30 °C and 40 °C were 72.3±5.73, 68.1±4.52 and 94.2±2.74 respectively (Figure 2.4).

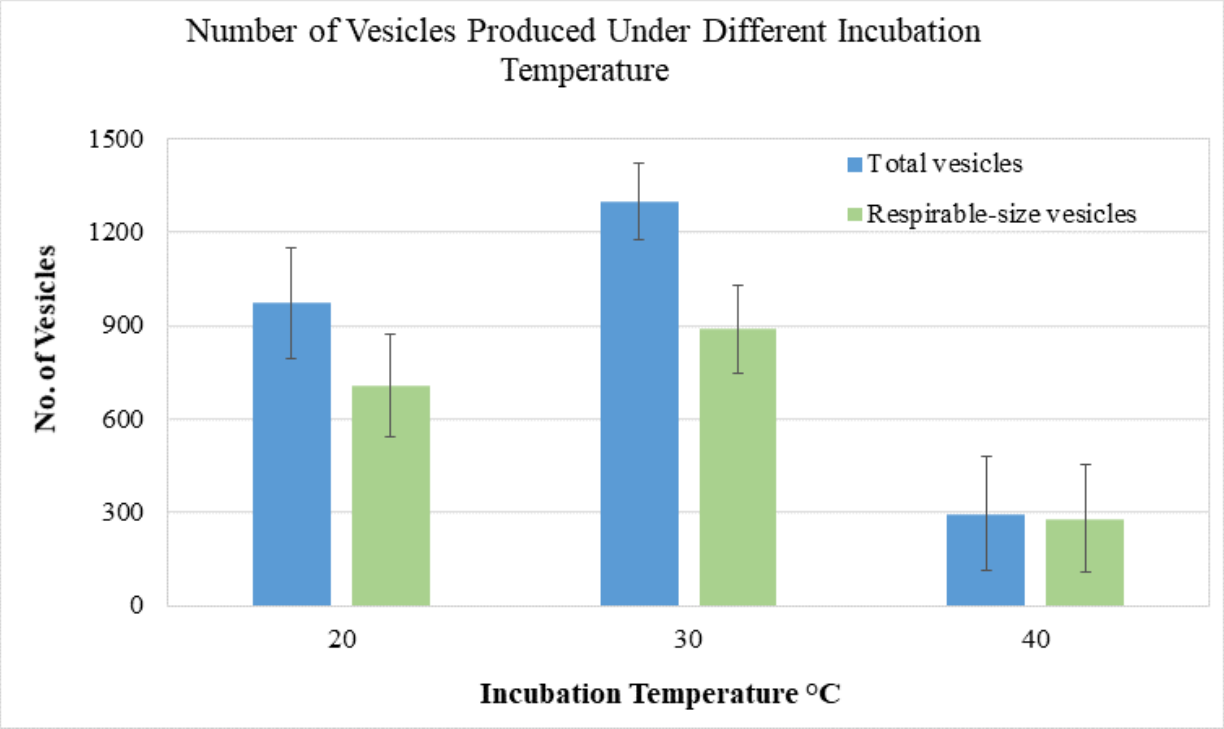

Figure 2.4: Total and respirable-size vesicles containing *L. pneumophila* produced at different temperatures. The error bars represent the mean standard deviation for three trials of the experiment.

Although the bacterial cells were tightly packed within the vesicles or vacuoles in amoeba trophozoites, they did not occupy the entire volume due to their random arrangement. Therefore, random close-packing density of spherocylindrical solid objects of similar length to diameter ratio of bacterial cell was taken into consideration to calculate a close approximate number of *L. pneumophila* cells in each vesicle. Vesicles with a diameter of 3 and 10 μm were estimated to contain up to 23 to 873 *L. pneumophila* cells respectively. Hence, large amoebae like, *W. magna* trophozoites (average diameter 70 μm) (Dey et al. 2009) have the potential to produce an astonishing number, 3×10^5 *L. pneumophila* cells when completely filled by the bacteria.

2.5 Discussion and conclusions

Drinking water is a very-low-nutrient medium for bacteria and other organisms to thrive, and most growth is assumed to occur within biofilms (Wingender and Flemming 2011). Naturally, in drinking water distribution systems and premise plumbing, microbial biofilm develops over time. Amoebae present in the drinking water are considered to graze on the bacterial biofilm components and amoeba-resisting bacteria like *Legionella* spp., and various non-tuberculous mycobacteria are able to prevent digestion by the amoebae (Thomas et al. 2008). *W. magna* supported intracellular growth of *L. pneumophila* in drinking water and produced vesicles of variable sizes containing *Legionella* cells, although many of the earlier studies could not demonstrate intracellular growth in water medium but in amoeba growth media (Newsome et al. 1985). The respirable-size vesicles are of great concern in terms of human health risk since these particles can reach deep down into the alveoli and therefore, have the potential to deposit the infectious bacterial cells directly to the site of infection. The respirable-

size vesicles may contain 23-873 *L. pneumophila* cells, which is within the likely median infectious dose, based on the available dose-response information (Armstrong and Haas 2007b, Hamilton and Haas 2016). Thus, a single vesicle aspirated deep into the alveoli might be enough to initiate an infection, which implies that water without having a high concentration of planktonic *Legionella* cells could have the potential to cause a LD outbreak if high concentrations of susceptible amoebae are present. Moreover, the growth of *L. pneumophila* in amoeba host cells enhances their stress resistance, virulence properties and invasion (Cirillo et al. 1994). Of particular concern is that current culture-based standard monitoring of drinking water for *L. pneumophila* would miss viable but non-culturable (VBNC) form of this bacterial cell (Al-Bana et al. 2014), but amoebae have the ability to transform VBNC cells to human infectious cells (Steinert et al. 1997). Furthermore, vesicle-bound cells may only produce a single colony on the culture plate as they are confined together within a vesicle. Hence, current monitoring methods likely underestimate the human health risk of *Legionella* from VBNC (Kirschner 2016)and vesicle-bound cells. Molecular techniques like qPCR, particularly the viability-qPCR method could be more appropriate in estimating the true health risk of waterborne *Legionella* spp. (Ditommaso et al. 2015) as it reduces the false risk alarm by not including the dead cells.

The vesicle membrane may also help *Legionella* spp. to survive through providing an additional barrier to protect it from harsh environmental factors like residual disinfectants maintained in water systems and desiccation during transmission in aerosols (Bouyer et al. 2007). Experimental evidence suggested that viable but non-culturable *Legionella* cells produced by heat treatment were only able to infect macrophages or alveolar epithelial cells if resuscitated within *A. polyphaga* (Epalle et al. 2015). Pathogenic *Legionella* spp. deploy the same virulence factors and infection strategies to grow within amoeba hosts as they do to destroy the human

innate immune system, macrophage cells and monocytes, which are the first line of defense to infection within human lungs (Escoll et al. 2013, Khweek and Amer 2010). Therefore, the vesicle-bound *L. pneumophila* may be more infectious due to the elevated level of expressed virulence factors resulting from intracellular growth in amoeba cells (Cirillo et al. 2002). The cluster of packaged bacterial cells may also provide a higher dose to macrophages and thus has a higher potential of initiating infection.

Therefore, the presence of amoebae in drinking water probably provides the environmental niche necessary to cause legionellosis. However, no drinking water quality regulatory agencies except the Australian National Water Quality Management Strategy (NHMRC and NRMMC 2011) discusses human pathogenic amoebae, but little about their control other than maintaining a chlorine residual. Our work adds weight to the suggestion that the amoeba concentration should be considered carefully as a tool for providing an early warning for a possible high concentration of *L. pneumophila* in drinking water distribution and premise plumbing systems (Codony et al. 2012). Due to the continuous increase in demand for water, water re-use is an inevitable choice for many parts of the world in the near future, if not for direct potable, at least for indirect potable uses like clothes washing, toilet flushing (Thomas and Ashbolt 2010). Therefore, more careful thought should be given to opportunistic pathogens and their intracellular growth supporting FLA and other protozoan hosts like ciliates (Berk and Garduño 2013), so as to communicate the human health risk from opportunistic pathogens in addition to the risk from pathogenic micro-eukaryotes.

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Chapter 3

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Chapter 3: Long-term persistence of *Legionella pneumophila* with free-living amoebae in drinking water biofilms

3.1 Abstract

Prolific growth of pathogenic *Legionella pneumophila* within drinking water distribution systems and premise plumbing, and human exposure to aerosols containing this bacterium results in the leading health burden of any water-related pathogen in developed regions. Ecologically, free-living amoebae (FLA) are an important group of the microbial community that influence biofilm bacterial diversity in the piped-water environment. Using fluorescent microscopy, the colonization of *L. pneumophila* in the presence of two water-related FLA species, *Willaertia magna* and *Acanthamoeba polyphaga* within drinking water biofilms was studied *in-situ*. During water flow as well as after periods of long-stagnation, the attachment and colonization of *L. pneumophila* to predeveloped water-biofilms was limited. Furthermore, *W. magna* and *A. polyphaga* showed no immediate interactions with *L. pneumophila* when introduced to the same natural biofilm environment. *A. polyphaga* encysted within 5-7 d after introduction to the tap-water biofilms and mostly persisted in cysts till the end of the study period (850 d). *W. magna* trophozoites, however, exhibited a time delay in feeding on *L. pneumophila* and were observed with internalized *Legionella* cells after 3 weeks from their introduction to till the end of the study period and supported putative (yet limited) intracellular growth. The culturable *L. pneumophila* in the bulk water was reduced by 2-log over two years at room temperature but increased (without a change in *mip* gene copies by qPCR) when the temperature was elevated to 40 °C within the same closed-loop tap-water system without any addition of nutrients or fresh water. The overall results suggest that *L. pneumophila* maintains an ecological balance with FLA within

the biofilm environment, and higher temperature improve the viability of *L. pneumophila* cells, and the intracellular growth of *Legionella* spp. is possibly cell-concentration dependent. Observing the preferential feeding behavior, it was hypothesized that an initial increase of FLA numbers through feeding on a range of other available bacteria could lead to a natural selection of *L. pneumophila*, and later "forced-feeding" of *Legionella* cells by the amoeba trophozoites resulting in rapid intracellular replication, and leading to problematic concentrations of *L. pneumophila* in drinking water. In order to find sustainable control options for legionellae and various other saprozoic, amoeba-resistant bacterial pathogens, this work emphasizes the need to better understand FLA feeding behavior and the range of ecological interactions impacting microbial population dynamics within engineered water systems.

Keywords: *Legionella* spp.; Free-living amoebae; biofilms; engineered water systems; premise plumbing

3.2 Introduction

Legionella pneumophila, a Gram-negative bacterium indigenous to natural and engineered water systems (EWS) (Atlas 1999, Orrison et al. 1981), has become the number one cause of drinking water-related disease outbreaks in many developed countries (Beaute et al. 2013, Beer et al. 2015, Gargano et al. 2017) and is ranked fourth highest by disease burden among the infectious diseases in Europe (Cassini et al. 2018). *Legionella pneumophila* causes Legionnaires' disease (LD), an acute pneumonia-like infection in humans with a high case-fatality rate (Edelstein 1993, Fraser et al. 1977), and also causes Pontiac fever, a rarely reported, self-limiting flu-like infection (Fields et al. 1990, Rowbotham 1980). According to the U.S. Centers for Disease Control and Prevention (CDC), *Legionella* spp. accounted for 57 % of reported outbreaks and all deaths associated with drinking water in the United States in 2013-14 (Benedict et al. 2017). However, the disease burden associated with *L. pneumophila* is considered higher, due to underreporting of Pontiac fever, for which the symptoms overlap with the common cold (Cunha et al. 2016, Garrison et al. 2014), and as the majority of cases of LD are sporadic and unreported (Che et al. 2008, England et al. 1981, Viasus et al. 2013). The source of exposures for most LD outbreaks has been reported to be EWS (Craun et al. 2010, Fields et al. 2002).

In natural and EWS, *Legionella* spp. coexists with other bacteria and microeukaryotes like free-living amoebae (FLA), ciliates and nematodes (Declerck et al. 2007, Koubar et al. 2011, Rasch et al. 2016, Taylor et al. 2009, van Heijnsbergen et al. 2015). Biofilms are considered to be the preferred niche where *Legionella* spp. interacts with other bacteria and potential eukaryotic hosts (Bryers and Characklis 1982). Micro-eukaryotes, including FLA feed on bacteria within aquatic/soil ecosystems (Parry 2004, Sherr et al. 1983) and bacterial adaptation

against protozoan predation (amoeba-resistant bacteria, ARB) (Greub and Raoult 2004) enables some of these bacteria to persist in the environment. This predator-prey interactions are thus considered responsible for increased environmental fitness of ARB like *Legionella* spp. (Cavalier-Smith 2002, Hahn and Höfle 2001) and shifting the environmental bacterial community towards higher ARB abundance (Raghupathi et al. 2017). *Legionella pneumophila* produces many effector proteins that help it to sustain and grow intracellularly within both amoebae and human macrophages (Segal and Shuman 1999) but their role in resisting phagocytosis have not been identified. Preferential bacterial feeding behavior of some aquatic protozoa has been studied (Amaro et al. 2015, Dopheide et al. 2011, Huws et al. 2005, Taylor et al. 2009), but it is not clear whether the same FLA species interact differently with *Legionella* spp. under different conditions (in the presence of other microorganisms within a water-biofilm environment). The most commonly reported FLA genera in EWS are *Acanthamoeba* spp.*, Vermamoeba (Hartmannella)* spp. and *Naegleria* spp. (Coşkun et al. 2013, Delafont et al. 2013), therefore this study focused on the interactions of *A. polyphaga* and *W. magna* (as a non-pathogenic species closely related to *N. fowleri* which is known to cause fatal meningoencephalitis in young children) (Robinson et al. 1989) with a pathogenic *L. pneumophila* strain, and its persistence within the tap-water biofilm environments. The positive correlation between the number of *L. pneumophila* and FLA in biofilm/sediment samples (Lu et al. 2015) suggested a close relationship between FLA and *L. pneumophila* in EWS. Intracellular growth in FLA provides both a rapid means of replication (Declerck et al. 2009, Murga et al. 2001) and seems responsible for the problematic concentrations of *L. pneumophila* that have been estimated to be about $3.5\times10^{6\text{-}8}$ cells$\cdot L^{-1}$ of water in premise plumbing (Schoen and Ashbolt 2011). FLA also provide protection from environmental stresses like water disinfectant products (Donlan et

al. 2005, Kilvington and Price 1990, Loret and Greub 2010, Storey et al. 2004, Thomas et al. 2004).

Bacterial species like *Pseudomonas aeruginosa* have been reported to interfere with *L. pneumophila*-FLA interactions, although the exact mechanisms are unknown (Declerck et al. 2005). *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* also interfere with the growth and persistence of *L. pneumophila* in biofilms (Kimura et al. 2009, Stewart et al. 2012). However, all these studies were carried out using pure culture or a mixture of two or three species but not in natural multispecies water-biofilms. The physical (e.g. flow rate, temperatures, etc.) and chemical factors (e.g. pipe materials, corrosion products and disinfectant residuals) also influence the persistence of *L. pneumophila* in EWS (Aggarwal et al. 2018, Buse et al. 2017, Dai et al. 2018a, Wang et al. 2012b). The last meters of pipe before the point of use are considered the most vulnerable regarding problematic growth of *Legionella* spp. due to periods of extended stagnation, warm temperature, low residual disinfection level and high organic carbon concentrations (Flemming et al. 2013). However, it is not clear how these conditions encourage this bacterium to grow intracellularly within FLA since these factors cannot directly support the planktonic or biofilm growth of this bacterium. Most often hot water pipes are identified as the site for the proliferation of these water-based pathogens, but it is the cold water that acts as a source of the bacterium and its FLA host (du Moulin et al. 1988). Yet the FLA-*Legionella* interactions within the water-biofilm environment are still largely unknown. Therefore, to understand the growth dynamics of *Legionella* spp. in EWS and to model risks, it is very important to know the details of the FLA-*Legionella* interactions within water-biofilms. The role FLA play in shaping the bacterial community and in supporting the intracellular growth of pathogens is currently a neglected factor in water regulatory guidelines and building codes

(ASHRAE 2015, WHO 2007), yet it is fundamental for estimating risk and for advising site-specific strategic management options for EWS. In this study, a water-flow system was used that did not simulate EWS, but rather satisfied some of the conditions like no residual chlorine level, relatively warmer temperature (22 to 40°C) and long stagnations that are considered to facilitate *Legionella* spp. growth and helped to observe FLA-*Legionella* in real-time within natural water-biofilms without interrupting the system (no staining for visualizing bacteria or amoebae, no isolation of biofilms). Fluorescent microscopy was used to observe long-term *in situ* interactions of pathogenic, green-fluorescent *L. pneumophila* with two common water-related FLA species, *W. magna* and *A. polyphaga* within tap-water grown biofilms. The main aims of this study were to identify the physical location of *L. pneumophila* (biofilm, planktonic or intracellular) and characterize the predatory interactions of FLA species with *L. pneumophila* within the natural drinking water biofilm environments.

3.3 Material and methods

3.3.1 Development of drinking water biofilms

A dual channel transmission flow-cell (each flow chamber acts as a viewing window and has two standard microscope glass coverslips, 22 mm × 60 mm with 2.3 mm space between them) (Biosurface Technologies Corporation, USA) was connected to a 20-L (HDPE Amber, VWR, USA) water reservoir with tubing (Cole-Parmer™ Masterflex™ Norprene™ 3/16" Black, Thermo Fisher Scientific) to allow the formation of natural water-biofilms (Figure S3.1A). Aged (to dissipate residual chlorine) drinking water was circulated at a rate of 0.5 mL·min^{-1} by a multi-channel peristaltic pump (Watson Marlow™ Peristaltic pump 205U/CA, 12 channel) through the flow-cell with an 8 h flow and 16 h stagnation cycle at room temperature (RT, about 22±1 °C)

for six months to obtain mature homogenous biofilms within the system. The source drinking water was collected from the municipal drinking water (mono-chloraminated) supply at the laboratory tap and stored at RT in a 20-L reservoir for 10-12 days to dissipate the residual chlorine, which was confirmed by analysis with a residual and total chlorine measuring kit (Hach free and total chlorine color disk test kit, USA). The flow-cell was kept in the dark and checked monthly by microscopy (EVOS Cell Imaging Systems, ThermoFisher Scientific) to observe biofilm formation *in-situ*.

3.3.2 Inoculating water-biofilms with *L. pneumophila*

L. pneumophila Lp02 (ATCC®33152) with a plasmid containing green fluorescence protein gene (GFP) (from Ann Karen Brassinga, University of Manitoba, Canada) was grown on BCYE (Buffered Charcoal Yeast Extract) agar plates without antibiotics at RT for 7-8 days (to avoid filamentous growth) (Piao et al. 2006). Several colonies were picked, re-suspended in filtered-sterile (Millipore Stericup® Filter unit, 0.22 µm) tap water (having no residual chlorine) and washed three times by centrifuging at 13000×*g* for 2 min, discarding the supernatant and re-suspending in the sterile tap water by vortex. The number of *L. pneumophila* in the final suspension was estimated by counting of the fluorescent cells with the aid of a haemocytometer (direct count) and confirmed by culture on BCYE plates, as described previously (Shaheen and Ashbolt 2018). The original *L. pneumophila* cell suspension along with the amoeba trophozoite suspensions (see method §3.3.3) were diluted with aged tap water (having no residual chlorine) in a 2 L-glass reservoir to have a final concentration of 10^5 cfu·mL^{-1} of the bacterium in the bulk water. The flow-cell with mature biofilms (6 months old) was disconnected from the 20 L-reservoir and connected to 2 L-reservoir with keeping all the tubing the same (Figure S3.1B).

The water containing *L. pneumophila* and amoebae was circulated as a closed-loop system at a rate of 0.5 mL·min^{-1} with the same flow regime (8 h flow and 16 h stagnation cycle) unless otherwise stated and the 2 L-reservoir was kept on a magnetic stirrer to avoid sedimentation of microorganisms. The water was recirculated till the study period (850 d) without adding any nutrient or fresh water to the system. The flow-cell was observed periodically (daily for the first month, weekly for the next three months and monthly for the rest of the study period) under the microscope (brightfield and fluorescence) to check for *L. pneumophila* adhesion to the pre-developed biofilm and interaction with amoeba trophozoites after stagnation periods *in-situ* (without disconnecting it from the water circulation system).

3.3.3 Introducing amoebae to water-biofilms

W. magna (ATCC®50035) was grown in Serum Casein Glucose Yeast Extract Medium (SCGYEM) and *A. polyphaga* (ATCC®30461) was grown in Peptone Yeast Extract Glucose (PYG) medium at 25 °C for 3 d in 25-cm^2 cell-culture flasks to provide a high yield of trophozoites. Trophozoites were harvested by centrifugation at 400×*g* for 10 min, washed three times with filtered-sterile tap-water (following the same procedure that was used for bacteria with the exception of centrifugation at 400×*g* for 10 min) and was re-suspended in sterile tap-water to a concentration of approximately 10^5 trophozoites·mL^{-1}. An equal number of each amoeba's trophozoites (20 mL of each amoeba suspension with a concentration 10^5 cfu·mL^{-1}) were introduced to 2 L-reservoir at the same time when *L. pneumophila* cells were introduced to make a final amoebae: *Legionella* ratio of about 1:50 (Concentration of *L. pneumophila* = 10^5 cfu·mL^{-1}, *W. magna* = 10^3 trophozoites·mL^{-1} and *A. polyphaga* = 10^3 trophozoites·mL^{-1} in a final water volume of 2 L). After introducing these organisms to the flow-cell system, the flow-regime

was interrupted to have variable stagnation periods (1 d, 3 d and 7 d of stagnation with 4 h of flow in between each stagnation period) to maximize the contact time for *L. pneumophila* to facilitate adherence to the pre-developed water-biofilms and also interaction with amoeba trophozoites.

3.3.4 Fluorescent microscopy and image processing

The flow-cell containing biofilms with amoebae and *L. pneumophila* was periodically (daily for the first month, weekly for next three months and monthly for the rest of the study period) observed by microscopy to check the amoebae-bacteria interactions and their physical locations (planktonic, biofilm and in food vacuoles of the trophozoites). Random bright field and fluorescent images and time-lapse videos of the flow-cell were taken throughout the study period. The images were further processed using ImageJ software (version 1.52g) (National Institutes of Health, US), if required.

3.3.5 Enumeration of *L. pneumophila*

Water samples were collected from the 2 L-reservoir bottle for three days immediately after introducing the *L. pneumophila*, for three consecutive days after the first month and daily (except the weekend) for the weeks mentioned in the supplementary Table 1. Samples were assayed immediately for *L. pneumophila* by culture and 10 mL water samples were filtered through a 0.22 µm filter (MicroFunnel™ Filter Funnels with Polycarbonate Membrane, Pall Corporation) and were stored at -80 °C for qPCR analysis. *Legionella pneumophila* were enumerated by direct plating (spread plate method) of 25 µL and 50 µL of water or appropriate dilutions to yield 30-300 colonies per plate on BCYE-PCV agar plate (BCYE agar with

Polymyxin B, Cycloheximide and Vancomycin) after incubation at 37 °C for 7 days. Since BCYE may support growth of other bacteria, to confirm legionellae, 60 random colonies of presumptive *Legionella* spp. were picked from 20 different BCYE plates of 10 different water samples (10 days) and checked for the presence of the *mip* (macrophage infectivity potentiator) gene by qPCR according to Mentasti et al. (Mentasti et al. 2015) (Appendix A: Supplementary method 1). The original inoculum strain of *L. pneumophila* (ATCC®33152) was used as a positive control and a pink colony-producing bacterium (*Methylorubrum* sp. NCBI Accession no. MK506269) co-isolated on the BCYE plate was tested as a negative control. Concentrations of *L. pneumophila* in the water samples were also estimated by quantifying *mip* gene copy number by qPCR and expressed as genome units (GU)·mL^{-1} (Mentasti et al. 2015). For molecular analysis, DNA was extracted from the filters (stored at -80 °C) following EPA Method 1611 (Appendix A: Supplementary method 2). The identification of the pink colonies was determined by 16S rRNA gene sequence analysis using a standard Sanger sequencing protocol (BigDye sequencing) with ReadyMade™ primers (Integrated DNA Technologies, Inc. Catalog# 51-01-19-06 and 51-01-19-07) and blasting to the NCBI database.

3.3.6 Recovery of amoebae from water

The amoebae from the bulk water were recovered on non-nutrient agar (NNA) plate with a lawn of live *E. coli* (One Shot® TOP10 Chemically Competent *E. coli*, Thermo Fisher Scientific, U.S.) (containing plasmid for Blue fluorescent protein gene provided by Prof. Robert Campbell, University of Alberta). Ten millilitres of water sampled directly from the 2 L-reservoir was centrifuged at 400 × *g*, and the pellet re-suspended in 100 µL of PAGE's saline (NaCl, 120 mg; $MgSO_4$, 4 mg; Na_2HPO_4, 142 mg; KH_2PO_4, 136 mg; $CaCl_2$, 4 mg; 1000 ml H_2O,

prepared by following instruction of ATCC Medium: 1323 PAGE's Amoeba Saline) and placed as 10 µL droplets on the NNA plate. The NNA plates were incubated at 30 °C for 5 d and observed by microscopy for amoeba trophozoites. Once spotted on NNA plate under a microscope, 100 µL of PAGE's saline was added on the amoeba trophozoites colony and collected back with a micropipette to a glass slide to enable further observation and identification of the trophozoites at higher magnifications by comparing the morphology with pure culture of the amoeba trophozoites and cysts.

3.3.7 Effect of temperature on *L. pneumophila* and amoebae in water-biofilms

The temperature of the entire flow-cell system with the water-reservoir was raised to 40 °C (±1 °C) from RT after 22 months by placing the entire system into an incubator maintained at 40 °C to observe the effects of a sudden change of the environmental condition. The flow regime was maintained the same and 11 mL (1 mL for culture + 10 mL for molecular identification) was collected every day for two weeks (Supplementary Table 3.1) to determine the total *L. pneumophila* by culture and qPCR (*mip* gene) and to observe the recovery of amoebae as described above. The flow-cell was also directly observed under a microscope (light and fluorescent) (without disconnecting it from the flow-system) for any changes in interactions of *L. pneumophila* and amoebae.

3.4 Results

3.4.1 Formation of water-biofilms and attachment of *L. pneumophila* to water-biofilms

Uniform, mature biofilms formation within the water flow system at RT was confirmed by observing the biofilms on the glass surfaces of the flow-cell (Figure 3.1). No FLA was

observed within the biofilms in the flow-cell during the first six months while running tap water only.

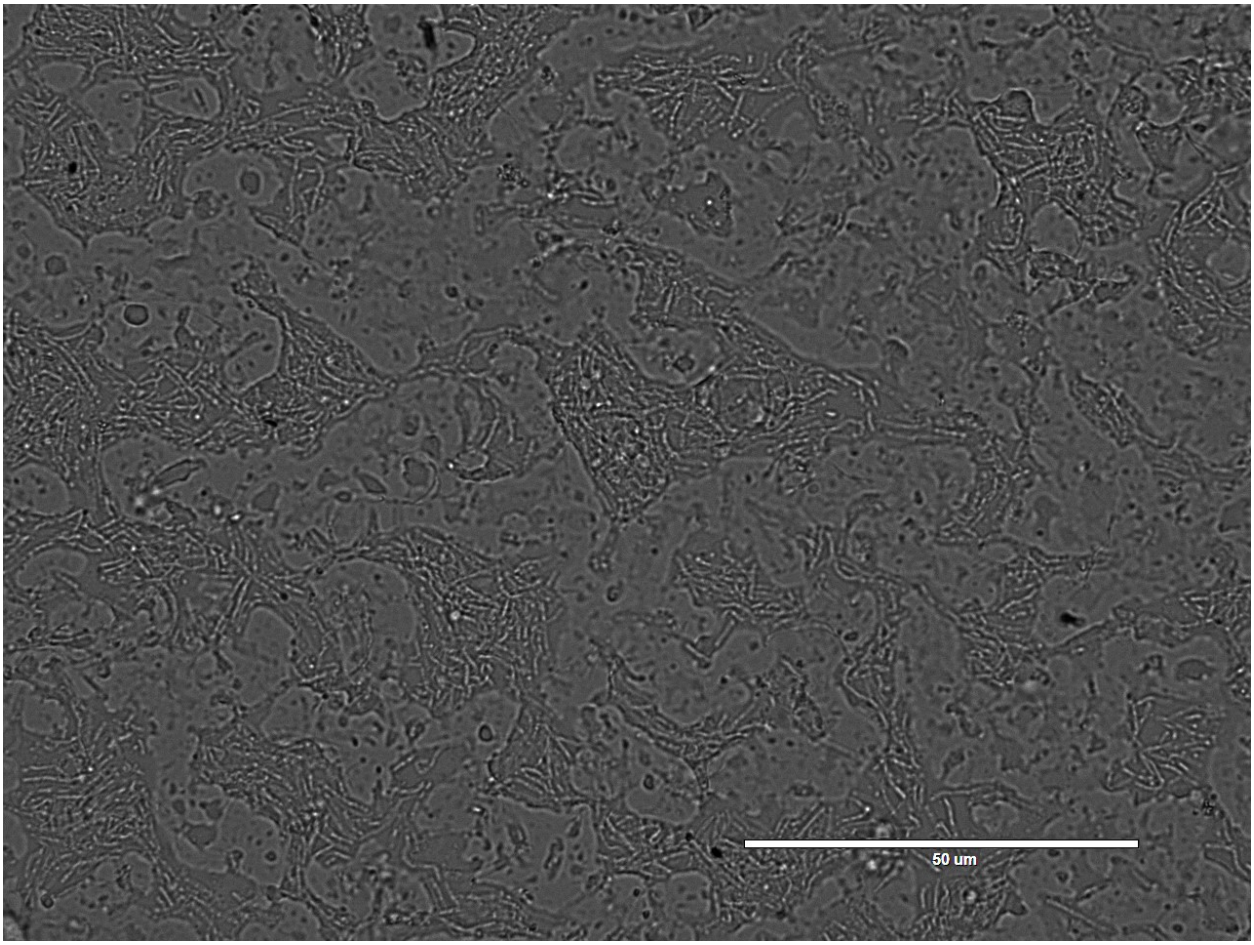

Figure 3.1: Near-surface image of the water-biofilms developed after six months on a glass surface in the flow-cells at RT by running aged tap water through the system.

Upon connecting the flow-cell to the 2 L glass reservoir (containing tap water with *L. pneumophila* and amoeba trophozoites inoculum), *L. pneumophila* cells were observed uniformly distributed on the pre-developed water-biofilms after stagnation periods. However, no strong adhesion to biofilm was observed for the *L. pneumophila* cells after 1 d, 3 d and 7 d of stagnation periods and in every instance, all the *L. pneumophila* cells that were observed within the biofilm during the stagnation period were washed away immediately when flow resumed. Only a few *L.*

pneumophila cells were adhered firmly to stay within the biofilm for a longer period during flow-condition (Figure S3.2 and S3.3). No micro-colony formation was observed by *L. pneumophila* during the entire study period (850 days). Free (planktonic) fluorescent *L. pneumophila* cells were drastically reduced (almost no cells) after a month following their introduction but the viable count on BCYE-PCV plates indicated their presence in high concentrations in the bulk water (3 days average 8.9×10^4 cfu·mL^{-1} that was close to the initial concentration of 1.07×10^5 cfu·mL^{-1}).

3.4.2 *L. pneumophila* - amoebae interactions in biofilms

Trophozoites and cysts of both *W. magna* and *A. polyphaga* were observed within biofilms under water-flow and stagnation conditions. The trophozoites and cysts observed in the system were the ones that were inoculated (based on the morphology and by the fact that no FLA was observed within the biofilms during its development and the presence of trophozoites within the biofilm in high number was observed only immediately after inoculating the two FLA species to the system) (Figure S3.4 and S3.5). *W. magna* were frequently observed moving freely on the surface of the biofilms (Figure 3.2A and S3.6). On the other hand, *A. polyphaga* appeared to be stressed and was mostly observed as rounded trophozoites and eventually as cysts within 5-7 days of its introduction. The flow-cell was observed every day for a month after 16 h of stagnation before resuming the flow, and no engulfment of *L. pneumophila* cells by the trophozoites was observed until the 20th day of their introduction (Figure 3.2B and S3.7). At this point *W. magna* trophozoites were observed to contain *L. pneumophila* cells within their food vacuoles with no indication of vigorous intracellular growth and amoeba trophozoites appeared healthy with no loss of mobility. In contrast, the same *L. pneumophila* strain when provided as a

pure culture with *W. magna*, grew intracellularly and caused loss of mobility of the amoeba trophozoites under laboratory co-culture conditions in sterile tap water at RT (Figure S3.8 and S3.9) (Shaheen and Ashbolt 2018).

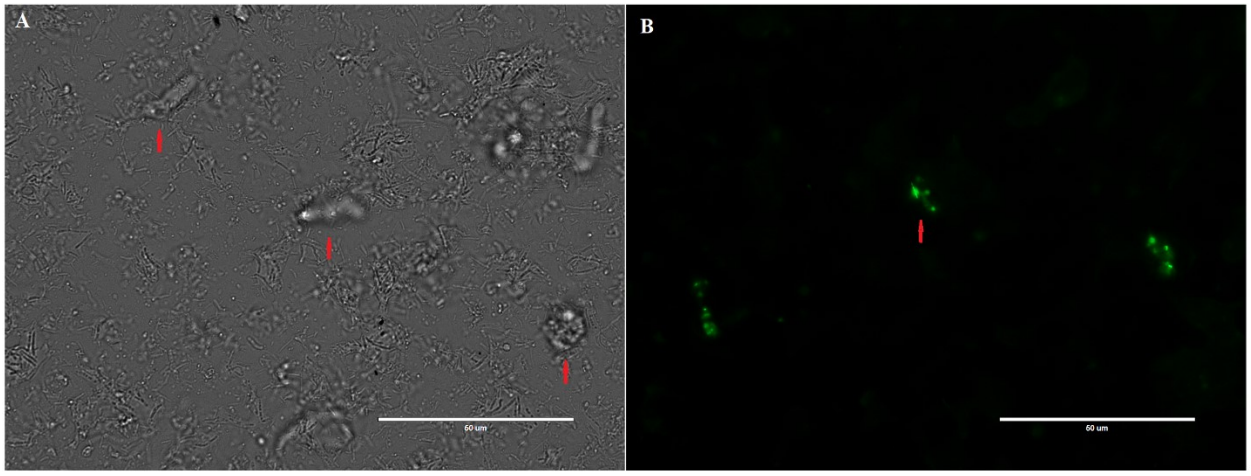

Figure 3.2: (A) *W. magna* trophozoites (Red arrows) within the drinking water biofilms at RT. (B) *W. magna* trophozoites containing *L. pneumophila* (Green) at RT after 20 days in the system (Right).

Putative intracellular growth of *L. pneumophila* and *Legionella*-containing vesicle formation in a few trophozoites (small green fluorescent patch with/ without visible trophozoite and no amoeboid movement) within the biofilms were also observed for a brief period immediately after the intracellular presence of *L. pneumophila* (Figure 3.3). The green fluorescent patch surrounded with no apparent membrane-like structure (Figure 3.3 right) could be a small cluster of *L. pneumophila* cells within a vesicle (or cells released in the biofilms from a vesicle) as also seen in pure culture of *Legionella-Willaertia* interactions (Figure S3.10) (Shaheen and Ashbolt 2018).

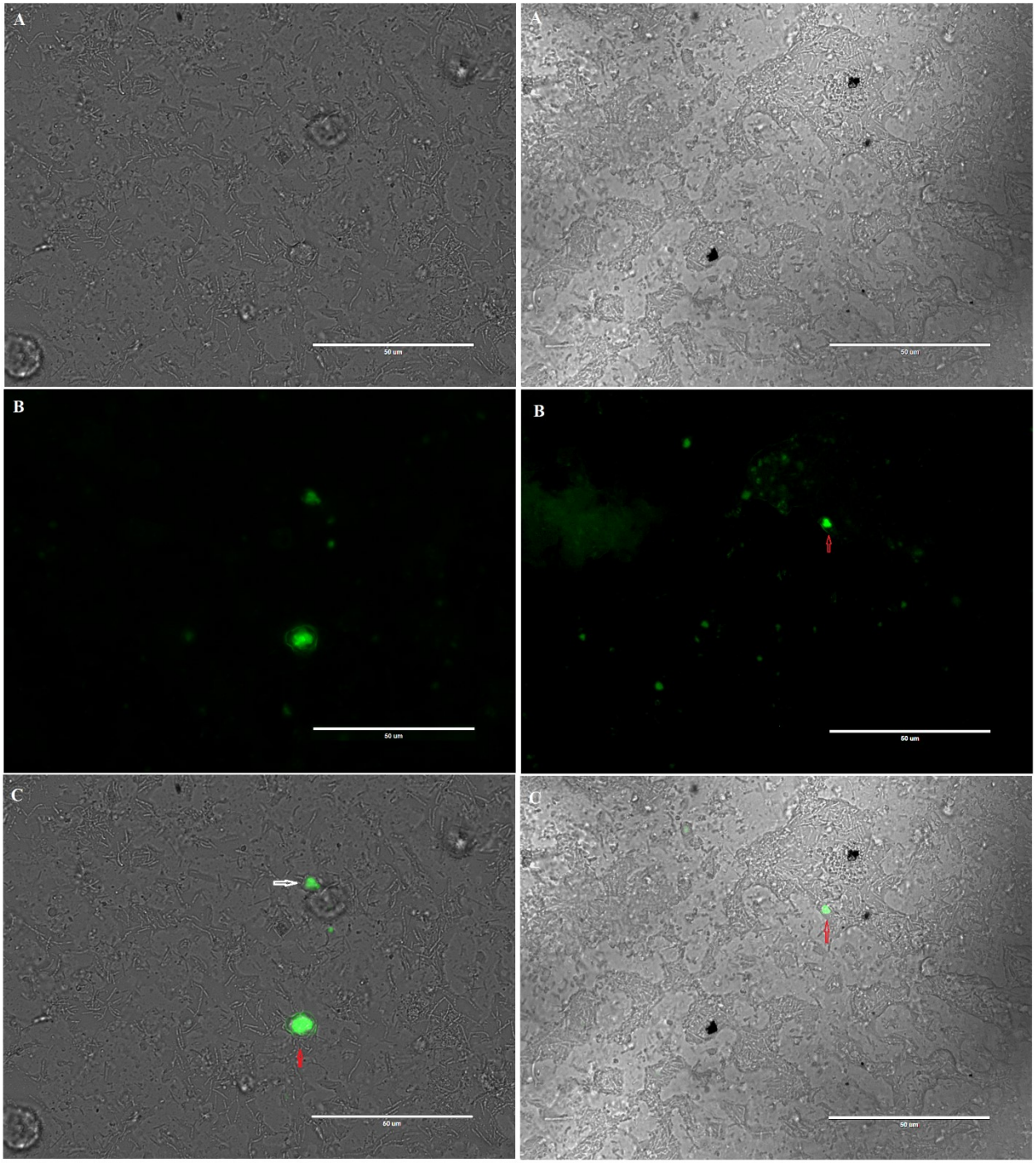

Figure 3.3: (Left panels) Intracellular growth of *L. pneumophila* within amoeba trophozoites (red arrow) and formation of vesicle containing *L. pneumophila* from an amoeba trophozoites within tap water biofilms (white arrow). (Right panels) A cluster of newly replicated *L. pneumophila* cells possibly released from amoeba or vesicles (Red Arrow). (A) Brightfield mono color image (B) Image under green fluorescent channel and (C) composite image of the two channels.

The size and number of *W. magna* trophozoites decreased over time, yet trophozoites containing fluorescent *L. pneumophila* cells were observed in the biofilms throughout the study period at RT (Figure S3.11 and S3.12) but not when the temperature of the system was increased to 40 °C.

3.4.3 Recovery of amoebae from the water

Both *A. polyphaga* and *W. magna* were always recoverable from the bulk water during the study period. The *E.* coli (Blue) within the food vacuoles of the amoeba trophozoites grown on NNA plates indicated active feeding of the amoebae (Figure 3.4). No GFP- *L. pneumophila* cell was observed in the food vacuoles of the recovered amoeba. Four weeks incubation of the entire water system at elevated temperature (40 °C) did not kill the amoebae, which also yielded trophozoites on NNA plates with an *E. coli* lawn. A high number of cysts were observed near the edge of the flow-cell where the velocity of the water could be lower than at the center of the flow-cell (Figure 3.4D).

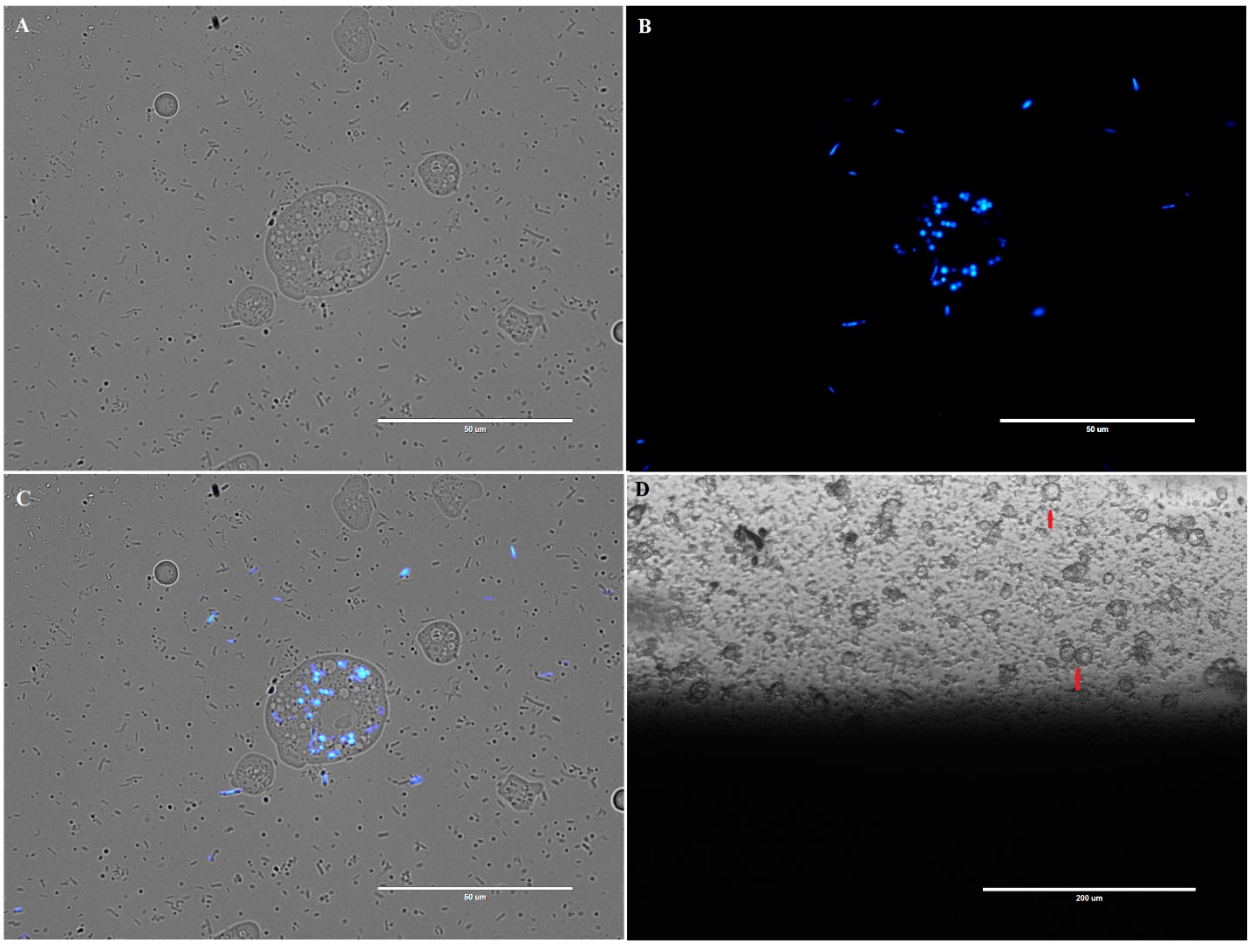

Figure 3.4: (A) Isolation of amoebae from the water samples of the flow-cell system on non-nutrient agar plates with a lawn of *E. coli* (Mono color brightfield image). (B) The amoeba (W. magna) trophozoites containing *E. coli* (blue fluorescent) demonstrating active feeding of bacteria by trophozoites (Image under blue channel). (C) Composite image of A and B. (D) Cysts (Red Arrow) accumulate near the edge of the flow-cell where the velocity of the water could be lower than at the center of the flow-cell (Left).

3.4.4 Enumeration of *L. pneumophila*

The number of culturable *L. pneumophila* was 1.07×10^{5} cfu·mL^{-1} (3 d average) in the bulk water, immediately after their introduction with amoeba trophozoites in the 2 L-reservoir

and was 8.9×10^4 cfu·mL^{-1} after the first month (3 d average). Interestingly, culturable *Legionella* spp. was observed even after 850 d from the time of introduction, with only about 2.0 log reduction in the total viable count (~1.5×10^3 cfu·mL^{-1}). Small variations in the culturable cell concentration of *Legionella* spp., determined continuously after 85 weeks of the study period indicated that the studied flow-system was in an ecological balance (Figure 3.5). However, incubation at elevated temperature (40 °C) disturbed the system and produced a significantly higher (about 4 times higher; $P<0.001$) culturable *Legionella* cell count compared to that at RT just prior to the upshift (two weeks plate count data of immediately before and after the temperature change was compared using t-test). When the temperature was lowered again to RT, the culturable legionellae count was reduced by 30 % ($P<0.001$) and remained in the range (~4.0×10^3 cfu·mL^{-1}) for the remaining 12 weeks of the study period.

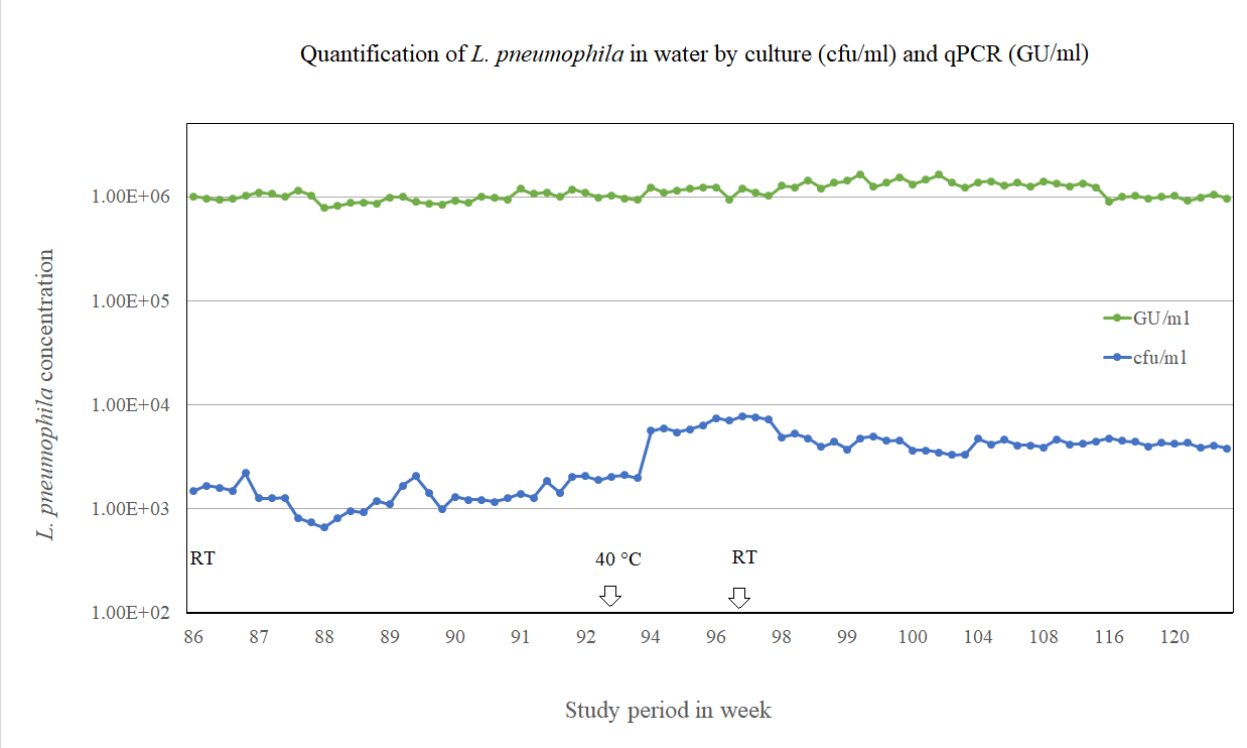

Figure 3.5: Concentrations of *L. pneumophila* in the flow-cell drinking water determined by culture and qPCR (mip gene) over the last 35 weeks of the study period. This long-term *L. pneumophila* cell count demonstrated a stable ecosystem with minimum changes in cell numbers. Sudden changes on the incubation temperature by increasing to 40 °C from room RT raised the culturable cell count without shifting the molecular quantification. This observation indicated that the bacteria could be more active/culturable at near optimum growth temperature. Decreasing the temperature again reduced the culturable counts indicated that lower temperature induced VBNC forms of the bacteria.

The randomly selected presumptive *Legionella* spp. colonies (non- fluorescent 60 colonies from 20 different BCYE plates representing ten days of water samples) were identified as *L. pneumophila* by qPCR assay for the *mip* gene. The ratio of the *L. pneumophila* concentrations obtained by direct quantification of the same water sample by molecular method (qPCR of *mip* gene) and culture method was supported by the linear relationship between $\log_{10}$ GU·mL^{-1} and $\log_{10}$ cfu·mL^{-1} reported for *L. pneumophila* in environmental water samples (Joly et al. 2006). In addition to the *L. pneumophila, Methylorubrum* spp. was consistently isolated from the water sample and grew with *L. pneumophila* on the BCYE-PCV agar plates. Even the two weeks incubation at 40 °C did not kill the *Methylorubrum* sp., which was consistently present in the water sample to the end of the study period.

3.5 Discussion

The glass-covered flow-cell provided a window to observe biofilm formation within the system in real-time and *in-situ*. The high concentration of fluorescent *L. pneumophila* and two

species of FLA trophozoites inoculated into the system enabled direct observation of FLA-*Legionella* interactions in real-time without using any staining protocol that could interfere with their behavior or damage cells. This study demonstrated that a sub-population of pathogenic *L. pneumophila* persisted as culturable cells in tap water/biofilms and within FLA trophozoites (intracellular) at RT for 850 days, which is the longest period ever reported for *Legionella* spp. to survive in drinking water. Pathogenic *Legionella* spp. has previously been reported to survive and remain viable for months to a year maximum in drinking water under laboratory conditions (Schofield 1985). However, there is very limited information available on how pathogenic *Legionella* spp. persists within a pre-developed competitive water-biofilm environment, especially in the presence of FLA (Taravaud et al. 2018, Wells et al. 2018).

Variable colonization of *Legionella* spp. to different plumbing materials exposed to sterile tap water inoculated with natural sludge containing an indigenous population of *Legionella* spp., as well as other bacteria and amoebae (from a Legionnaires' disease outbreak source) has been reported, however biofilms developed on the pipe materials after 24 h and after a month had very similar concentrations of *Legionella* (Rogers et al. 1994a, b). Colonization of *Legionella* spp. within known multi-species bacterial biofilms has also been reported in potable water, but the bacterial biofilm was developed with rich microbiological media (Murga et al. 2001). In contrast, any strong adherence of *L. pneumophila* to predeveloped natural drinking water-biofilms was not observed in this study.

Since pathogenic *L. pneumophila* are able to grow intracellularly in FLA and disperse in water as free cells or vesicle-bound cluster of cells (Dupuy et al. 2016, Fields et al. 1989, Shaheen and Ashbolt 2018), the current work suggested that the *L. pneumophila*-amoebae interactions may not be the same in a multi-species water biofilm environment (containing both

prokaryotes and eukaryotes). The time delay in feeding (phagocytosis) of *L. pneumophila* by *W. magna* provided an impression that given a choice, FLA may not prefer to feed on pathogenic *Legionella* spp. in water-biofilms. It raises a question if common water associated FLA are reluctant to feed on *L. pneumophila* when given a choice, how intracellular growth is initiated to reach the critical concentrations of the bacteria required to cause LD outbreaks (Ashbolt 2015, Bonadonna et al. 2009, Donohue et al. 2014, Hamilton and Haas 2016, Lu et al. 2015). The FLA-bacteria ecological interactions could be more complicated in true biofilm environments and an understanding of the FLA initial food selection process could help to predict prolific growth conditions for *Legionella* spp. and its control. Non-*Legionella* bacteria have been reported to have no effects on the uptake of *L. pneumophila* by two FLA species *A. castellanii* and *N. lovaniensis*, and the authors suggested highly specific and efficient uptake mechanisms of the amoeba species for *L. pneumophila* (Declerck et al. 2005). In contrast, our results indicate that biofilm bacterial community members may affect the FLA-bacteria interactions (could be general or FLA species-specific) and the selective feeding behavior of FLA may lead to the natural selection of amoeba-resisting human pathogens, thus changing the dynamics of drinking water-biofilm bacterial community compositions. This observation also indicates that bacterial feeding could be more actively controlled from the FLA side. Therefore, it appears that the predation by FLA in drinking water biofilms is very specific and environmental factors (such as availability of food, temperature) could manipulate this food selection and ultimately lead to *Legionella* internalization - the initial step of the intracellular growth.

The presence of viable, pathogenic *Legionella* spp. in the food vacuoles of trophozoites for more than two years suggested that a more mutualistic FLA-bacteria relationship is possible, which is supported by the report that amoebae harbor bacterial symbionts to resist the

intracellular growth of *Legionella* spp. (Maita et al. 2018, Okubo et al. 2018). The intracellular growth of *L. pneumophila* only in a few *W. magna* trophozoites also suggested that the intracellular growth could be concentration-dependent (quorum sensing or simply higher concentration of effector proteins of *Legionella* spp.) (Schell et al. 2014, Simon et al. 2015, Tiaden et al. 2007). Putative quorum-sensing gene clusters in *L. pneumophila*, homologous to *Vibrio cholerae*, have been reported to facilitate phagocytosis by protozoa and macrophages (Spirig et al. 2008, Tiaden et al. 2007) and modulate host cell migration (Simon et al. 2015). Thus, low concentration of *L. pneumophila* in amoeba trophozoites may turn the amoeba cell into a protective reservoir by impairing their digestion process. Also, the presence of fluorescent *L. pneumophila* within amoeba trophozoites indicate a common environmental pressure that forced the bacteria to maintain the GFP plasmid, since the free (planktonic) *L. pneumophila* cells became non-fluorescent in water-biofilms within a month from its introduction to the system. *L. pneumophila* strains with a chromosomal fluorescent protein gene may have been a better choice to observe their physical location in a long-term study. Still, the plasmid GFP provided an additional indication that the continuous stresses on intracellular *Legionella* cells by amoeba may be responsible for the higher environmental fitness of cells of amoeba origin. This observation also implies a balance between the cellular responses by the amoeba and intracellular *Legionella* to maintain their persistence in the water-biofilm environment.

The higher culturable *Legionella* cell counts with no significant change in *mip* gene copy numbers at 40 °C compared to the respective counts at RT suggested that low temperature might induce viable but non culturable (VBNC) forms of this bacterium, similar to other bacteria like *V. cholerae* that more likely become VBNC at 15 °C (Lutz et al. 2013). Exposure to a higher temperature (40 °C) also seemed to improve the overall fitness and viability of *L. pneumophila*

cells, as lowering the temperature back to the RT only resulted in a 30 % reduction in culturable cell counts. Since the studied system was a closed water-flow system (no addition of nutrient), the presence of amoeba in their trophozoite state and culturable *Legionella* cells after more than 850 days from their introduction emphasizes a nutrient recycling within the system and a balanced relationship between FLA and *Legionella* at RT.

Further research is still required to resolve FLA-*L. pneumophila* interactions better in more controlled environments with multiple known bacterial species to characterize the differential feeding behavior of FLA with regards to water-based respiratory pathogens. Nonetheless, it is apparent from the current study that FLA species may recognize extracellular chemicals and/or surface markers of virulent *L. pneumophila* and either do not feed on or expel the engulfed bacteria in vesicles so as not to allow for intracellular growth. Chemotaxis movement of amoebae toward certain bacterial cell lysate supports their selective behavior for food (Dopheide et al. 2011) however, when the system is homogenous, chemotaxis may be less effective. Immediate phagocytosis of *L. pneumophila,* when presented as pure culture to the studied FLA suggested a 'must-feed-on' condition when FLA have no other options but to feed on *Legionella* spp. (Shaheen and Ashbolt 2018).

Considering all the above, a modified model for hypothesized growth of *Legionella* spp. within the water-biofilm environments (Figure 3.6) is proposed and described that the conditions like high carbon concentration or sudden nutrient inflow, optimum growth temperature and low or no residual disinfectants increase biofilm formation that encourages FLA growth from selective feeding on non-pathogenic, preferred bacteria of the biofilm communities. This differential feeding by FLA could cause a localized selection of water-based amoeba-resistant pathogens, which increases the likelihood for a must-feed-on-*L. pneumophila* condition (by

increasing the relative abundance of *Legionella* spp.). The *Legionella*-amoeba ratio can also be increased during stagnation periods due to the settling of free-floating planktonic *Legionella* cells on biofilms (S3.1). Under this condition of 'force-feeding', high number of intracellular *L. pneumophila* accumulated within amoeba trophozoites, which may initiate explosive growth/lysis cycles leading to problematic concentrations of *L. pneumophila* released into bulk water.

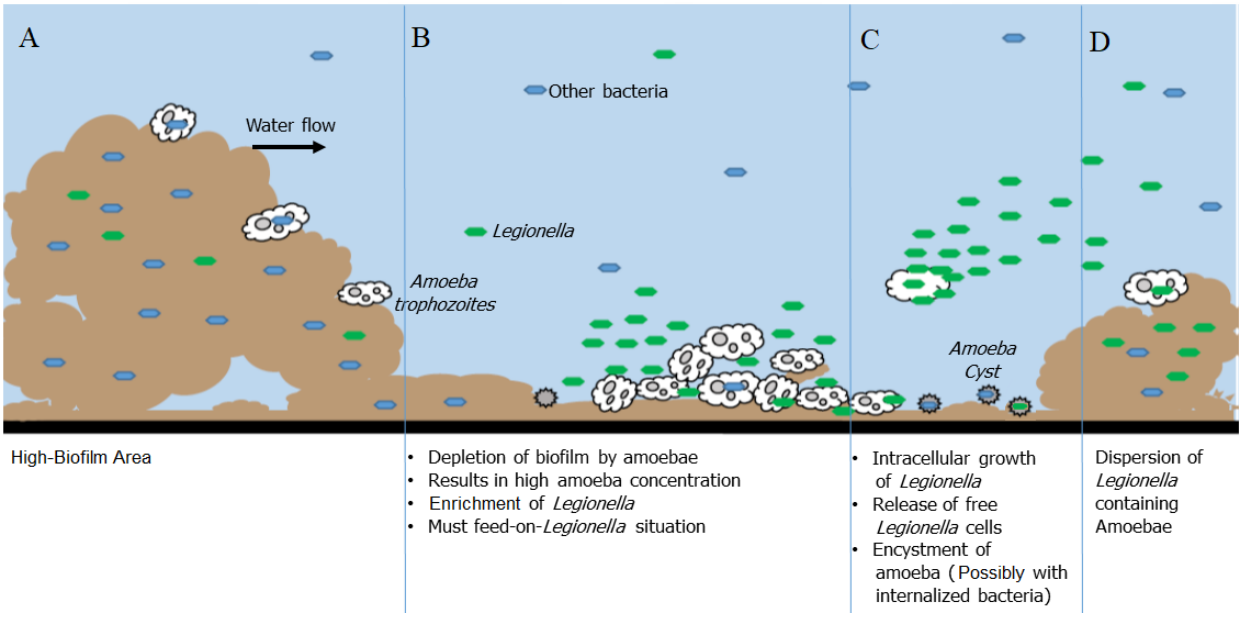

Figure 3.6: Hypothetical model for FLA-bacteria interaction in drinking water pipe environment for *Legionella*'s growth. Zone A: High biofilm formation (due to nutrients inflow or other reasons) encouraging FLA grazing and multiplication. Zone B: Depletion (diversity of food source) of biofilm by FLA grazing, an increase of FLA numbers and selective grazing leaving a must-feed-on-*Legionella* condition for FLA. Zone C: Force-feeding on pathogenic *Legionella* spp. may cause high concentration of *Legionella* cells through intracellular growth. Due to the nutrient limitation some trophozoites may form cyst with entrapped ARB in the cysts. Zone D: Dispersal of newly replicated pathogenic *Legionella* cells and amoeba cysts with/without internalized bacteria.

In the presence of comparatively less virulent bacteria, FLA's reluctance to graze on *L. pneumophila* indicates that a probiotic approach could be useful to control the intracellular growth of *L. pneumophila* in EWS. Ecological interactions such as competition, antagonism and obligate parasite-host relationships have been described for potential targets for probiotic control of opportunistic pathogens in premise plumbing systems (Wang et al. 2013). In fact, upstream microbiota has been described to have a profound effect on the downstream biofilm bacterial compositions (Lu et al. 2014, Pinto et al. 2012). Thus, understanding the ecology of *Legionella* spp. and its relationship with FLA could be key to identify a useful monitoring target for predicting the near-future growth-potential of *Legionella* spp. in order to develop its proactive control strategies. Overall, this study provided insight into how pathogenic *Legionella* spp. possibly behave in a multispecies water-biofilm environment and their growth dynamics.

3.6 Conclusions

L. pneumophila is a naturally occurring opportunistic human pathogen of major public health concern. Understanding its ecology, especially its interaction with FLA leading to intracellular growth, will not only improve human health risk estimation but should also aid the identification of effective, sustainable and site-specific control strategies and balanced resource management of EWS. Identifying the conditions for bacterial community shifts towards the enrichment of water-based pathogens driven through interacting with FLA could be a better predictor for the problematic concentration of *L. pneumophila*. The current approach for ensuring public health safety by monitoring *Legionella* spp. using a culture-based method is unable to determine the true concentration of infectious *Legionella* cells and by not considering FLA may be missing the critical conditions that promote *Legionella*'s growth. Thus, a more nuanced

understanding of drinking water microbial ecology may help to develop better water quality monitoring tools by identifying other targets, such as FLA, microbial population shifts and conditions favorable for an explosive growth of water-based pathogens.

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Chapter 4

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Chapter 4: Differential bacterial predation by free-living amoebae may result in blooms of *Legionella* spp. in drinking water systems

4.1 Abstract

Intracellular growth of pathogenic *Legionella* spp. in free-living amoebae (FLA) results in the critical concentrations that are problematic in engineered water systems (EWS). However, being amoeba-resistant bacteria (ARB), how *Legionella* spp. becomes internalized within FLA is still poorly understood. Using fluorescent microscopy, the preferential feeding behavior of three water-related FLA species, *Willaertia magna, Acanthamoeba polyphaga,* and *Vermamoeba vermiformis* regarding *Legionella pneumophila* and two *Escherichia coli* strains was investigated in real-time. Although all the studied FLA species supported intracellular growth of *L. pneumophila*, they avoided this bacterium to a certain degree in the presence of *E. coli* and mostly fed on it when the preferred bacterial food-sources were limited. Moreover, once *L. pneumophila* were intracellular, it inhibited digestion of co-occurring *E. coli* within the same trophozoites. Altogether, based on FLA–bacteria interactions and the shifts in microbial population dynamics, it is proposed that FLA's feeding preference leads to an initial growth of FLA and depletion of prey bacteria, thus increases the relative abundance of *Legionella* spp. and creates a "forced-feeding" condition facilitating the internalization of *L. pneumophila* into FLA to initiate the cycles of intracellular multiplication. These findings imply that monitoring of FLA levels in EWS could be useful in predicting possible imminent high occurrence of *Legionella* spp.

Keywords: Free-living amoebae, *Legionella* spp., engineered water systems, Legionnaires' disease

4.2 Introduction

Legionella pneumophila, a Gram-negative bacterium, indigenous to natural and engineered water systems (EWS) (Atlas 1999, Orrison et al. 1981), has become the number one cause of drinking water-related disease outbreaks in developed countries (Beaute et al. 2013, Beer et al. 2015, Gargano et al. 2017). Engineered water systems including building water systems and cooling towers, are often reported as the source of exposure to pathogenic *Legionella* spp. (Craun et al. 2010, Fields et al. 2002). *Legionella* spp. coexists in natural and EWS with other bacteria and microscopic eukaryotes like free-living amoebae (FLA), ciliates, and nematodes (Declerck et al. 2007, Koubar et al. 2011, Rasch et al. 2016, Taylor et al. 2009, van Heijnsbergen et al. 2015), as bacteria serve as a major source of food for FLA and other microeukaryotes (Parry 2004, Sherr et al. 1983). Growth of pathogenic *Legionella* spp. in EWS is considered to occur predominantly via intracellular growth within the susceptible FLA hosts (Declerck et al. 2009, Murga et al. 2001, Shaheen et al. 2019), to very high concentrations considered necessary for causing infections through aerosol exposures (Schoen and Ashbolt 2011).

Over millennia various bacteria that have developed mechanisms to protect against protozoan predation and digestion (König et al. 2017, Mou and Leung 2018, Okubo et al. 2018, Schmitz-Esser et al. 2010), and even to replicate within predatory host cells (Erken et al. 2011, Jousset 2012, Matz and Kjelleberg 2005). These bacteria are referred to as amoeba resistant bacteria (ARB). The increased environmental 'fitness' of *Legionella* spp. has been considered to have resulted from amoeba-bacteria interactions (Cavalier-Smith 2002, Hahn and Höfle 2001). About one-third of *L. pneumophila*'s genome encodes effector proteins that are required to prevent digestion and to grow intracellularly in amoebae and, coincidently, in human

macrophages (Gomez-Valero et al. 2019, Segal and Shuman 1999). *L. pneumophila* effector proteins are functionally redundant (presumably to deal with a wide range of predatory FLA), as elimination of one or many does not affect its overall pathogenic behavior (Al-Quadan et al. 2012, Luo 2011). However, the Type IV secretion system called the Dot/Icm system is essential for *L. pneumophila* pathogenicity, to resist digestion by amoebae and to replicate within various host cells (Segal et al. 1998, Vogel et al. 1998). However, the question is whether pathogenic *Legionella* spp. are effective in evading phagocytosis or whether FLA avoids phagocytosing pathogenic *Legionella* spp. in a multispecies aquatic environment, and what conditions make the FLA able to phagocytose *Legionella* spp. to enable them to grow intracellularly.

Protozoa, including FLA appear to have recognition mechanisms to choose particular food sources. While the preferential feeding behavior of some protozoa has been studied (Dopheide et al. 2011), there is limited information on these selection processes and feeding preferences of amoeba-resistant bacteria (ARB) in multi-species environments. It is apparent that preferential predation by amoebae would affect the biofilm microbial compositions and play an important role in shaping biofilm bacterial communities (Raghupathi et al. 2018), but the mechanisms and microbial dynamics are not well understood (Amaro et al. 2015, Dopheide et al. 2011, Huws et al. 2005, Taylor et al. 2009). Certain bacterial species like *Pseudomonas aeruginosa* has been reported to promote *L. pneumophila* uptake by amoeba hosts (Declerck et al. 2005). In contrast, amoeba-symbionts were presumed to resist intracellular growth of *L. pneumophila* (Okubo et al. 2018), although the mechanisms is unknown. *Pseudomonas aeruginosa* and *Klebsiella pneumonia* have also been reported to interfere with the growth and persistence of *L. pneumophila* in biofilms (Kimura et al. 2009, Stewart et al. 2012).

Low-level presence of FLA and *Legionella* spp. are expected in natural and EWS (Falkinham et al. 2015, Thomas and Ashbolt 2011) but it is unclear who (prey or predator) play the primary role in internalizing the *Legionella* cells in FLA. In addition, there is some uncertainty in how *Legionella* cells are picked-up by the amoeba trophozoites and whether the number of *Legionella* cells in the trophozoites is sufficient to initiate intracellular growth. Given the complex interactions of *L. pneumophila* with FLA in water-biofilms, fluorescent microscopy was used to observe *in situ* the interactions of three FLA species with *L. pneumophila* in the presence of two *E. coli* K12 strains to explore the microbial selection processes through amoeba-bacteria interactions. Using two very similar *E. coli* strains also help to determine the precision in this selection process. Using bacteria that express different fluorescent proteins (different colors) facilitated locating (intracellular/planktonic) the cells *in-situ* and in real-time. Overall, this work demonstrated how the predatory preference of FLA species may cause problematic concentrations of *L. pneumophila* in EWS.

4.3 Material and methods

4.3.1 *L. pneumophila* culture

L. pneumophila Lp02 (ATCC®33152) with a plasmid containing green fluorescence protein (GFP) (from Ann Karen Brassinga, University of Manitoba, Canada) was grown on BCYE (Buffered Charcoal Yeast Extract) agar plates without antibiotics at room temperature (RT, 22±1 °C) for 5-7 days (to avoid filamentous growth) (Piao et al. 2006). *L. pneumophila* cell suspension was prepared by following the procedure described previously (Shaheen and Ashbolt 2018). When appropriate, heat-killed *L. pneumophila* (GFP) cells were also used in co-culture experiments. The *L. pneumophila* (GFP) cell suspension in tap water was heated at 75 °C for 10

min in a heating-block to kill bacteria (confirmed by culturing on BCYE agar plate at 37 °C for 7 days).

4.3.2 *E. coli* culture

E. coli TOP10 (Invitrogen) cells were transformed with pBad-EBFP2 plasmid (provided by Prof. Robert E. Campbell, University of Alberta) to express a blue fluorescent protein. The other *E. coli* K-12 strain (MG 1655, genotype: F^-, λ^-, rph-1) contained the plasmid (pTV-mCherry) expressing a red fluorescent protein (provided by Dr. Tracy Raivio, University of Alberta). These two *E. coli* strains were grown on LB agar plates at 37 ºC for 24 h. Cell suspensions of each bacterium were prepared in filtered-sterile tap water and the cell concentration was estimated by checking the optical density at 600 nm and confirmed by culture method.

4.3.3 Amoebae culture

W. magna (ATCC®50035) was grown in Serum Casein Glucose Yeast Extract Medium (SCGYEM) at RT for 3 d in 25-cm^2 cell-culture flasks to obtain trophozoites. *A. polyphaga* (ATCC®30461) and *V. vermiformis* (ATCC®50237) were grown separately at RT in Peptone Yeast Extract Glucose (PYG) medium for 2 days in 25-cm^2 cell-culture flasks for trophozoites. The trophozoites of each amoeba species were harvested individually by centrifugation at 400×*g* for 10 min, washed three times with filtered-sterile, dechlorinated tap water (or PAGE's saline when appropriate) and re-suspended in the same medium to a concentration of approximately 10^5 trophozoites mL^{-1}.

4.3.4 Amoeba- bacteria co-culture

All bacterial strains were mixed together at equal concentrations and added to individual FLA species (and in mixed FLA species) to make a final ratio of bacteria:trophozoites of 300:1 in filtered-sterile tap water (or PAGE's saline when appropriate). *L. pneumophila* cells were also mixed separately with different FLA species trophozoites at a ratio of 100:1 in filtered-sterile tap water. Five milliliters of these bacteria-amoeba suspensions were dispensed in 25-cm^2 cell-culture flasks (about 4.0×10^5 trophozoites per flask). The mixed bacterial suspension was also dispensed in 25-cm^2 cell-culture flasks and diluted to 5 mL to have a final concentration of 4.0×10^7 cells of individual strains in sterile tap water in each flask (as an amoeba-negative control) to observe whether the different bacterial species have any effect on each other. Heat-killed *L. pneumophila* cells were added with viable *E. coli* cells in amoeba co-culture in a similar experimental setup. The experiments were undertaken at RT and in triplicate.

4.3.5 Fluorescent microscopy and image processing

The amoebae-bacteria co-cultures were observed at multiple time points (at 5 and 30 min, 24, 48, 72 and 96 h of incubation) to check the amoebae-bacteria interactions and physical locations (intracellular in amoeba trophozoites and extracellular outside the trophozoites in the medium) of different bacteria using an EVOS Cell Imaging Systems (ThermoFisher Scientific). When required (for microscopy and before antibiotic treatment), the co-cultures were washed with the filtered-sterile tap water, to reduce the planktonic bacterial cell number by gently changing the water without interrupting the surface-adhered amoeba trophozoites to observe the intracellular (food vacuoles) location of the bacteria better. Random bright field and fluorescent images were taken from each 25-cm^2 cell-culture flasks at different time points. The images were

further processed using ImageJ software (version 1.52e), if required and the number of trophozoites with and without internalized bacteria (different strains) in each field of view was counted.

4.3.6 Determining the intracellular bacteria

To enumerate the intracellular bacteria, only two bacterial strains (*L. pneumophila* and *E. coli* TOP10) were mixed together at equal concentrations and added to *W. magna* trophozoites to make a final ratio of bacteria:trophozoites of 200:1 in filtered-sterile tap water in 25-cm^2 cell-culture flasks and incubated at RT. At different time points (0.5 h, 24 h, 48 h and 96 h) of the co-culture, the medium was aspirated gently from the flasks (in duplicate) and replaced once with 3 ml of sterile water to reduce the planktonic bacterial cell number (as much as possible without disturbing the adhered trophozoites to the flask bottom). Three millilitres of filtered-sterile tap water containing gentamicin (200 μg/mL) were added to the cell-culture flask containing amoeba trophozoites with mostly internalized bacteria and incubate at RT for 1 h to kill the remaining planktonic bacterial cells. The trophozoites were harvested from the flasks after 1 h and re-suspended in 1.5 mL water in 2-mL tubes. The trophozoites were washed three times by centrifuging at 2000 × *g* and re-suspending in water. Finally, the trophozoites were lysed by passing through (back and forth) a 23-gauge needle five times to release the internalized bacteria. Appropriate dilutions of these cell suspensions were plated (spread plate technique) on BCYE-PCV plate and LB agar plate to determine the number of *L. pneumophila* and *E. coli* TOP10 cells, respectively. The plates were incubated at 37 °C overnight for *E. coli* TOP10 and 5 days for *L. pneumophila.*

4.3.7 Statistical analysis

The number of trophozoites were counted from 12 random fields of view images taken under different fluorescent and bright field channels for visualizing internalized bacteria (studied bacteria produce green, red and blue fluorescent proteins). A Student's t-test was carried out to compare the feeding preference of the amoeba trophozoites for different bacterial strains.

4.4 Results

4.4.1 Differential feeding preference of *W. magna*

Large number of *E. coli* cells were found accumulated in the food vacuoles of *W. magna* trophozoites as early as five minutes of co-culturing them in sterile tap water at RT. Even though *W. magna* fed on both the *E. coli* strains, there was a clear preference (visual observation of microscopic images) for *E. coli* TOP10 cells over *E. coli* MG1655 (as determined by the apparent number of food vacuoles containing bacteria and the intensity of fluorescence) (Figure 4.1).

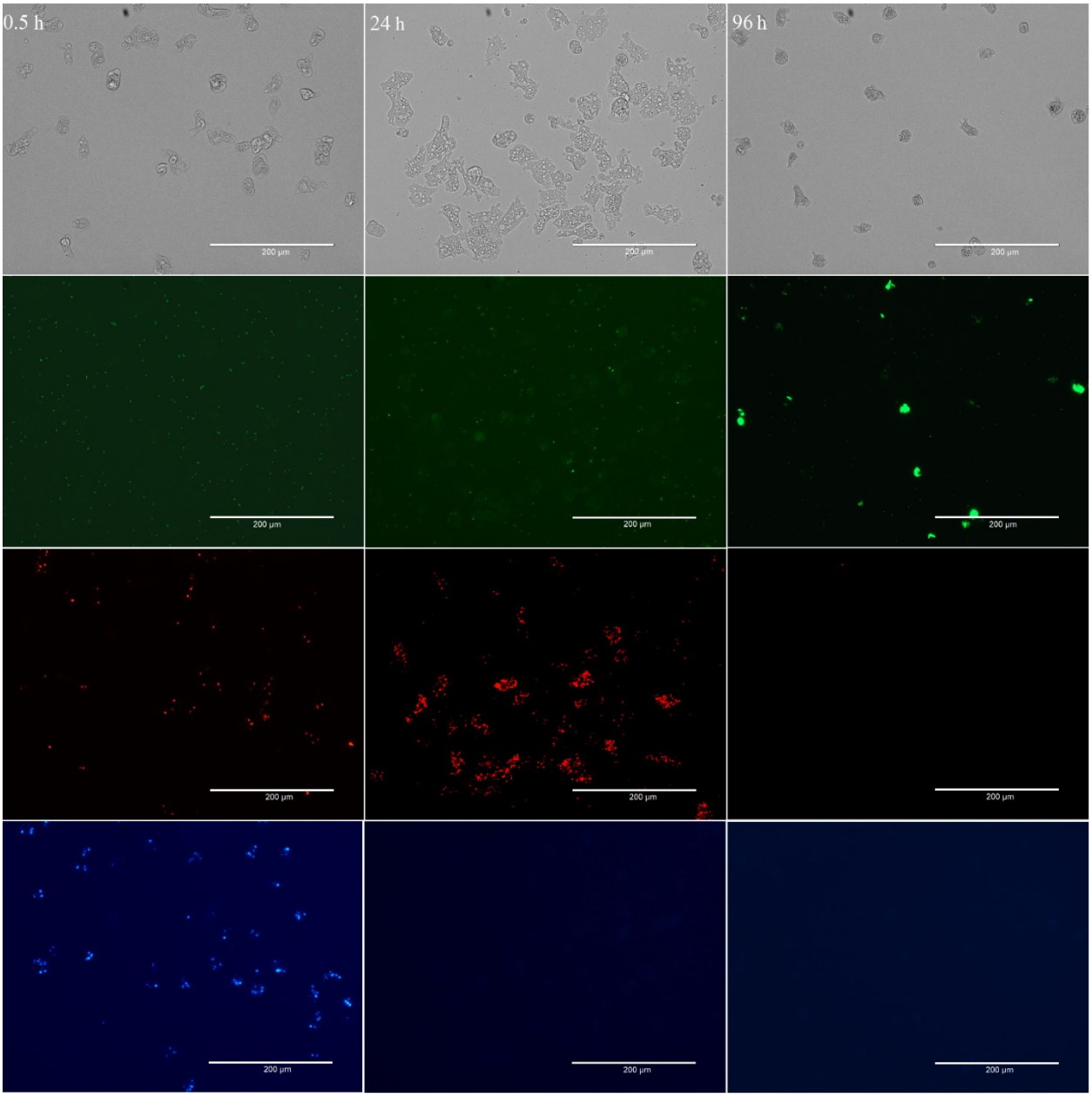

Figure 4.1: Preferential feeding on bacteria by *W. magna* at RT at different time points (0.5, 24 and 96 h) of co-culture. The four series of horizontal images (from top to bottom) represent the same field of view under different fluorescent light channels, Mono-color transmission light channel, Green fluorescent channel to observe GFP-*L. pneumophila*, Texas-Red channel for mCherry-*E. coli* MG1655, and DAPI channel for BFP-*E. coli* TOP10 respectively. The clusters of color dots in images indicate the presence of different intracellular bacteria in the food vacuoles of *W. magna* trophozoites. The scattered color dots (smaller in size) indicate planktonic bacterial cells in the medium.

After 0.5 h of co-incubation, 87.1 ±9.0 % of the total *W. magna* trophozoites contained *E. coli* TOP10 cells, 69.7 ±9.0 % *E. coli* MG1655 and none contained *L. pneumophila*, despite being present in equally high numbers in close proximity to the amoeba trophozoites (Figure 4.2). Although both the *E. coli* strains were present in the same trophozoites, the number of food vacuoles containing *E. coli* TOP10 cells was much higher than that contained the *E. coli* MG1655 cells.

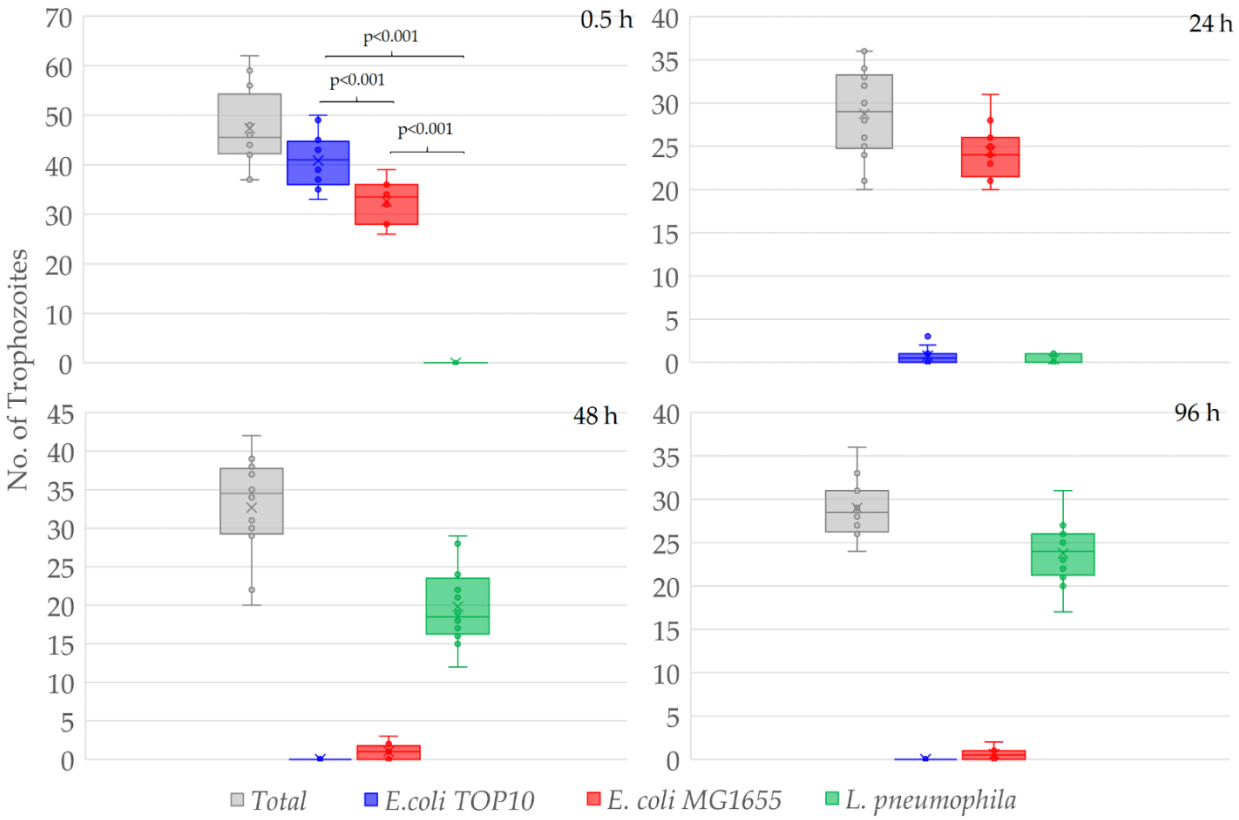

Figure 4.2: The number of *W. magna* trophozoites containing *E. coli* TOP10, *E. coli* MG1655 and *L. pneumophila* cells at different time points of co-culture. The number of trophozoites were counted from four fields of view of three replicates (total 48 images). The Whiskers represent standard deviations of the mean, the box represents 25 to 75 percentiles of the data and the horizontal line within the box represents the median. The t-test indicates significant differences in prey preference ($p < 0.001$).

After 24 h of co-incubation, no *E. coli* TOP10 cells were observed in the medium and hardly any in the food vacuoles of the trophozoites (*E. coli* TOP10 cells were digested), however, *E. coli* MG1655 cells were numerous within food vacuoles. After 24 h 2.8 ±2.8 % of the total amoeba trophozoites contained *E. coli* TOP10 cells, 87.0 ±10.1 % *E. coli* MG1655 and 1.5 ±1.9 % contained *L. pneumophila*. Hence, *E. coli* MG1655 appeared to be the second preferred food by *W. magna* under the study conditions and exhibited some resistance to amoeboid digestion in compare to *E. coli* TOP10. However, both were eventually digested by the amoeba trophozoites within 48-72 h of co-culture. After 48 h and 96 h of co-culture no *E. coli* TOP10 cells were observed within trophozoites, only a few (3.5 ±3.1 % and 2.1 ±2.1 % respectively) contained *E. coli* MG1655 and most (61.0 ±8.6 % and 81.8 ±6.5 % respectively) contained *L. pneumophila*. The intracellular concentrations of different bacteria at different time points confirmed the preferential feeding of bacteria by *W. magna* (Appendix B: Figure S4.1). *W. magna* also showed similar preferential feeding behavior with heat killed *L. pneumophila* when provided in presence of the *E. coli* strains (Appendix B: Figure S4.2).

Thus, *W. magna* only appeared to phagocytose *L. pneumophila* when other bacterial (*E. coli*) were unavailable in the co-culture due to prior predation and after 72 h, intracellular growth of *L. pneumophila* was observed in many of the trophozoites. None of the *E. coli* strains produced any apparent adverse effect on *W. magna* growth and activity (i.e., all demonstrated regular gliding movement. The gliding movement of the trophozoites with fluorescent food vacuoles also confirmed the intracellular status of the targeted cells. *W. magna* even avoided heat-killed *L. pneumophila* when presented with the two *E. coli* strains in the same culture. However, *W. magna* phagocytosed *L. pneumophila* within 24 h of co-culture in sterile tap water, when it was provided as a single bacterial prey. Interestingly, trophozoites that had recently

acquired *L. pneumophila* also subsequently phagocytosed *E. coli* strains upon adding them to the culture (Figure 4.3), but could not digest the them as quickly as they could without having the intracellular *L. pneumophila*.

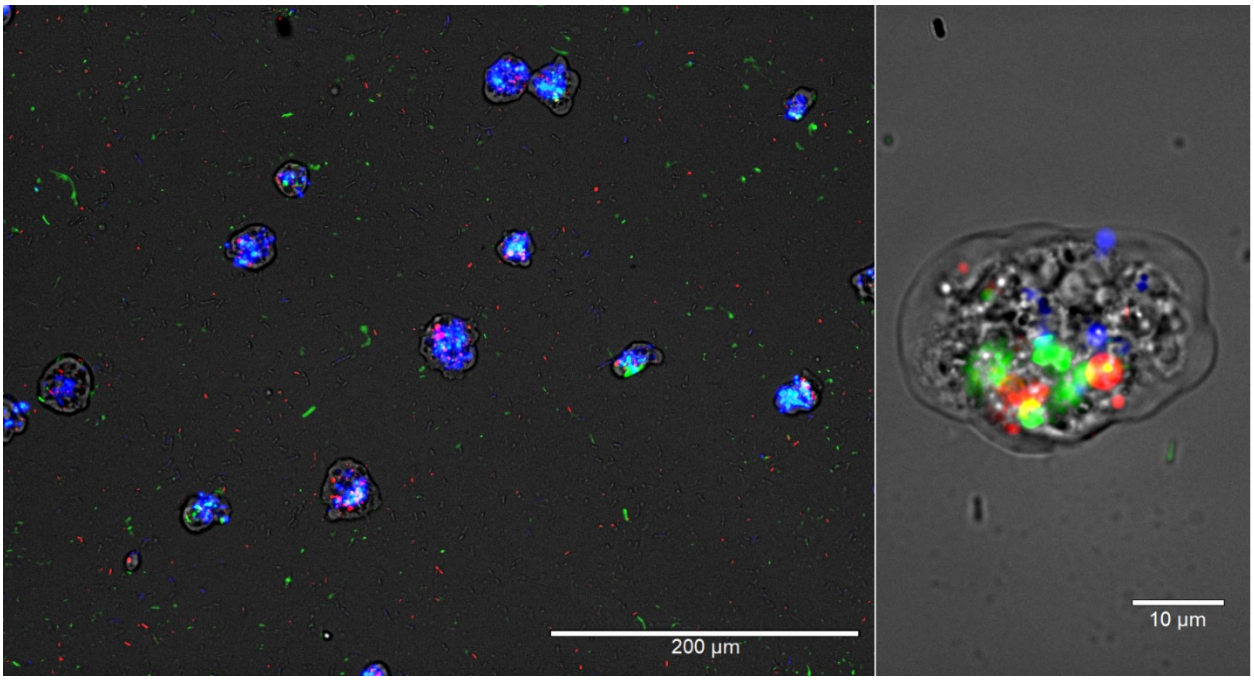

Figure 4.3: Intracellular *L. pneumophila* interferes with *W. magna*'s digestion process. *W. magna* and *L. pneumophila* were co-cultured at RT and after 24 h, *E. coli* TOP10 and *E. coli* MG1655 were added to the culture. The images represent overlaid images of the same field of view under four light channels, Mono-color transmission light channel, Green fluorescent channel, Texas-Red channel and DAPI channel after 26 h of co-culture. *L. pneumophila* cells are green, *E. coli* MG1655cells are red and *E. coli* TOP10 cells are blue in color.

Hence, internalized *L. pneumophila* seemed to interfere with the overall digestion process of trophozoites and may lead to long-term intracellular persistence of the bacteria without initiating active intracellular growth at RT. All the bacterial species remained fluorescent and no reduction in their numbers were observed in amoeba negative cultures for up to 4 days in sterile

tap water at RT. No adverse effect was observed on each other by the studied bacterial species (Appendix B: Figure S4.3).

4.4.2 Interactions of *A. polyphaga* and *V. vermiformis* with bacteria

Acanthamoeba polyphaga and *V. vermiformis* exhibited a higher preference for both the *E. coli* strains when all three bacterial species were present in co-culture in sterile tap water or PAGE's saline at RT (Appendix B: Figure S4.4 and S4.5). However, *A. polyphaga* did not avoid *L. pneumophila* as strongly as *W. magna* and *V. vermiformis*. In tap water *A. polyphaga* tended to form cysts within 48 h and therefore PAGE's saline was used. *E. coli* TOP10 was readily phagocytosed and mostly digested within 24 hours by *V. vermiformis,* and therefore relatively few *E. coli* TOP10 cells were found in the medium as well as in the food vacuoles of the trophozoites after 24 h of co-culture. However, due to the presence of internalized *L. pneumophila* in *A. polyphaga* trophozoites, some *E. coli* cells were still observed in the food vacuoles after 24 h. Due to the rapid encystation of *A. polyphaga* and *E. coli* MG1655's moderate resistance to the amoeboid digestion, the bacteria stayed in the cysts, most likely in between the two outer layers of the cysts (Figure 4.4). Releasing of vesicles during encystation was also observed in *A. polyphaga*, as reported previously for the protozoan *Giardia* (Benchimol 2004, Marti and Hehl 2003). Both the amoebae phagocytosed *L. pneumophila* when this bacterium was provided as a single culture and supported intracellular growth.

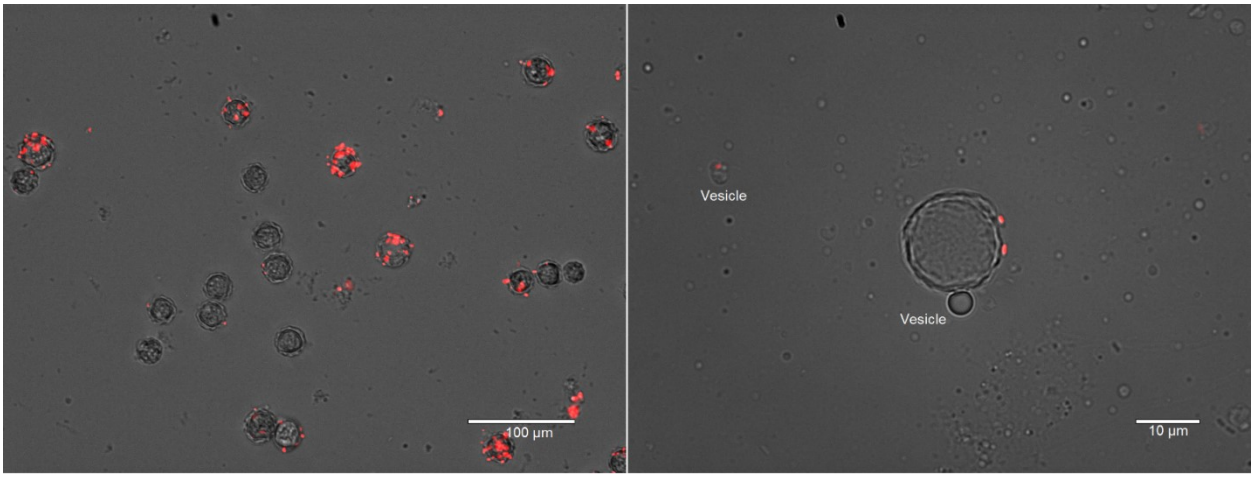

Figure 4.4: Intracellular locations of *E. coli* MG1655 (red) in *A. polyphaga* cysts at 96 h of co-culture. Many of the cysts did not contain any *E. coli* MG1655 cells, which indicate that the digestion may depend on the intracellular load of the bacterial cells and encystation time. Releasing of vesicle during encystation and vesicle-containing *E. coli* MG1655 after 48 h of co-culture (Right image).

4.4.3 Amoebae-bacteria interactions

When all three species of the amoebae (trophozoites) were co-cultured with the studied bacterial species in sterile tap water at RT, the amoebae phagocytosed and digested the *E. coli* strains first before effectively engulfing the *L. pneumophila*. Of particular note, the co-presence of the amoebae species did not appear to affect the feeding preferences of individual strain. However, in water *A. polyphaga* and *V. vermiformis* underwent encystation earlier than *W. magna*, resulting in *E. coli* MG1655 cells being entrapped in cysts (Appendix B: Figure S6 and S7), as previously reported for *L. pneumophila* (Greub and Raoult 2003). *W. magna* was also observed to phagocytose cysts of the other amoeba species present under the studied conditions (Appendix B: Figure S4.8).

4.5. Discussion

It is well known that FLA support the intracellular growth of pathogenic *Legionella* spp. and other similar opportunistic water-based human pathogens. In fact, the ability to grow within amoebae has led to the evolution of *L. pneumophila* as a human pathogen (Amaro et al. 2015). However, very limited information are available on how pathogenic *Legionella* spp. interacts with FLA in its natural water-biofilm environment. The previous study suggested that *L. pneumophila* may not be the preferred prey for *W. magna* within the drinking water-biofilms (Shaheen et al. 2019). This study provided visual evidence to support this earlier observation by showing the interactions of different FLA species and *L. pneumophila* in the presence of other bacteria in real-time, *in situ*. Accumulation of large numbers of *E. coli* cells and no *L. pneumophila* in food vacuoles of *W. magna* trophozoites within five minutes of co-incubation indicated a very fast, effective and precise recognition mechanisms since the bacteria were present in a homogenous equally high concentration mixture. The sequential feeding order of the two *E. coli* strains and *L. pneumophila* by *W. magna* also indicated that amoeba played the active role in recognizing food through a highly selective manner. Although the two *E. coli* strains (TOP10 and MG1655) are very similar and originated from a common ancestor (*E. coli* K12). It was surprising to observe that *W. magna* displayed a feeding preference between these two strains. The higher tolerance of *E. coli* MG1655 to amoeboid digestion implied that the resistance to digestion was bacterially-driven. *W. magna* was reluctant to feed on *L. pneumophila* until it had no other choice, therefore, these amoebae-bacteria interactions caused a selection of *L. pneumophila* followed by a "forced-feeding" condition which insist FLA to phagocytose *L. pneumophila* as the only remaining food source (Shaheen et al. 2019). Phagocytosing of *L. pneumophila* by *A. polyphaga* and *V. vermiformis* in the presence of other bacteria indicated that

the recognition systems for bacteria were different and might not be very specific among different amoeba species as no apparent effect of non-*Legionella* bacteria on the uptake of *L. pneumophila* by *A. castellanii* and *N. lovaniensis* was reported earlier (Declerck et al. 2005).

Further research is required to characterize FLA-*L. pneumophila* interactions in complex natural environments where other organisms and biofilms are present. Nonetheless, it is apparent from the current study that FLA may recognize extracellular chemicals and/or virulence-associated surface markers of *L. pneumophila* since *W. magna* was even unwilling to phagocytose heat-killed *L. pneumophila* in the presence of *E. coli*. Chemotaxis movement of amoeba toward certain bacterial cell lysate supports this observation (Dopheide et al. 2011). Phagocytosis of *L. pneumophila*, when presented in pure culture, suggested a "forced-feeding" condition when FLA had no choice but to feed on *L. pneumophila*, despite being detrimental to them. Larger amoeba trophozoites also phagocytosed other amoeba cysts which could be another example of "forced-feeding". Thus, this study strengthens the hypothesis that selection of *L. pneumophila* through preferential feeding of FLA creates conditions when *L. pneumophila* becomes the main available food; thus, FLA must feed on them and lead to *L. pneumophila*'s ultimate rapid growth (Shaheen et al. 2019).

The prolong presence of undigested *E. coli* in trophozoites containing *L. pneumophila* indicates that *L. pneumophila* actively interferes with the amoeboid digestion process. Hence, low intracellular concentrations of *L. pneumophila* may render the amoeba cell a reservoir of the bacteria by potentially 'intoxicating' the amoeba trophozoites and impairing their digestion process. Similarly, the trapped bacteria within the amoeba cysts also could serve as a source of contamination when the cysts germinate. Moreover, the cysts protect the internal bacteria from the harsh environment and chemical disinfectants (Kilvington and Price 1990). These cysts with

L. pneumophila may help explaining the recurrent LD outbreaks within hospital plumbing systems with clonal strains over decades (Bernander et al. 2003, Bernander et al. 2004, Oberdorfer et al. 2008, Stout et al. 2007). Vigorous treatment of water systems in case of an LD outbreak may remove the planktonic or biofilm-associated cells to some extent but leave behind the cysts with bacteria which may act as a source for subsequent LD outbreaks (Phin et al. 2014).

Although the Dot/Icm, type IV secretion system of *L. pneumophila*, is essential for replication within the amoebae and kills them (Segal et al. 1998, Vogel et al. 1998), the lack of adverse effects on *E. coli* suggested that the Dot/Icm system has no antibacterial activity as reported for type VI secretion system of *V. cholerae* (MacIntyre et al. 2010). The type VI secretion system of *V. cholera*e is also required to kill the protozoan, *Dictyostelium discoideum* (Miyata et al. 2011).

Overall, this study supports our previous hypothesis that preferential feeding of FLA might be the driving force for rapid growth of pathogenic *Legionella* spp. and other ARB in EWS. Although it is well known that the pathogenic *L. pneumophila* can grow intracellularly and disperse in water as free or vesicle-bound cluster of cells (Dupuy et al. 2016, Fields et al. 1989, Shaheen and Ashbolt 2018), the current work has described a possible mechanism of attaining the critical concentrations of *L. pneumophila* needed to initiate an infection. Ultimately, this work describes the ecological perspective of *L. pneumophila*'s growth in EWS where its numbers are usually very low. FLA's reluctance to graze on *L. pneumophila* in the presence of non-ARB also suggests that a probiotic approach could work to control the pathogenic *Legionella* spp. in water. Ecological interactions such as competition, antagonism and obligate parasite-host relationships have been described for potential targets for probiotic control of opportunistic pathogens in EWS (Wang et al. 2013). In fact, upstream microbiota has been

described to have a profound effect on the downstream biofilm bacterial compositions in water pipes (Lu et al. 2014, Pinto et al. 2012).

4.6 Conclusions

Since intracellular multiplication in FLA is the major means for *L. pneumophila*'s growth, understanding the ecology of *L. pneumophila*, especially its interactions with FLA are fundamental to better management of water in EWS and to ensure public health safety. This study provided visual evidence of how FLA selects and supports the growth of pathogenic *Legionella* spp. Hence, the current monitoring of *L. pneumophila* without any consideration on FLA appears to be a weakness in water quality monitoring for EWS. Since FLA appears to be a major driving force for bacterial community shifts towards enrichment of opportunistic water-based pathogens, more research on other FLA and opportunistic pathogens like non-tuberculosis mycobacterium and *Pseudomonas* spp. is required to develop generalized approaches for novel monitoring and control strategies for these pathogens.

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, rph-1) with the plasmid (pTV-mCherry) respectively. The authors declare no conflict of interest.

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Chapter 5

Chapter 5. Effects of temperature, pipe material and probiome on drinking water biofilm bacterial communities and pathogenic *Legionella* colonization

5.1 Abstract

Premise plumbing is vulnerable to colonization by *Legionella* spp. and other water-based opportunistic pathogens, and therefore, a pivotal site to control from a public health perspective. Yet there is a poor understanding of the ecological relationship of pathogenic *Legionella* spp. colonization and other microorganisms within pipe biofilms. Using partial 16S-rRNA gene amplicon sequencing and bioinformatics analysis, the effects of introduced *Legionella pneumophila* and an upstream selector (probiome) on bacterial community compositions (BCC) of naturally developed mature water-biofilms on PVC and copper (Cu) pipe materials were examined. The biofilm BCC varied with pipe materials and temperatures and was possibly influenced by the source water and residual monochloramine. Although PVC pipe material supported higher biofilm bacterial diversity than Cu at 40 °C, the Cu-biofilms appeared to have higher *L. pneumophila* colonization. *Quipengyuania* was the most prevalent genus present in PVC and Cu biofilms grown at RT, whereas *Blastomonas* and *Phreatobacter* predominated on both pipe materials at 40 °C. Temperature was the stronger determinant than pipe materials for biofilm BCC. Nevertheless, the upstream probiome significantly reduced the downstream *L. pneumophila* colonization within biofilms on Cu but not on PVC surface. Although *L. pneumophila* colonization was transient in both annular reactors (with and without upstream probiome), its sudden introduction to predeveloped biofilms shifted the bacterial community structures, which did not reset to the pre-spiked state within the study time of four months. The difference between the BCC of biofilms developed within annular reactors running with water

having no residual monochloramine and in an exhumed building Cu water pipe under the influence of the disinfectant residual suggested that a microbial community profile map of a premise plumbing system could help to identify compromised (poorly disinfected) sites. Overall, this study demonstrated that the colonization of bacterial species from the source water to form biofilms depends on temperature and pipe material (PVC & Cu), and Cu pipe at high temperature appeared more vulnerable to *L. pneumophila* colonization, but partly could be mitigated by an upstream PVC-grown probiome.

5.2 Introduction

Legionella pneumophila, the primary aetiological agent of Legionnaires' disease (LD) is a Gram-negative bacterium commonly present in natural and engineered water systems (EWS) (Atlas 1999, NASEM 2020, Orrison et al. 1981). Water-biofilms are considered to be a major growth niche for *Legionella* spp. and similar water-based opportunistic pathogens (OPPPs) that interact with other bacteria and eukaryotic hosts present in water (Bryers and Characklis 1982, Flemming et al. 2016, Wingender and Flemming 2011). Biofilms develop naturally in drinking water distribution systems (DWDS), from water treatment facility to customer connections, and within premise plumbing (PP) and affect the biological stability of drinking water (Laurent et al. 2005, Lautenschlager et al. 2013, Prest et al. 2016). Thus, biofilms modulate the health risk associated with water and even may have the potential to affect the gut microbiota (Dias et al. 2018). Abiotic factors including pipe materials, flow regimes and temperature, along with water chemistry and residual disinfectants may all affect bacterial colonization, biofilm development, intra and inter-species interactions and OPPPs growth within drinking water (Buse et al. 2014b, Ji et al. 2015, Learbuch et al. 2019, Proctor et al. 2016, Rhoads et al. 2015, Shen et al. 2016, Wang et al. 2012b, Yu et al. 2010).

PP refers to the pipe and fixers associated with drinking water after the water meter within the built environment, such as large building complexes and individual houses (Wang et al. 2017). Usually, PP has pipes for hot and cold water that may have intermittent flow regimes (e.g., periods of stagnation), dead-ends and conditions where cold water may warm to ambient temperatures within buildings (around 20 °C) and the hot water may stagnate in pipes at 40-45 °C (despite being initially heated > 55 °C) (Ji et al., 2017). Hence, it may not be uncommon that PP water temperatures are favorable for the growth of water-based OPPPs like

L. pneumophila, nontuberculous mycobacteria (NTM) and *Pseudomonas aeruginosa* as frequently identified in PP systems (Codony et al. 2002, Kuroki et al. 2017, Lu et al. 2016, Wang et al. 2012a). The PP has much higher surface area to volume ratio than the DWDS (Neu and Hammes 2020) and at the distal parts, the residual disinfectant concentrations may be negligible, making the PP a more favorable hot-spot for microbial growth and interactions (Council 2006, Feazel et al. 2009). Building hot water systems have frequently been reported to be associated with LD outbreaks, but a few studies have focused on the microbiomes to understand the ecology of pathogenic *Legionella* spp. within hot water plumbing systems (Dai et al. 2018b, Ji et al. 2018, Proctor et al. 2017).

Although biofilms are problematic for PP and water quality, it is practically impossible to eradicate biofilm or inhibit its formation (Wingender and Flemming 2011). Risk-based approaches, including quantitative microbial risk assessment (QMRA) (Ashbolt 2009), hazard analysis critical control point (HACCP) (ASHRAE 2015) and water safety plans (WSP) (Tsoukalas and Tsitsifli 2018, WHO 2013) inform where to reduce human health risks from pathogens in DWDS and PP and advise control strategies to minimize biofilm development. Many physical and chemical treatments used to control biofilm formation, however, are not without their own limitations (Flemming 2020). More recently, a biological (probiotic) approach has been proposed whereby a microbiome (probiome) with selected 'protective' microorganisms could be used to manipulate the biofilm microbial communities in the downstream PP as a strategy to control water-based OPPPs colonization (Wang et al. 2013). In order to define and successfully engineer a drinking water probiome and to ensure system robustness for public health protection, a discovery process through monitoring, predictive modeling and process validation are required. However the inherent stochasticity of water biology and chemistry are

the challenges to ensuring probiome functionality and public health safety of water supply (Schroeder et al. 2015).

Under current regulatory requirements, the microbiological safety of drinking water is assessed by the absence of fecal indicators. In some cases, a heterotrophic plate count (HPC) is included to evaluate the disinfection efficacy and biological stability (HPC ≤ 500 cfu/mL) (WHO 2011). Water safety plans that address the drinking water safety from source to tap are becoming common best management practice (Gunnarsdottir et al. 2012). However, monitoring of *Legionella* spp. is generally not a routine practice for PP but may be considered during an outbreak or case investigations (Hamilton et al. 2019). Many bacteria, including OPPPs and some eukaryotes are usually present in very high-quality drinking water systems, especially at the point of use but less so within the upstream DWDS (Lu et al. 2016, van der Kooij et al. 2018, Wullings et al. 2011). *Sphingomonas*, *Methylobacterium, Sediminibacterium, Gemmatimonas, Bradirhizobium* and *Rhodobacter* are some of the most commonly reported genera present in drinking water, but are considered of no immediate public health risk (Buse et al. 2014a, De Sotto et al. 2020, Kelley et al. 2004, Proctor et al. 2018). However, the current knowledge about the role of these organisms for water quality is extremely limited and it is not known whether they have any link (negative or positive) to the colonization of OPPPs. Moreover, the nutrient-limiting (oligotrophic) conditions of drinking water and eukaryotic cell-associated growth of OPPPs may provide them a competitive advantage within biofilms (Thomas et al. 2010, Wingender and Flemming 2011).

Next-generation DNA sequencing technology and bioinformatics analytical platforms are rapidly evolving and enable the analysis of *in situ* microbial community compositions of niches like PP or water system biofilms in built environments. Sequencing of partial 16S rRNA genes

(variable regions) is a widely used method and, along with available databases enables the identification of bacterial community members. However, the high spatiotemporal heterogeneity and limited reports on microbial diversity for drinking water systems (Bautista-de los Santos et al. 2016, Holinger et al. 2014, Oh et al. 2018, Stanish et al. 2016) make it challenging to generalize interpretations about drinking water microbiomes. Also, increasing population growth and urbanization are adding pressure to safe drinking water access to reduce disease outbreaks (from water-born and water-based pathogens). Therefore, understanding the drinking water microbiome should be given priority to optimize current and future infrastructure development to protect public health.

The aims of this study were to: 1) compare the bacterial community compositions (BCC) of biofilms developed on Cu and PVC pipe materials in annular reactors (ARs) at temperatures representative of cold and hot water pipes in buildings; and 2) compare these microbiomes when modulated with an upstream predeveloped natural biofilm (probiome) to see how it may influence the downstream bacterial community following the introduction of *L. pneumophila* into the ARs. Overall, this study will help to improve the knowledge on premise plumbing bacterial ecology, its relationship to opportunistic pathogen colonization, and to identify possible surrogate bacteria for monitoring and predicting conditions that could lead to OPPPs outbreaks.

5.3 Material and methods

5.3.1 *L. pneumophila* culture

L. pneumophila Lp02 (ATCC 33152) with the green fluorescent protein (GFP) gene on a plasmid (from Ann Karen Brassinga, University of Manitoba) was grown on BCYE (Buffered Charcoal Yeast Extract) agar plates without antibiotics at room temperature (RT) (22±1 °C) for 7

days. A *L. pneumophila* cell suspension was prepared in tap water (filtered-sterile and contains no residual chlorine) by following the procedure described previously (Shaheen and Ashbolt 2018).

5.3.2 Up-stream microbiome (probiome) development

PVC pipe (white opaque, internal diameter 1 inch from a local store) was cut into sections (6 sections, length 12 inches/ section) and decontaminated with 70% ethanol and sterile water. Aseptically the pipe sections were filled with virgin PVC granules (Geon™ Vinyl Rigid Extrusion 8700X raw materials for PVC pipe for potable water, collected from Drader Manufacturing Industries Ltd., Edmonton) to maximize the surface area for biofilm development. The pipe sections were then sealed with PVC endcaps connected to Norprene® tubing (Masterflex® tube, Col-Parmer, USA) and placed upright in groups of three sections in series (total biofilm surface area of approx. 0.6 m^2) to build one probiome module. Tap water (unchlorinated) was pumped at a rate of 30 mL/ h (8 h flow and 16 h stagnation) through each module for six months at RT. After 6 months, one module was connected upstream of four in-house constructed 200 mL annular reactors (ARs) (§ 2.3) (one with 10 PVC coupons, one with 10 Cu coupons at RT and 40 °C [HT]) (Figure S5.1); another set of four ARs had no upstream probiome module connected. *L. pneumophila* (5×10^6 cfu) was inoculated at the first pipe section of one of the probiome modules and the water was run for 5 h (one water volume change of three pipe sections/ 6 h) to distribute the *Legionella* cells across the other pipe sections; then stopped for 7 d (stagnation to help colonization of *L. pneumophila* to predeveloped water-biofilms). After seven days, water flow resumed through both probiome modules (one without *L. pneumophila* to serve as a probiome for downstream biofilms and other under the influence of *L. pneumophila*)

by maintaining the initial flow regime for another 4 months. The probiome modules were always kept at RT. After four months, the PVC pipe sections were cut open by maintaining aseptic technique and the biofilms from PVC granules were harvested by and stored at -80 °C for microbial community analysis.

5.3.3 Annular reactor biofilms and water samples

Municipal tap water was collected in 40 L HDPE (sunlight protected high-density polyethylene) carboys and kept in the dark for 10 days to dissipate the residual chlorine (checked every day with HACH chlorine test kits [Lot No. A5218]). The water was pumped (Watson-Marlow Sci-Q 205U with 12 channel pump head) through Norprene (Masterflex® tube, Col-Parmer, USA) tubing at a flow rate of 30 mL/ h individually to eight ARs (in-house made with plexiglass, polymethyl methacrylate, Appendix C: Figure S5.1), each containing 10 coupons of the same pipe material (Cu or PVC of 1×10 cm^2) with a hydraulic retention time of about 7 h. The pump was run for 8 h per day, to simulate purging of pipe flow in buildings. Four ARs were kept at RT and another four at HT during the 10 months study period and the pipe coupons were on continuous rotation at 30 rpm throughout the study period. The ARs were mostly kept in the dark with occasional exposure to room light for sampling or other lab activities. Biofilms were developed for six months and then the first samples (from duplicate coupons) were collected from each AR. The biofilms were harvested aseptically from each coupon using a flocked swab (BD ESwab® collection and transport system, USA) by stroking in one direction across the coupon. Two 100 mL aliquots of water from each AR were passed through 0.22 µm filter (MicroFunnel™ Filter Funnels 100 ml, Pall Corporation, USA) to analyze the BCC of water. The biofilm and filter samples were stored at -80 °C for further analysis. The harvested coupons

were decontaminated by rinsing with ethanol followed by sterile tap water (5-6 times) and replaced (marked harvested) into their original annular reactor. *L. pneumophila* was inoculated to each annular reactor to a final concentration of 10^5 cfu/mL in the bulk water after the first sampling. The water flow to ARs was stopped for 7 d and then resumed with the regular flow regime (8h flow, 16 h no-flow). Duplicate coupons were collected from the ARs for the next four months (monthly) to harvest biofilms from all the 10 coupons from each AR.

5.3.4 PP biofilm samples

Actual premise plumbing biofilm samples (duplicates, DW1Jun12 and DW2Jun12) were collected from a cold-water Cu pipe on-site with flocked swabs (one-time sampling) immediately after a drinking water fountain was uninstalled (close proximity to the lab). The water of the fountain site had residual disinfectant (Total Chlorine 1.8 ppm and Free chlorine 0.3 ppm. HACH chlorine kits). The biofilm samples were stored at -80 °C for BCC analysis.

5.3.5 DNA extraction and Illumina MiSeq sequencing

DNA was extracted from the thawed biofilm samples using DNeasy PowerBiofilm Kit (Qiagen) by following the manufacturer instructions. The DNA samples were then quantified by Qubit dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific) and sent to Centre d'innovation Génome Québec et Université McGill for sequencing (Illumina MiSeq System). The degenerate primer set 515F-Y (5' - GTGYCAGCMGCCGCGGTAA - 3') and 926R (5' - CCGYCAATTYMTTTRAGTTT - 3') were used to amplify the V4-V5 region of the 16S rRNA gene. Pair-end sequencing was performed using Illumina MiSeq PE 250 sequencing chemistry.

5.3.6 Sequencing data processing

The Illumina MiSeq sequence reads for biofilm and water samples were processed and annotated against the SILVA reference database using Mothur (version 1.44.1) by following the MiSeq SOP (https://mothur.org/wiki/miseq_sop/). Before using the Mothur tool, the primer sequences were removed from the Illumina reads and the length of the forward and reverse reads were truncated based on quality information using DADA2 (version 1.16). Merging of the forward and reverse reads by Mothur yielded 4,510,181 sequences and after completing all the processing steps, removing chimera and classifying against the SILVA reference database (Mothur version of SILVA 138 SSU), 4,080,060 qualified sequences were processed for BCC analysis by following the Phylotype method (Mothur 1.44.1). The phylotype method bins the sequences based on their taxonomic classification; therefore it depends on the existing reference database and produced a smaller number of individual taxonomic features. The sequences were clustered by aligning with the reference database and annotated to genus level identification. The usual OTU (Operational Taxonomic Unit) (0.03 cut-off) or ASV (Amplicon Sequence Variant) (0.02 cut-off) based analysis was avoided here to minimize the effect of sequencing errors.

5.3.7 Bacterial community analysis and statistics

After annotating all genera present in the samples, the representative bacterial members were determined by removing all the singletons, low abundance OTU based on prevalence (Low count filter: minimum count 4, minimum prevalence 20%) and low variance OTU based on inter-quantile range (IQR, Low variance filter: removed 10%). The low count filter was used to eliminate OTUs that had very low counts and were present only in a few samples assuming that these features were created due to sequencing errors. A low variance filter was applied to remove

features that were close to constant in all the samples and not affected by different experimental conditions and the inter-quantile range was used to remove the low variant features.

The OTU-sequence data for each comparison group (HT vs RT or PVC vs Cu) was pulled out of the total sequence-data set and normalized using Mothur commands to determine the lowest sequencing depth of the samples and the sample coverage. The alpha diversity indices were measured for the individual samples and compared between the groups with T-tests. The alpha diversity is a measure of average species diversity of a particular sample, specific area or environment. Two diversity indices were examined: Chao1 and Shannon to characterize the bacterial communities. The Chao1 estimates the richness by considering all the rare OTUs that may have been lost due to under-sampling, while Shannon measure both the number of OTUs and their abundance.

The beta diversity measure provides a way to compare the diversity or composition of two microbial communities, and a non-phylogenetic index (Jaccard index) was used in this study. The dissimilarity matrices were compared by PERMANOVA with PCoA analysis. The bacterial community analysis was performed using Mothur tools as well as the Mothur output data on the MicrobiomeAnalyst web server (https://www.microbiomeanalyst.ca/) (Chong et al. 2020, Dhariwal et al. 2017). Hierarchical clustering was performed by taking into consideration the Bray-Curtis distance matrix with Ward's linkage clustering algorithms at the genus level and expressed as dendrograms. Microsoft Excel was used for plotting or modifying graphical outputs if required.

The core microbiome was identified for each pipe material under HT and RT conditions and with/without the effect of an upstream probiome. Prevalence (80% within the sample group) and relative abundance (RA) (cut-off= 0.01) were taken into consideration to identify the core

microbiome for each group of samples analyzed. The correlation between different bacterial members within groups of samples was determined using the SparCC algorithm at the genus level (SparCC permutation=100, p-value threshold = 0.05 and correlation threshold = 0.5) and expressed as network map.

The LEfSe (Linear discriminant analysis Effect Size) analysis was performed to determine the bacterial members that were responsible for the variation in biofilm BCC due to different parameters. LEfSe analysis performs a non-parametric factorial Kruska-Wallis rank-sum test to identify the OTUs with significant variation in abundance with respect to experimental conditions followed by Linear Discriminant Analysis (LDA) to determine the effect size of each differentially abundant OTUs. This study used Log LDA score cut-off 2.0 and p-value cut-off = 0.05.

5.4 Results

5.4.1 Bacterial community composition (BCC) at phylum and class levels

As most previous studies report drinking water biofilms BCC at the phylum or class level, first the biofilm bacterial communities were examined at these higher taxa levels. Alphaproteobacteria within the phylum Proteobacteria dominated both the biofilm and water samples, as reported previously (Buse et al. 2017, Gerrity et al. 2018, Lu et al. 2014, Potgieter et al. 2018, Stüken et al. 2018). However, Gammaproteobacteria were the most dominant class (86% of the total bacteria) in the probiome samples. The RA of Gammaproteobacteria in Cu and PVC biofilms was increased immediately after the introduction of *L. pneumophila* to the ARs (partly due to colonization of *L. pneumophila*, a member of the Gammaproteobacteria), but decreased over the subsequent months. Moreover, the introduction of *L. pneumophila* increased

the RA of Planctomycetes and Actinobacteria in Cu-biofilms, especially within the probiome at HT in samples collected during later months. *L. pneumophila* also increased the RA of Planctomycetes and Bacteroidia and decreased the Alphaproteobacteria in PVC-biofilms with and without the probiome at HT but the effect was stronger without the probiome (Appendix C: Figure S5.2).

5.4.2 Baseline biofilm and water bacterial community compositions (BCCs)

The baseline bacterial community profiles of biofilms on PVC and Cu coupons were determined by analyzing the first-month samples (M1) at two temperatures (RT and HT). A total of 206 OTUs were identified from 989,663 SILVA annotated sequences for 16 biofilms (BF) and 16 water counterparts (W) (water samples from the AR containing PVC or Cu coupons). By removing all the singletons, low abundance features, 45 OTUs were identified. Normalization of the data by subsampling (24,267 sequences/sample) showed that the sample coverages were all above 99.9%, which indicated that the sequencing depth captured almost the full richness of all the samples (Appendix C: Table: S5.3).

The biofilm BCC varied with respect to pipe material as well as to temperature. The BCC of water samples (planktonic bacteria and slough-off biofilm) were also different from their biofilm counterparts (Figure 5.1). At HT, the two genera *Blastomonas* and *Phreatobacter* dominated in Cu biofilms, and *Quipengyuania*, *Blastomonas* and *Sediminibacterium* in PVC biofilms. However, the water samples from ARs containing Cu or PVC coupons at HT had similar BCC with high abundance of *Quipengyuania*, *Sphingomonas* and *Blastomonas* except for *Gemmatimonas,* which was more dominant in water with PVC coupons. At RT, *Quipengyuania* and *Sphingomonas* dominated in all the water and biofilm samples (PVC and Cu). However,

Quipengyuania was more abundant in biofilms on Cu than on PVC, whereas *Aquabacterium* was more abundant on PVC. An unidentified bacterial genus (uncultured genus of Gemmataceae family) was present at a high RA in both Cu and PVC biofilm samples compared to their water counterpart at RT. All replicates (Cu1 and Cu2 or PVC1 and PVC2) had very similar RA patterns for bacterial community members except one PVC biofilm sample, which had *Methylobacterium_ Methylorubrum* at an unusually high proportion in one of the duplicates (Figure 5.1, 2HTPVC2Mar29, shown with a red arrow).

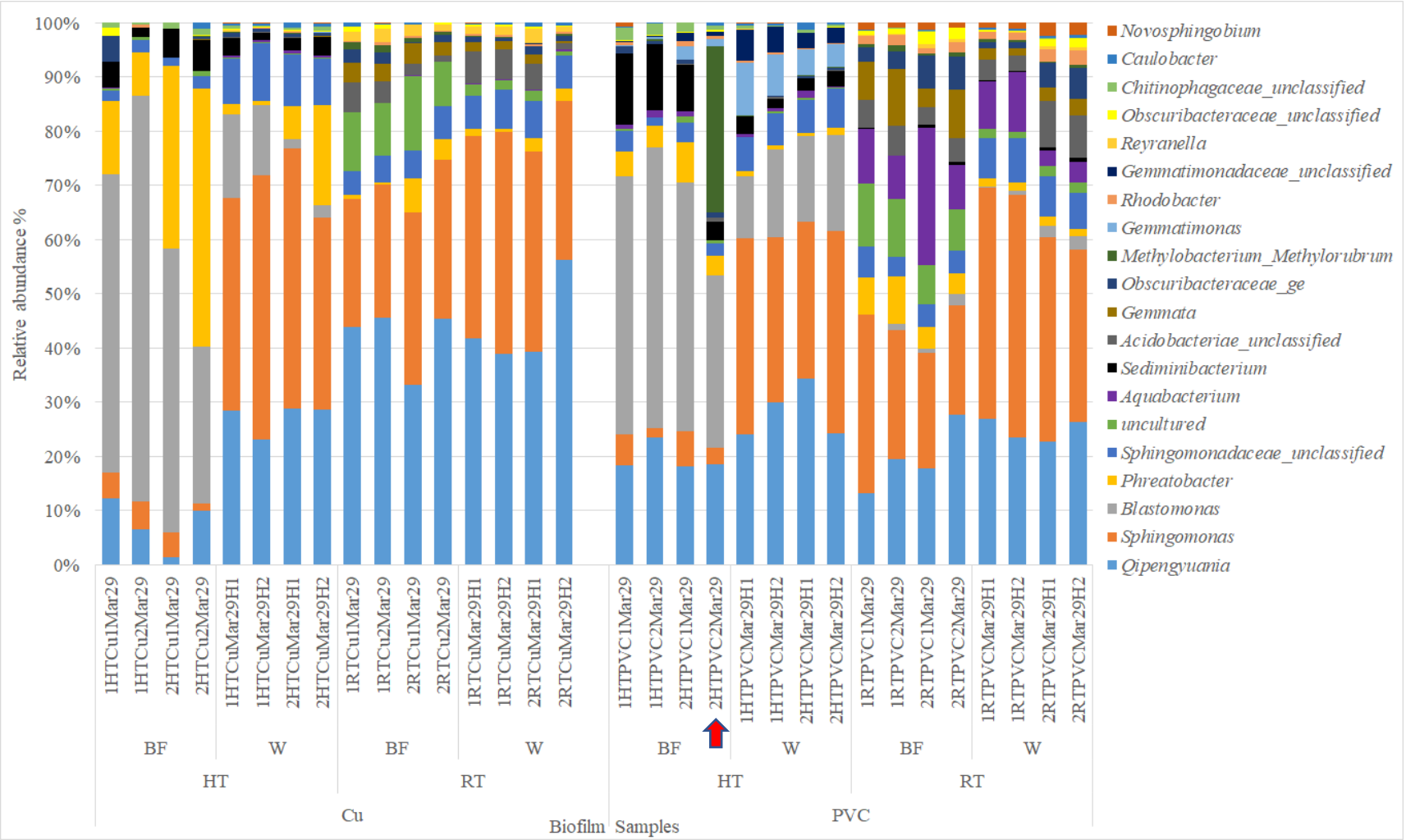


Figure 5.1: Baseline bacterial community composition (20 most dominant genera) of water (W) and biofilms (BF) on Cu and PVC coupons at RT and HT as of 1st month (M1, March 29) prior to *L. pneumophila* and upstream probiome being introduced. The red arrow indicates *Methylobacterium_ Methylorubrum* at an unusually high proportion in one of the duplicates.

5.4.3 Probiome (selector) bacterial community profiles and *L. pneumophila* colonization

The probiome samples (24 samples from 6 PVC pipe sections) produced 1,318,142 sequences (average 32,954 sequences/sample) that belong to 180 genera. Thirty-eight bacterial genera were analyzed further after filtering out all the singletons, low abundance and low variance features. In both sample groups (Lp [challenged with *L. pneumophila*] and Con [Control]) the class Gammaproteobacteria within the phylum Proteobacteria represented 94% of the total bacteria. Interestingly, a single genus, *Aquabacterium* represented 86% of the total Proteobacteria and 83% of the total bacteria of the biofilms (Appendix C: Figure S5.4). The introduction of *L. pneumophila* affected the Alphaproteobacteria compositions with increased RA of *Qipengyuania* and *Reyranella*, and decreased *Sphingomonas, Phreatobacter* and *Methylobacterium_Methylorubrum* in Lp samples in comparison to control samples (Appendix C: Figure S5.5 and Table: S5.6). However, the genus *Legionella* was found in both probiome modules (Con and Lp) at a very low concentration with a similar RA (0.1% of the total biofilm bacteria).

5.4.4 Effect of water temperature on biofilm bacterial communities

PVC biofilm samples (from 20 coupons at RT and 20 at HT) generated 12350905 SILVA annotated sequences of 191 OTUs. After data filtering, as described previously, 50 OTUs were considered for determining the effect of temperature on BCC of PVC biofilms. *Blastomonas*, *Phreatobacter* and *Sediminibacterium* dominated at HT and *Qipengyuania* at RT in PVC biofilms (Appendix C: Figure S5.7).

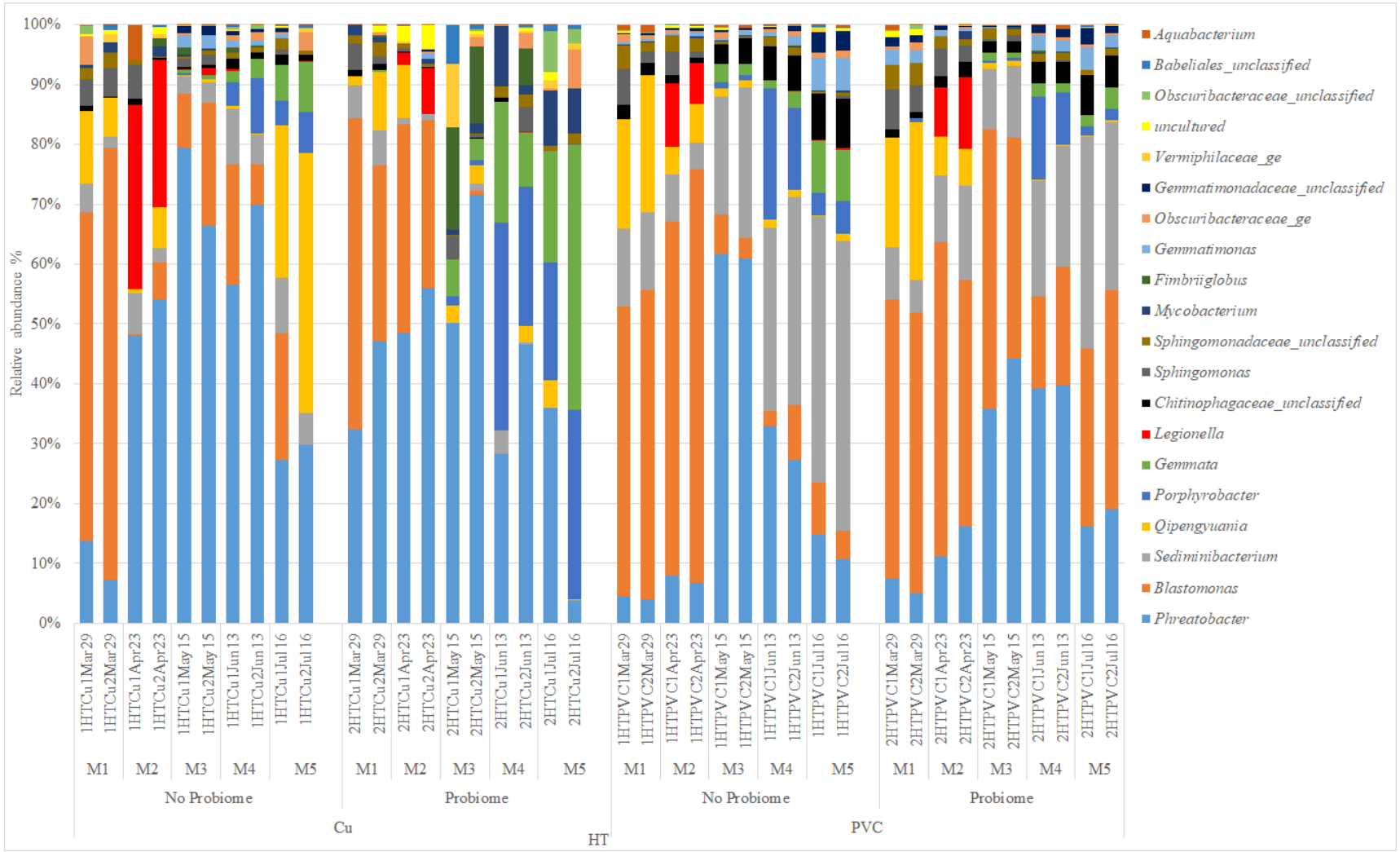
Similarly, 1318142 annotated sequences from Cu biofilms generated 180 OTUs and 37 OTUs were finally considered to determine the effects of temperature on the BCC. *Blastomonas*

and *Phreatobacter* were prevalent at HT and *Qipengyuania* and *Sphingomonas* at RT in Cu biofilms. The genus *Legionella* was identified relatively in high proportion in the second month (M2) (the first samples after the introduction of *L. pneumophila* to the ARs) than in the subsequent months and in higher abundance at HT than at RT in both PVC and Cu biofilms.

5.4.5 Effects of pipe materials, *L. pneumophila* and probiome on predeveloped biofilm bacterial community compositions

To determine the effects of pipe materials on BCC, the SILVA annotated sequences of the biofilms were grouped based on the temperature. *Blastomonas* was the most dominant bacterial genus in both Cu and PVC biofilms at HT before the introduction of *L. pneumophila* (in M1 samples). The genus *Legionella* was found in its highest abundance in M2 samples of both Cu and PVC biofilms and reflected the introduction of *L. pneumophila* to the AR. Importantly, the upstream probiome minimized the colonization of *L. pneumophila* in Cu biofilms (as seen in M2 samples) at HT but the same effect was not observed in PVC biofilms. A differential change in biofilm BCC occurred on PVC and Cu surfaces after the introduction of *L. pneumophila* with a significant decrease in *Blastomonas* abundance in both biofilm types. Although the introduced *L. pneumophila* did not persist well in the predeveloped biofilms on both pipe materials, the abundance of *Blastomonas* did not recover to the original level till the end of the study period; instead, *Phreatobacter* increased transiently to a very high abundance (M3 samples without probiome) (Figure 5.2). Interestingly, post *L. pneumophila* introduction, probiome altered the BCC of Cu biofilms by replacing *Blastomonas* completely with *Porphyrobacter* and *Gemmata*. Surprisingly, the probiome was also found to increase the abundance of *Mycobacterium* in Cu biofilms. Although the effect of *L. pneumophila* was strong, it appeared that the bacterial

communities were on a trajectory to their original balance, as the RA of *Blastomonas* was increased and *Phreactobacter* decreased in the Cu biofilms without priobiome in the subsequent months (M3-M5). However, the disruption in the BCC had long-term effects as seen by the M5 samples still varying from the M1 samples by higher abundance of *Quipengyuania, Porphyrobacter* and *Gemmata*.


Relative abundance %
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1HTCu1Mar29
1HTCu2Mar29
1HTCu1Apr23
1HTCu2Apr23
1HTCu1May15
1HTCu2May15
1HTCu1Jun13
1HTCu2Jun13
1HTCu1Jul16
1HTCu2Jul16
2HTCu1Mar29
2HTCu2Mar29
2HTCu1Apr23
2HTCu2Apr23
2HTCu1May15
2HTCu2May15
2HTCu1Jun13
2HTCu2Jun13
2HTCu1Jul16
2HTCu2Jul16
1HTPVC1Mar29
1HTPVC2Mar29
1HTPVC1Apr23
1HTPVC2Apr23
1HTPVC1May15
1HTPVC2May15
1HTPVC1Jun13
1HTPVC2Jun13
1HTPVC1Jul16
1HTPVC2Jul16
2HTPVC1Mar29
2HTPVC2Mar29
2HTPVC1Apr23
2HTPVC2Apr23
2HTPVC1May15
2HTPVC2May15
2HTPVC1Jun13
2HTPVC2Jun13
2HTPVC1Jul16
2HTPVC2Jul16
M1
M2
M3
M4
M5
No Probiome
Probiome
Cu
HT
PVC
Aquabacterium
Babeliales_unclassified
Obscuribacteraceae_unclassified
uncultured
Vermiphilaceae_ge
Gemmatimonadaceae_unclassified
Obscuribacteraceae_ge
Gemmatimonas
Fimbriiglobus
Mycobacterium
Sphingomonadaceae_unclassified
Sphingomonas
Chitinophagaceae_unclassified
Legionella
Gemmata
Porphyrobacter
Qipengyuania
Sediminibacterium
Blastomonas
Phreatobacter

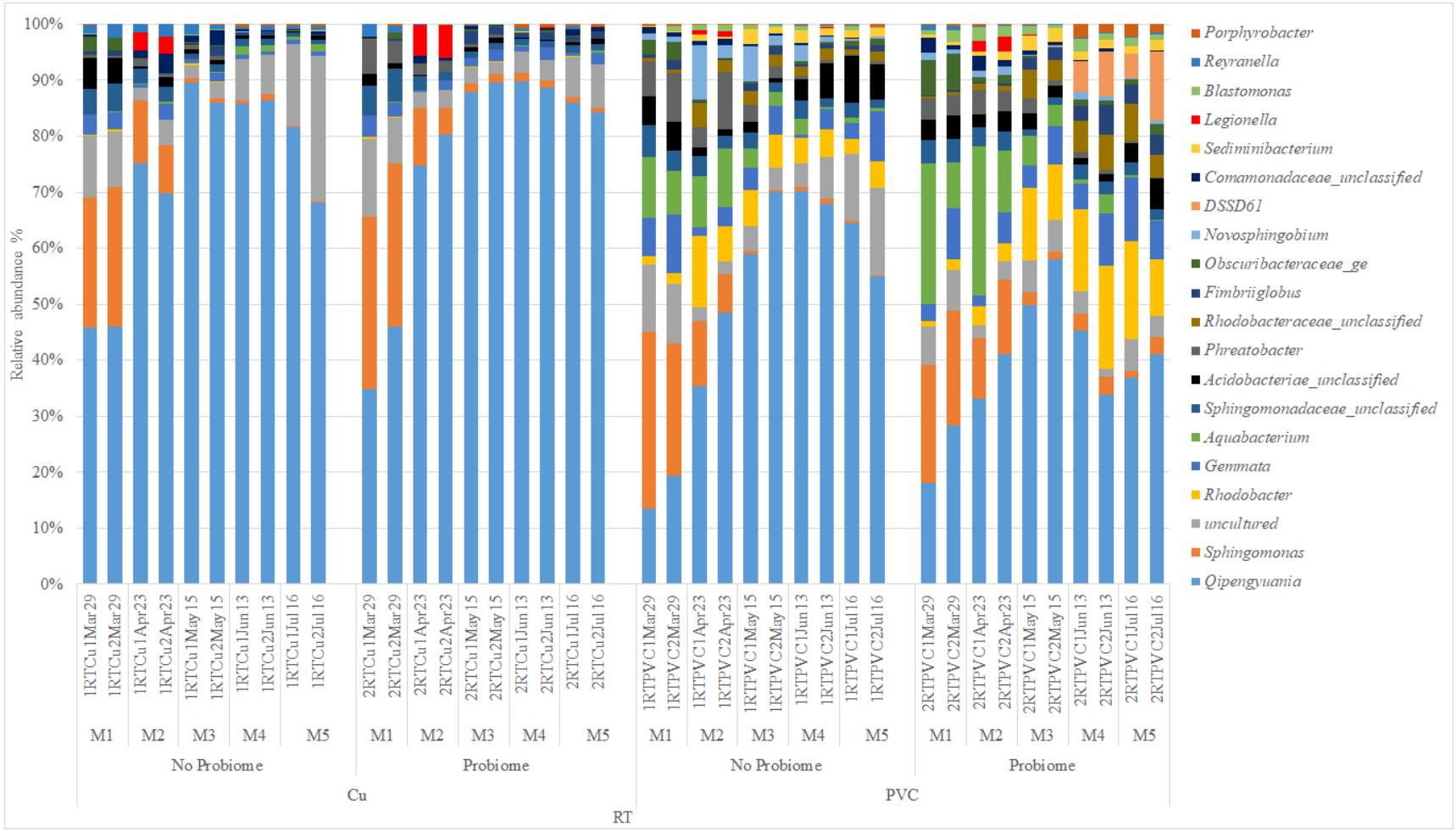


Figure 5.2: Bacterial community shifts in Cu and PVC- biofilms at HT (Top) and RT (Bottom) due to introduced *L. pneumophila* and probiome. *L. pneumophila* and probiome were introduced to the system after M1 sampling.

The genus *Legionella* was present in higher abundance in both M2 PVC-biofilm samples (with and without the probiome). The introduced *L. pneumophila* increased the abundance of *Phreactobacter* and *Sediminibacterium* and decreased *Blastomonas* drastically in M3 PVC-biofilm samples without the probiome, whereas the effects were milder with probiome. The RA of *Phreactobacter* was gradually reduced in the subsequent months (M4-M5) and the abundance of *Blastomonas* almost recovered with an upstream probiome; however, *Qipengyuania* was diminished from both PVC and Cu biofilms at HT.

At RT, *Qipengyuania* was the most prevalent genus on both pipe materials, but its RA was higher on Cu than on PVC and *Aquabacterium, Gemmata* and *Rhodobacter* were only in higher abundance on PVC (Figure 5.2). At RT, the introduction of *L. pneumophila* impacted the BCC by mostly removing *Sphingomonas* from both Cu and PVC biofilms. The probiome had no significant effects on downstream Cu biofilms but surprisingly caused a slight increase in *Legionella* abundance. However, the probiome increased the RA of *Rhodobacter,* DSSD61 and some unclassified *Sphingomonadaceae* and *Rhodobacteraceae* in PVC biofilms. Interestingly, the introduction of *L. pneumophila* to PVC biofilms also decreased the RA of *Aquabacterium* which was not recovered by the presence of probiome, although 86% of the probiome community was *Aquabacterium.*

5.4.6 Building pipe vs. annular reactor biofilms

True PP Cu biofilm samples were compared with Cu biofilm samples from AR (M1 samples at RT) to investigate the potential reactor artifacts. In PP Cu biofilms, *Methylobacterium_Methylorubrum* was present in relatively higher abundance with no *Qipengyuania* being present. *Nitrosomonas, Pirellula*, and *Hyphomicrobium* were only detected

in PP samples and some unidentified bacteria (Gemmataceae family), as well as unclassified *Acidobacteriae, Blastomonas, Afipia, Rhodobacter* and *Gemmata* were only found in AR Cu samples. *Sphingomonas* was present in both sample types but in higher abundance in PP biofilms (Appendix C: Figure S5.8).

5.4.7 PVC, norprene™ biofilms, *Legionella* and amoebae

Biofilms that developed on the Norprene™ tubing (feeding tube to the ARs, Con) surface was compared with the Norprene™ tubing (WmApLp) that was kept under the influence of *L. pneumophila* and two amoeba strains *Willaertia magna* (ATCC 50035) and *Acanthamoeba polyphaga* (ATCC 30461) from another experiment (under same RT condition) (Shaheen et al. 2019). The BCC of Norprene (Con) and probiome biofilm samples seemed very similar and had a very high abundance of *Aquabacterium* and an unclassified *Comamonadaceae*; however, the RA of *Sphingomonas* varied significantly (Appendix C: Figure S5.8). The BCC of Norprene (WmApLp) biofilms with amoeba and *L. pneumophila* was different from that of Norprene (Con) and was dominated by *Qipengyuania, Reyranella, DSSD61* and *Sediminibacterium*.

5.4.8 Alpha diversity

The alpha diversity was measured with the original number (without rarefaction) of sequences of each sample. The alpha diversity indices (Chao1 and Shannon indices) of Cu-biofilm bacterial communities were higher with the probiome at HT (HTC vs HTP) and lower at RT (RTC vs RTP), however the effects of probiome on bacterial communities were not statistically significant (p-value > 0.05). The difference between the alpha diversity indices (Chao1 and Shannon indices) of Cu-biofilm bacterial communities at HT and RT (HTC vs RTC)

were statistically significant (p-value < 0.05). The higher Chao1 index and lower Shannon index at RT compared to HT implied that the bacterial communities at RT could be more diverse but only few members dominated within the biofilms. The alpha diversity indices (Chao1 and Shannon indices) of PVC-biofilm bacterial communities were higher with the probiome at both HT (HTC vs HTP) and RT (RTC vs RTP), however the effects of probiome on bacterial communities were only statistically significant (p-value < 0.05) at RT. The alpha diversity indices (Chao1 and Shannon indices) of PVC-biofilm bacterial communities were lower at HT than RT (HTC vs RTC), but the differences were not statistically significant (p-value > 0.05) (Figure 5.3). When comparing the effects of pipe materials on bacterial community, the alpha-diversity was slightly higher in PVC samples in comparison to Cu samples at both HT and RT (Table: S5.9). Introduction of the *L. pneumophila* to the probiome had no statistically significant effect on the BCC (Two sample T-test of α-diversity indices, Chao1: p-value: 0.75294 and Shannon: p-value: 0.62982).

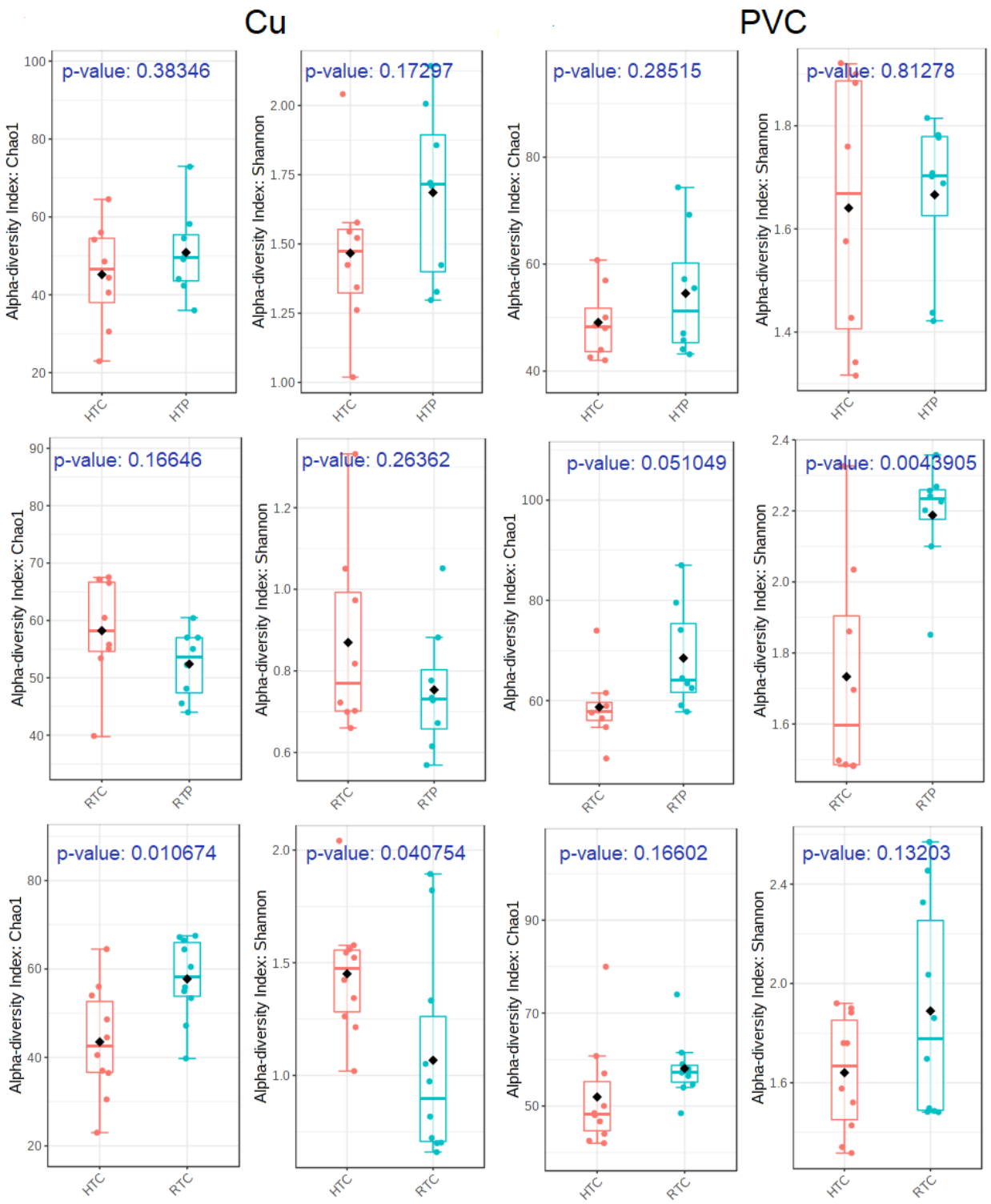


Figure 5.3: Alpha diversity indices (Chao1 and Shannon indices) of Cu and PVC biofilms with/without upstream probiome under HT (Control=HTC, probiome=HTP) and RT (RTC and RTP).

5.4.9 Beta diversity

The PCoA analysis with the Jaccard distances showed that after the introduction of *L. pneumophila* to the Cu-biofilms, the probiome changed the bacterial community membership. The PERMANOVA analysis indicated that the differences between the bacterial communities on Cu with and without the probiome were statistically significant at HT (p-value < 0.04) but not at RT (p-value < 0.182). The temperature played a major role in selecting the BCC on Cu surface. The biofilm samples on Cu clustered separately for HT and RT and the differences in bacterial community membership were statistically significant (p-value < 0.001). Moreover, the introduction of the *L. pneumophila* affected the BCC more at HT than at RT. Interestingly the differences between the bacterial communities on PVC with and without the probiome were statistically significant at RT (p-value < 0.008) but not at HT (p-value < 0.111). However, similar effects of temperature on the BCC of PVC biofilms were observed. The biofilm samples on PVC clustered separately for HT and RT and the differences in bacterial community membership were statistically significant (p-value < 0.001). The introduction of the *L. pneumophila* also affected the BCC in PVC biofilms more at HT than at RT (Figure 5.4).

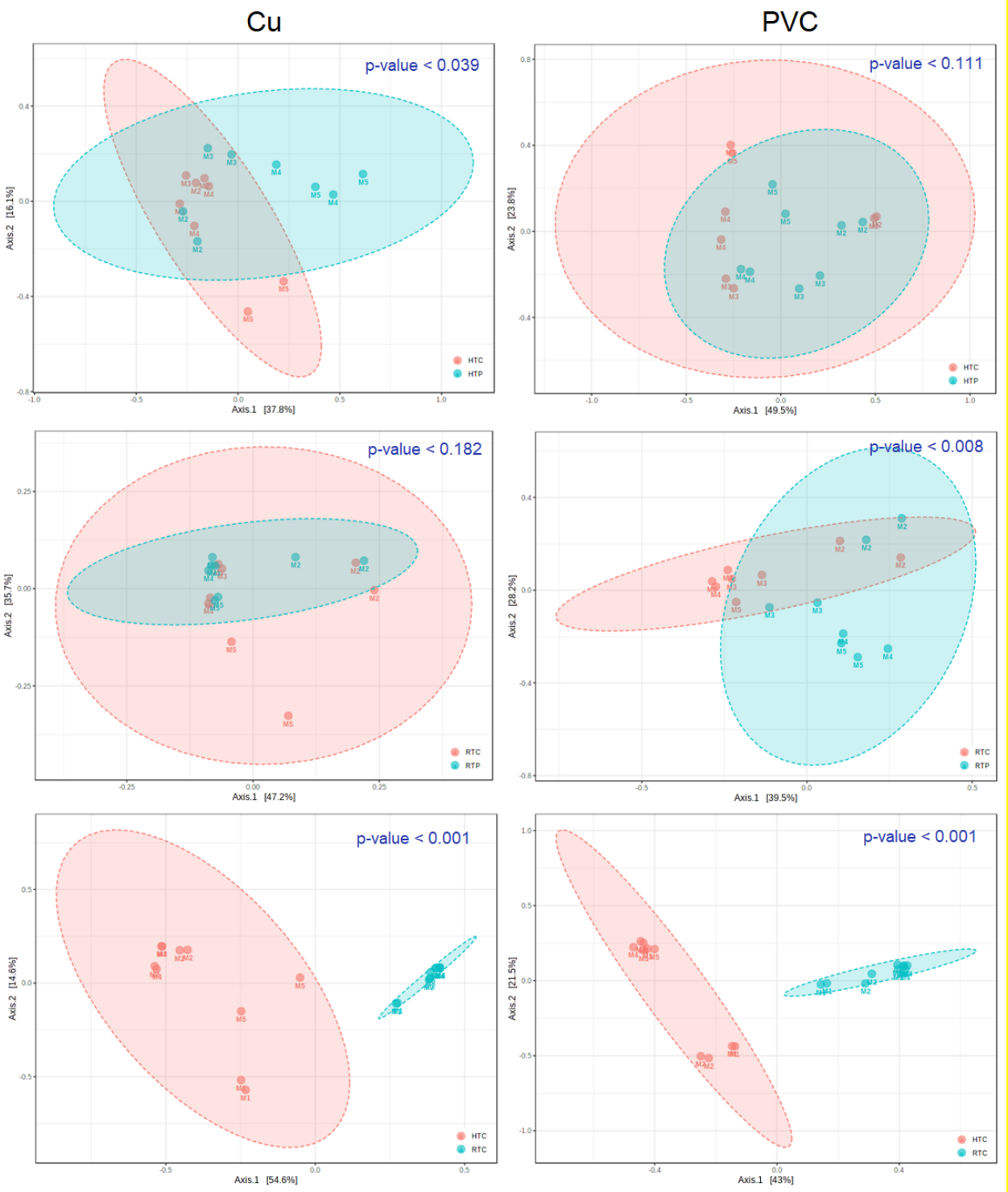


Figure 5.4: Beta diversity (PCoA plot of the Jaccard index) of Cu and PVC biofilms (on different months M1-M5) with/without upstream probiome at HT (Control=HTC, probiome=HTP) and RT (Control=RTC and probiome=RTP).

The month-wise comparison of the beta diversities of Cu and PVC biofilms at HT and RT conditions revealed that the introduction of *L. pneumophila* shifted the biofilm BCC on both pipe materials and the shifts were stronger at HT that at RT. Moreover, at RT the BCC stayed very similar during the subsequent months (M2-M5) with or without the probiome but at HT the effect of *L. pneumophila* continued and shifted the BCC further. The PCoA plots for the Jaccard index (Cu: F-value: 1.6259, R-squared: 0.1567, p-value < 0.083; PVC: F-value: 2.1533, R-squared: 0.19749, p-value < 0.016) with PERMANOVA analysis indicated statistically significance in clustering pattern in ordination plots (Appendix C: Figure S5.10). The 5th month (M5) PVC biofilm BCC showed a reverse shift at HT condition. There was no significant change in the BCC of the probiome biofilm due to the introduction of the *L. pneumophila* as shown by the PCoA with PERMANOVA analysis using Jaccard index (F-value: 1.385; R-squared: 0.059227; p-value < 0.231).

5.4.10 Clustering, core microbiome and population level analysis

The HT and RT samples of both Cu and PVC biofilms were clearly separated in two clusters (Figure 5.5). Moreover, the first month samples (M1=Mar29) of both Cu and PVC biofilms at RT and HT (except one Cu biofilm sample 2HTCu2Mar29) were clustered closely in a separate sub-branch in the dendrogram - indicating very small variations within the replicates (between coupons and also between ARs).

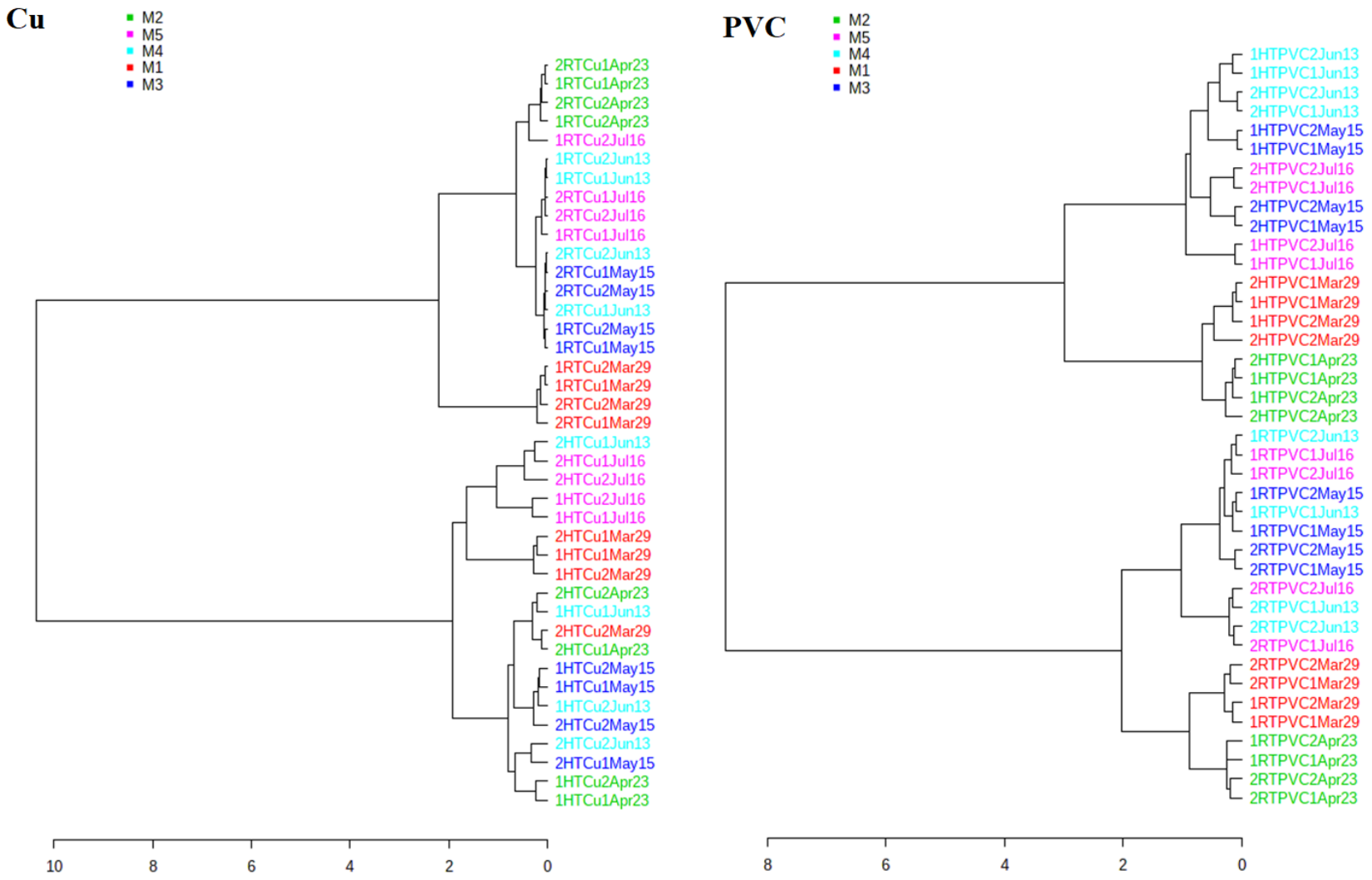


Figure 5.5: Hierarchical clustering of Cu and PVC biofilms at HT and RT. The biofilm samples at HT and RT were clustered on different branch for both pipe materials. The colors indicate the sampling months.

The second month samples (M2= Apr23) of PVC biofilm at RT and HT also formed their own clusters and have close branch distances from the respective M1 clusters. The PVC biofilms at RT and HT on the other months were clustered on the distant branch from the M1 and M2 clusters with M4 the most distant from the M1 and M5 near to M3 and M1 at HT. For Cu biofilms, the effect of *L. pneumophila* was quite drastic and shifted the BCC to cluster the M2 samples to the most distant branch from M1 cluster under both temperatures. The Cu biofilms also exhibited a strong recovery from the shock of *L. pneumophila* introduction as the M5 samples for both with/without probiome had a close branch distance from the M1 samples.

The core microbiome analysis identifies the community members that are consistently present within the specific group of samples (Appendix C: Figure S5.11). The heat map of different sample groups showed the differential prevalence of biofilm bacterial members on different pipe materials, under two temperatures and with/without the probiome. In Cu-biofilms without probiome at HT, *Legionella* was present as a core member of the bacterial community, but not in samples under the effect of probiome. However, *Mycobacterium* and *Fimbriiglobus* were present as core microbiome only in the samples with probiome. At RT, *Reyranella* and *Aquabacterium* were replaced by the probiome with *Phreatobacter* and *Fimbriiglobus*. In PVC-biofilms at HT and RT, the probiome did not affect the core microbiome except introducing the genus *DSSD61* and *Blastomonas* to biofilm community at RT.

The correlation network analysis at genus level for Cu-biofilms at HT showed reduced *Legionella* and significantly higher presence of *Mycobacterium* with probiome, although they did not have any inter-correlation. In PVC-biofilms at HT, no affect of probiome on *Legionella* and *Mycobacterium* was observed and there was no inter-correlation between these two genera. However, the probiome increased the concentration of *Methylobacterium_Methylorubrum* which

had a positive correlation with an unclassified Beijerinckiaceae. Interestingly, *Legionella* showed a negative correlation with this Beijerinckiaceae (Appendix C: Figure S5.12).

The population level study, LEfSe analysis (P= < 0.05) revealed only a few genera that were significantly different in Cu and PVC biofilms due to the probiome at HT (Only HT samples were analyzed since *Legionella* colonized better at this temperature) (Figure: 5.6). Eleven OTUs were significantly different (P= < 0.05) in Cu biofilms due to probiome. The probiome used in this study decreased the *Legionella* colonization but interestingly increased the concentration of *Mycobacterium.* In PVC biofilms only two OTUs were significantly different (P= < 0.05) and the probiome increased the *Methylobacterium_Methylorubrum* and decreased a genus of *Obscuribacteraceae* in the biofilms.

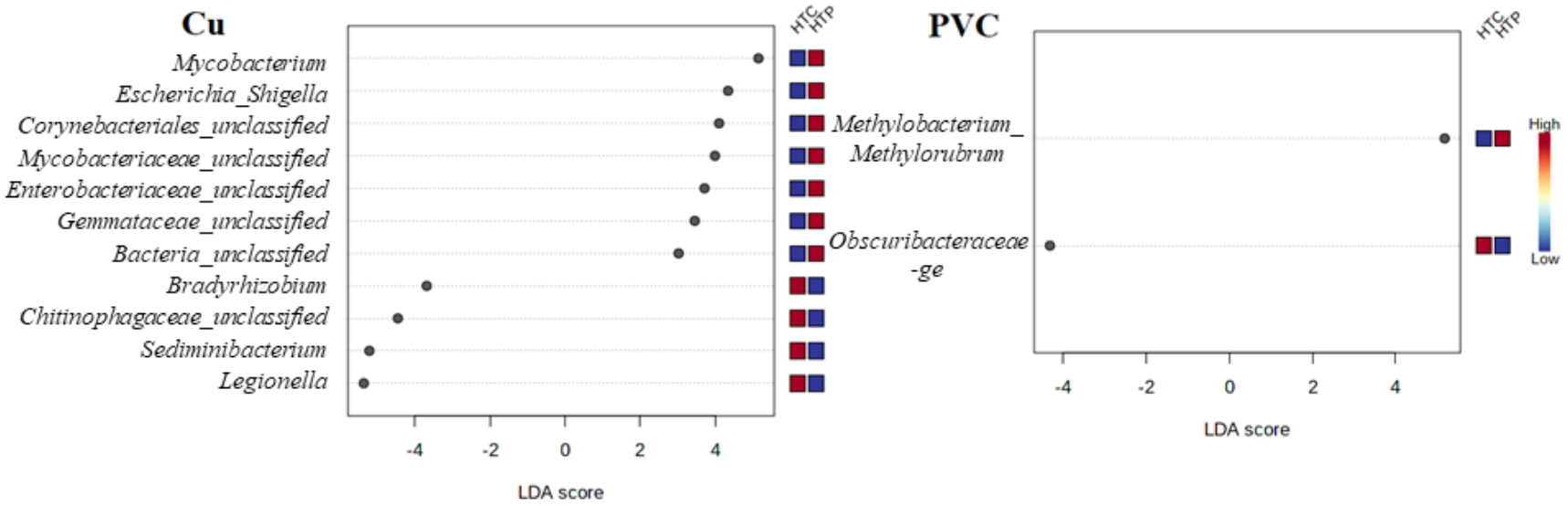


Figure 5.6: LEfSe analysis indicating the genera occurred differently (P= <0.05) due to probiome in Cu and PVC biofilms at HT.

5.5 Discussion

A comprehensive understanding of the microbial ecology within PP is critical to develop sustainable strategies to control the growth of opportunistic premise plumbing pathogens (OPPPs). In the current study, a holistic approach was taken to compare the BCC at the genus-level of water- biofilms on the most commonly used pipe materials, Cu and PVC, at temperatures representing cold and hot water systems and to follow up the impacts of a simulated influx of pathogenic *Legionella* spp. To assess mitigation measures, the effects of a probiotic intervention was studied on biofilms on Cu and PVC surfaces predeveloped in AR, based on earlier evidence that colonization of *L. pneumophila* was not favored in PVC biofilms (Buse et al. 2014b).

Finding Alphaproteobacteria as the most dominant bacterial community members in the biofilm samples was consistent with previous studies (Buse et al. 2017, Buse et al. 2014a, Gerrity et al. 2018). The coverage for all the samples was above 99.9% which indicates an adequate sequencing depth was achieved for the samples. Although the same water source (same container) was used for feeding both Cu and PVC annular reactors at HT and RT, the difference in BCC of water samples suggests that the source bacterial community was modulated by pipe materials and temperature (Figure 5.1). Moreover, the high prevalence of *Aquabacterium* only in water samples from ARs with PVC at RT indicated that the biofilms also affected the water, presumably through growth and erosion/sloughing of bacteria from biofilms to water. A high abundance of *Aquabacterium* in PVC biofilms was also interesting given this genus has a putative ability to use vinyl chloride as a carbon source (Wilson et al. 2016). Cu and PVC differentially supported colonization of the water bacteria to develop biofilms and temperature modulated this colonization further - a similar observation has been reported (Buse et al. 2017).

Phreatobacter, a very common water bacterium (Baek and Choi 2019, Perrin et al. 2019), was found in Cu-pipe biofilms at a very high RA under HT. *Phreatobacter* has been isolated from ultrapure water from a power plant and from a piece of wood in a lava cave and has high tolerance to disinfection (Li et al. 2020a, Ma et al. 2020) and high temperature (Lee et al. 2017, Tóth et al. 2014). The higher richness (Chao1 index) in water than in biofilms suggests that not all bacteria present in the water did not colonize to develop biofilms. However, the Shannon indices suggest that the dominant taxa were equally present in water and biofilms.

It was interesting that the probiome biofilms (grown on PVC granules) had a bacterial community profile dominated by Gammaproteobacteria (94% of the total bacteria), with a high abundance of *Aquabacterium* (86% of the Gammaproteobacteria). Previous studies has reported Alphaproteobacteria as the most dominant class in water biofilms on commonly used pipe materials as seen for the PVC and Cu biofilms of this study (Buse et al. 2017, Gerrity et al. 2018, Lu et al. 2014, Potgieter et al. 2018, Stüken et al. 2018). The high presence of *Aquabacterium* in the probiome could be due to the fact that the virgin PVC granules were different from PVC pipe as it has not gone through the process required to make pipe with it. Nonetheless, *Aquabacterium* is a common member of drinking water biofilm and has also been reported in natural spring water (Bachmann and Edyvean 2006, Chen et al. 2012, Kalmbach et al. 2000). The genus *Legionella* was present in both probiome modules (Con and Lp-Probiome) but only at a RA of 0.1% of the total biofilm bacteria; suggesting that the introduced *L. pneumophila* did not persist within the predeveloped biofilms (van der Kooij et al. 2017) for greater than four months but its introduction had a long-term effect on the BCC of the probiome biofilms, as indicated by the variation in Alphaproteobacteria compositions of the two probiome modules. Interestingly, there was no apparent impact observed on *Aquabacterium,* the most prevalent Gammaproteobacteria

member in the probiome. A similar minimal colonization by introduced *L. pneumophila* in biofilm has been reported previously for unplasticized PVC at RT (Buse et al. 2014b, van der Kooij et al. 2017).

Another consistent finding with previous studies was that PVC supported more microbial richness than Cu pipe material (Bucheli-Witschel et al. 2012, Buse et al. 2017, Buse et al. 2014a, Jang et al. 2011, Lehtola et al. 2005, Lu et al. 2014, Mathys et al. 2008, Morvay et al. 2011, Yu et al. 2010). *Qipengyuania, Phreatobacter, Blastomonas, Sphingomonas, Aquabacterium, Sediminibacterium* and *Gemmata* etc. were the most prevalent genera in the biofilm samples and have been reported as the common drinking water bacterial community members. *Qipengyuania* has been reported to be isolated from permafrost in China (Feng et al. 2015) and other cold temperature environments (Li et al. 2020b), thus reflect the drinking water source of the current study given that the source originated in the Canadian Rocky Mountains. Both *Sediminibacterium* and *Aquabacterium* have been linked to vinyl chloride biodegradation (Wilson et al. 2016) and were dominant bacterial members in PVC biofilms compared to Cu biofilms. *Aquabacterium* was mainly present at RT and *Sediminibacterium* at HT, suggesting different optimum growth temperatures and possibly different metabolic pathways for vinyl chloride utilization. At HT, decreased *Sediminibacterium* in PVC biofilms with upstream probiome compared to without probiome was interesting since *Sediminibacterium* would be expected to colonize better for its capability.

Sphingomonas was a dominant genus in water and biofilms, especially at RT and *Sphingomonas* spp. isolated from drinking water has been reported to have high intrinsic antibiotic resistance (Vaz-Moreira et al. 2017). Horizontal gene transfer of antimicrobial resistance is possible between actively growing biofilm bacteria (Abe et al. 2020, Schwartz et al.

2003), while an assessment of the resistome (antibiotic resistance genes) was not part of the current work but the need to address this issue is noted. *Methylobacterium* was found at higher abundance in PVC biofilms than on Cu and has been recently described as an emerging OP (Kressel and Kidd 2001, Szwetkowski and Falkinham 2020) and therefore, its biology in drinking water requires a further study.

Compared to the previous study (Buse et al. 2014a), it was very interesting that the introduction of *L. pneumophila* caused a major disruption in the BCC of both Cu and PVC biofilms at HT with a significant change in RA of the most dominant genera. This observation supported our hypothesis about the possibility of monitoring alternative bacteria as a better tool for determining LD outbreak conditions. It was also interesting to observe that the probiome recovered the original balance of the bacterial community in PVC biofilm better in comparison to samples without probiome at HT. However, the probiome completely altered the dominance in bacterial genera in Cu biofilms at HT and was also reflected by the increased alpha diversity indices of the biofilm samples with probiome. At HT, decreased *Sediminibacterium* in PVC biofilms with probiome compared to without probiome was interesting since it was expected that probiome grown on PVC granules would increase the RA of *Sediminibacterium*.

Significant increase of *Gemmata, Porphyrobacter* and *Mycobacterium* due to the introduction of *L. pneumophila* and probiome in Cu biofilms should be investigated further, since *Mycobacterium* is a members of public health concern OPPPs and *Gemmata* has been reported to be found frequently in hospital water (Aghnatios and Drancourt 2015) and potentially phagocytosed by common amoebae present in drinking water (Kaboré et al. 2018). *Porphyrobacter* is considered as a secondary colonizer in PP (Douterelo et al. 2014) and utilizes metabolites from initial colonizers (Szewzyk et al. 2000). At RT *Aquabacterium* was decreased

significantly in PVC biofilms due to the introduction of *L. pneumophila*, especially with probiome, although the probiome had *Aquabacterium* as the most dominant genus. The high presence of *Legionella* in M2 samples at HT could be due to the transient colonization of introduced *L. pneumophila*. At HT, Cu appeared to support more legionellae as reported previously for *L. pneumophila* that colonized with FLA on Cu surface better at 37 °C than at RT (Buse et al. 2017) and potentially grew in a water heater at 39 °C (Rhoads et al. 2015).

It was interesting that the probiome BCC (on PVC granules) was more similar to that of Norprene™ tube than on PVC pipe. In Norprene™ *Aqubacterium* and an unclassified Comamonadaceae were found in abundances similar to a previous report (Lu et al. 2014) and in the probiome biofilms, whereas *Sphingomona*s was dominated on PVC pipe material- suggesting a presence of common accessible chemicals in Norprene™ and PVC granule that selected *Aqubacterium*.

Comparing the biofilm BCC of building Cu pipe (PP) with Cu coupons in ARs at RT (grown without a residual disinfectant) most likely revealed the effect of residual chlorine (from monochloramine). *Nitrosomonas, Methylobcterium* (Order: Rhizobiales) observed in PP biofilms have been reported to be the most abundant genera in chlorinated drinking water (Potgieter et al. 2018, Stüken et al. 2018). DSSD61 is a non-culturable genus of Nitrosomonadaceae family, commonly present in drinking water. Presence of *Nitrosomonas* in building water was also indicative of monochloramine use as residual disinfectant since *Nitrosomonas* can oxidize ammonium which is required to generate monochloramine from free chlorine and is also a product of monochloramine decay in water (Maestre et al. 2013).

The alpha diversity indices suggested that the probiome affected the Cu and PVC biofilms at RT and HT differently and probiome decreased the number and abundance of

dominant genera in Cu biofilm at RT and increased in PVC biofilms at RT and HT. It was hypothesised that the probiome developed on PVC granules at RT (different from on PVC coupons) introduced more genera that colonized to downstream PVC biofilms but interfered with Cu biofilm bacterial genera (competition among the same optimum growth temperature bacteria). But surprisingly, probiome increased the alpha diversity indices of Cu biofilm at HT, and possibly due to secondary colonization of some of the genera through an availability of different nutrients (providing more nutrients as RT bacteria decomposed or from their metabolites). The beta diversity indices and hierarchical clustering suggested that the bacterial community membership was modulated by temperature strongly and the probiome affected the predeveloped biofilms more at HT for Cu samples and at RT for PVC samples and helped to restore to some extent the original balance of the bacterial communities (Figure 5.4 and 5.5). The population level study suggested that Cu pipe could be more vulnerable to well-known OPPPs like *Legionella* and *Mycobacterium* whereas PVC pipe could be to emerging OPPPs like *Methylobacterium* at HT (Figure 5.6 and S5.12).

5.6 Conclusion

Overall, this study demonstrated that the same source water could produce different biofilms in hot and cold-water pipe surfaces in PP by differential colonization of the source water bacteria and the colonization could be further modulated by the pipe materials. Poorly managed hot water PP system should be of more concern for pathogenic *Legionella* as warm temperature (around 40 °C) favoured its colonization and Cu pipe increased the risk further. The biofilm bacterial communities tend to restore the original balance if disrupted due to growth or introduction of pathogenic legionellae. However, the strong bacterial community shifts by the

introduced *Legionella* suggested that there is scope for developing alternative monitoring (targeting fast-growing and less resource-demanding surrogate bacteria) strategies to predict high *Legionella* or other OPPPs presence in water, although a considerable amount of work is necessary to optimize and validate. A microbial community profile map of a system could also be useful to identify vulnerable zones within a PP and provide the foundation for developing better monitoring strategies for large building by applying the HACCP/WSP concept.

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Chapter 6

Chapter 6: Concluding remarks

6.1 Summary and conclusion

This study was intended to improve the understanding of the health risks associated with pathogenic *Legionella* spp. (that may be generalized for other saprozoic water-based opportunistic pathogens like non-tuberculous *mycobacteria* and *Pseudomonas* spp.) and to identify possible options for improved monitoring and control of these pathogens by understanding their ecology in engineered water systems. Novel aspects of this work included demonstrating in real-time the feeding behavior of different FLA species within the drinking water biofilm environment and uptake of pathogenic *Legionella* spp. in a multispecies complex environment, health significance of amoebal-released *Legionella*-containing vesicles and potential probiotic control of *L. pneumphila* within engineered water systems. Real time monitoring of FLA-*L. pneumophila* interactions within water-biofilms and in presence of other known bacteria indicated a possible mechanism how opportunistic water-based pathogens are being selected, enriched and grow within drinking water systems. The effects of abiotic factors of PP like the water-pipe materials, residual disinfectant and water temperature along with high presence of *L. pneumophila* on the water-biofilms microbial community suggested that PP microbial mapping for possible hazards or risk factors and to re-engineer such systems to minimize risks associated with these opportunistic respiratory pathogens. This research demonstrated the interactions of pathogenic *Legionella* spp. with common FLA and other bacteria in true water-biofilm environments and explained some of these interactions by experiments with controlled parameters and specific microorganisms.

Chapter 2 showed that FLA not only support pathogenic *Legionella* spp. by providing means for intracellular growth but also potentially assisting in transmission and initiating

infection by producing respirable-size vesicles containing numerous *Legionella* cells. These vesicle-bound *Legionella* are more protected from environmental stresses (desiccation kills *L. pneumophila* within 90 min (Katz and Hammel 1987) and hold enough bacterial cells to potentially serve as a single human dose for initiating infection. Thus, the current gold standard for determining *Legionella* spp. concentration in water by culture methods may also underestimate the vesicle-bound *Legionella* cells in addition to the inability to determine the VBNC cells. Although, qPCR may overestimate the risk by considering dead bacterial cells, it would be more practical to use this method as the VBNC *Legionella* cells are infectious and the FLA are the main growth support for pathogenic *Legionella* spp. in drinking water. Other enumeration methods like immunofluorescence-based assays capable of distinguishing total and viable cells were developed for *L. pneumophila* but never implemented as standard practice (Delgado-Viscogliosi et al. 2005, Parthuisot et al. 2011). The intracellular growth of *Legionella* spp. may occur at 20-40 °C and are affected by the optimal growth temperature of both the FLA and *Legionella* spp., but more by the FLA since it has to be in trophozoite state to support bacterial growth. The FLA-bacteria interactions should be more carefully considered for designing water systems for green buildings since reduced water temperatures and long holding periods may encourage these problematic interactions. The growth dynamics of FLA within engineered water systems and how they support the intracellular growth of pathogenic *Legionella* spp. and maintain an ecological balance remains unknown.

Chapter 3 demonstrated the long-term persistence of *Legionella* spp. in tap water and a possible preferential feeding behaviour of common water FLA which led to a hypothetical model of *Legionella* spp. persistence, growth and the cascade of events that could lead to the critical concentrations of *L. pneumophila* within drinking water biofilms in the water-pipe environment.

Although, common water FLA support the intracellular growth of pathogenic *Legionella* spp., they may not preferably feed on *Legionella* spp. in water-biofilm environments and play more active role in making *Legionella* cells intracellular within the trophozoites. This study also indicated a dose-response and possible environmental stimulus (temperature) to initiate the intracellular growth, since the intracellular *L. pneumophila* had been observed during the entire period of study.

Chapter 4 confirmed the preferential feeding behaviour of common FLA with known bacterial species, *E. coli* and *L. pneumophila* and demonstrated that *L. pneumophila* was not a preferred prey when other bacteria were present. The real-time tracking of the bacteria and their physical locations (intracellular or extracellular to FLA trophozoites) helped to identify in detail the periodic events of FLA-bacteria interactions and the fate of the bacteria and FLA trophozoites. It also strengthened the observation that FLA played the major role in initiating the FLA-bacteria interactions by preying on the bacteria, and only engulfed *L. pneumophila* if other non-amoeba resisting bacteria were unavailable as a food source.

Chapter 5 showed the effects of temperature and common water pipe materials on biofilm BCC and the colonization and persistent of *L. pneumophila* on predeveloped water-biofilms on different pipe materials at two different temperature. The variation in water-biofilm BCC due to temperature and residual chlorine indicated that a building-wise PP microbial mapping would be very helpful from a HACCP point to determine the robustness of the system and to help identifying vulnerable sites. It also demonstrated how a high concentration of *L. pneumophila* could modulate the water-biofilm BCC on different pipe materials and provided valuable information for potential of surrogate bacteria monitoring as an alternative monitoring strategy for system vulnerability. A novel aspect of this work was to demonstrate the influence of a

natural mature water-biofilms (a probiotic approach) on predeveloped mature biofilms on common PP pipe materials and *L. pneumophila* colonization and indicated that a fabricated well-designed probiotic may help to reduce *L. pneumophila* colonization and to maintain the health of PP.

6.2 Limitations and future directions

This study identified some of the key feature of *L. pneumophila* interactions with FLA and water biofilm bacteria. Chapter 2 showed the significance of vesicle-bound *Legionella* cells in initiating infection by providing a potentially infectious single human dose. The expression level of virulence genes varies in planktonic, biofilm, FLA-grown cells and in VBNC cells of the same *Legionella* spp. (Abu Khweek and Amer 2018, Mou and Leung 2018) and thus it is difficult to determine the true risk of a *Legionella* spp. by current risk assessment models (Kirschner 2016). A comprehensive analysis of the virulence genes expression by different *Legionella* spp. within aquatic environments (PP or EWS) and in different FLA would provide fundamental understanding of the true pathogenic potentials of *Legionella* spp. in association with environmental factors (biotic and abiotic).

Identifying all the controls in a long-term experiment is always difficult. *L. pneumophila* persists in water-biofilms in the presence of FLA for long time but it is still difficult to identify whether the persistence of *Legionella* spp. in water biofilms with FLA is virulence/intracellular growth mediated. A mutant of the same *L. pneumophila* strain (incapable of intracellular growth and containing different fluorescent markers) along with a virulent strain could help to address this question. Although culturable *L. pneumophila* was isolated from the water, the fluorescent signal of the planktonic or biofilm-associated bacteria was weak or lost after three months;

therefore, a constitutive chromosomal fluorescent gene (different color) would provide additional information. Moreover, a threshold level of effector proteins is required to initiate intracellular growth in trophozoites, thus a dose-response information of pathogenic *Legionella* spp. for different susceptible amoeba species could help to understand the growth dynamics in water environments.

A significant limitation in the current study is that only one *L. pneumophila* strain was used, therefore, it was not possible to make any firm generalized conclusions including the hypothesized growth model. Feeding preference of different FLA strains for various pathogenic and non-pathogenic species of *Legionella* spp. would help to understand the stochasticity of FLA-*Legionella* interactions.

The water-biofilm BCC was found to be influenced by abiotic factors like temperature and pipe materials in this study. however, the biofilms were developed with water containing no residual chlorine and chlorine does have effects on BCC as identified from PP biofilms. Therefore, setting up a set of annular reactors with common pipe material coupons would help to compare the effect of chlorine on biofilm BCC and on predeveloped probiome biofilms. The cost associated with annular reactors and overall bench-top water flow systems is a major limitation for designing a comprehensive experimental setup with more control and more replicates. Moreover, the biofilms studied here were developed with water from a single source and having a history of monochloramine treatment and hence, the microbiome could be biased to the source and the treatment process. In many countries, like in Netherlands, different disinfection processes (ozone or UV-disinfection) are used to treat drinking water and which have no residual activity in the distribution system, and also low concentrations of assimilable organic carbon (AOC) are maintained in water in distributions. Therefore these systems may have very different

microbial composition in water (Smeets et al. 2009). A large-scale coordinated drinking water microbiome project have been proposed for fostering collaborations across geographical boundaries and water utility settings for developing the best practices for drinking water microbiome research (Hull et al. 2019). The focus of this project is to ensure accuracy and reproducibility of meta-omics techniques to obtain a comprehensive understanding of the drinking water systems and to control drinking water microbiomes by developing interventions for better public health protection. However, the lesson learned from this study would be very valuable in the ecological characterization of DWDS and PP through a microbial community composition mapping with abiotic factor information and all together, this will help to develop a HACCP plan for EWS for emergency preparedness, ensuring water quality and public health safety. Moreover, the biofilm BCC and corresponding physicochemical conditions of water could help to identify the emerging problems associated with Green buildings in terms of water quality and OPPPs and to make design changes to obtain the goal of ensuring water safety.

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Appendices

Appendix A: Supplementary information for chapter 3

Material and methods

1. qPCR assay for *L. pneumophila*

The assay targeting the macrophage infectivity potentiator gene (*mip*) was used to quantify the *L. pneumophila*. 20 µL qPCR reactions were performed on the ABI 7500 Fast Real Time thermocycler containing 15 µL Mastermix (final concentrations of 1X PrimeTime Gene Expression Master Mix (IDT), 200 µg/mL Bovine Serum Albumin (Sigma), 300 nM forward and reverse primers, 100 nM probe, nuclease free water (Life Technologies)) plus 5 µL of template using MicroAmp Optical 96-well Reaction Plates (Life Technologies). The results were analysed with the threshold set to 0.05 and setting the reporter/quencher as VIC/TAMRA using v2.0.6 of the ABI 7500 software. The cycling conditions: 95°C for 3 minutes (holding) then 40 cycles of (95°C for 5 seconds & 60°C for 30 seconds). The assay includes standards of known copy number Standards = pIDT Smart Mentasti mip-1 (50,000/5000/500/50/5/0.5 copies/rxn, each in triplicate) and no template control for each PCR reaction to assure the reaction is contamination free. The LOD50 (limit of detection) is 2.8 and the LOD95 is 10.

Final concentrations of forward primers (*F*), reverse primers (*R*), and hybridisation probes (*H*) used in this study:

Name	Sequence	Final Concentration
L.pneu_Mentasti_mip-F	GAAGCAATGGCTAAAGGCATGC	300 nM
L.pneu_Mentasti_mip-R	GAACGTCTTTCATTTGYTGTTCGG	300nM

L.pneu_Mentasti_mip-P	HEX-CGCTATGAGTGGCGCTCAATTGGCTTTA-BHQ1	100 nM

Primers, probe, mastermix are all from IDT

2. DNA extraction from filters using EPA method 1611

The water sample filters from the -80 °C freezer were transferred to appropriately labeled fresh 1.5–ml tubes containing glass beads (Gene-rite Sorenson Sigma # G-1277). 600 µl of AE buffer (Qiagen Cat. No. 19077) was pipetted to each tube. The tubes are then secured on the tube holder of the Bead Mill (Bead Mill 24, Fisher scientific) and ran for 1 cycle of 1 min at a speed 3.1 m/s. 350 µL of supernatant from the bead tube(s) was transferred to the corresponding labeled 1.5 mL tube(s). To remove glass beads (may be transferred during this step), the tubes were centrifuged for 5 minutes at 12,000 x g. 250 µL of supernatant was transferred from the 1.5 mL tube(s) into the final labeled 1.5 mL tube(s) for qPCR assay. Appropriate amount of salmon testes DNA (10 µg/mL) was used as an internal control to determine the extraction efficacy.

Table 1:

Sampling of water from 2.0 L-glass water reservoir

Time	Volume	Total volume	Tests
Week 0	3 days × 5 ml	15 ml	Plate count (CFU)
Week 5	3 days × 5 ml	15 ml	Plate count (CFU)
Week 86	1 day × 40 ml	40 ml	Recovery of Amoeba

Week 86	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Week 87	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Week 88	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Week 89	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Week 90	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Week 91	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Week 92	1 day × 40 ml	40 ml	Recovery of Amoeba
Week 92	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Week 94	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Week 96	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Week 98	1 day × 40 ml	40 ml	Recovery of Amoeba
Week 98	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Week 99	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Week 100	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Week 104	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Week 108	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Week 116	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Week 120	1 day × 40 ml	40 ml	Recovery of Amoeba
Week 120	5 days × 11 ml	55 ml	Plate count (CFU) + qPCR of *mip* gene (GU)
Water withdrawn		1070 ml	
Water remained		930 ml	

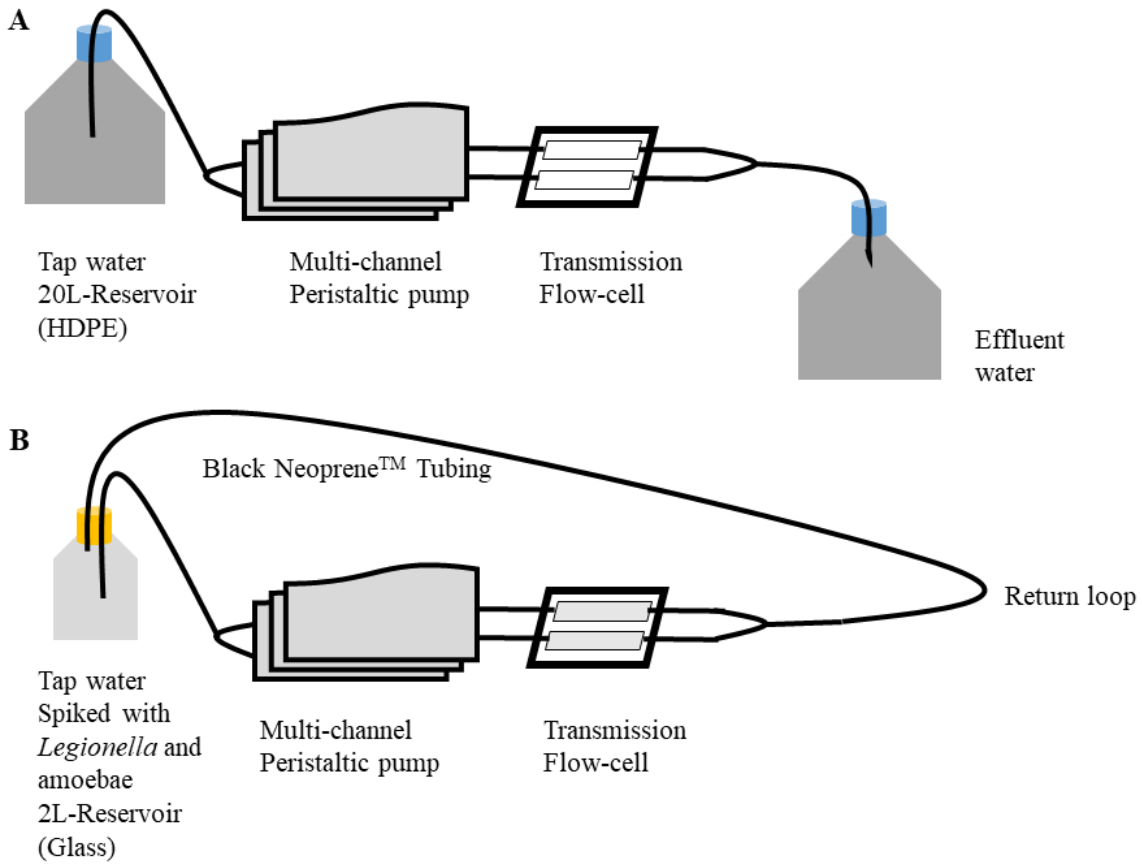

Figure S3.1: (A) The water-flow system with a dual channel transmission flow-cell. The tap water (with no residual chlorine) was pumped from a 20 L- reservoir through the flow-cell to developed natural tap water biofilms. (B) The water-flow system with the flow-cell and tubing was disconnected from the 20 L-reservoir after 6 months and connected to a 2 L-reservoir containing *L. pneumophila* and two amoeba species and the water was circulated as a closed-loop system.

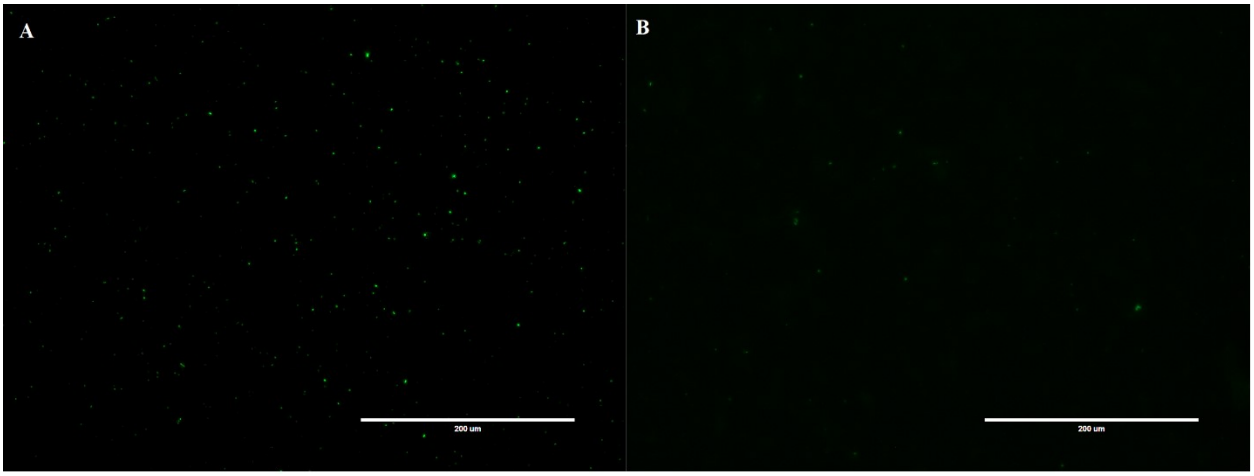

Figure S3.2: High number of *L. pneumophila* cells (green) distributed evenly on the water-biofilms due to sedimentation of free planktonic cells after 7 d of stagnation. (B) Most of the deposited *L. pneumophila* cells (green) on the biofilms were washed away 15 min after resuming the water flow.

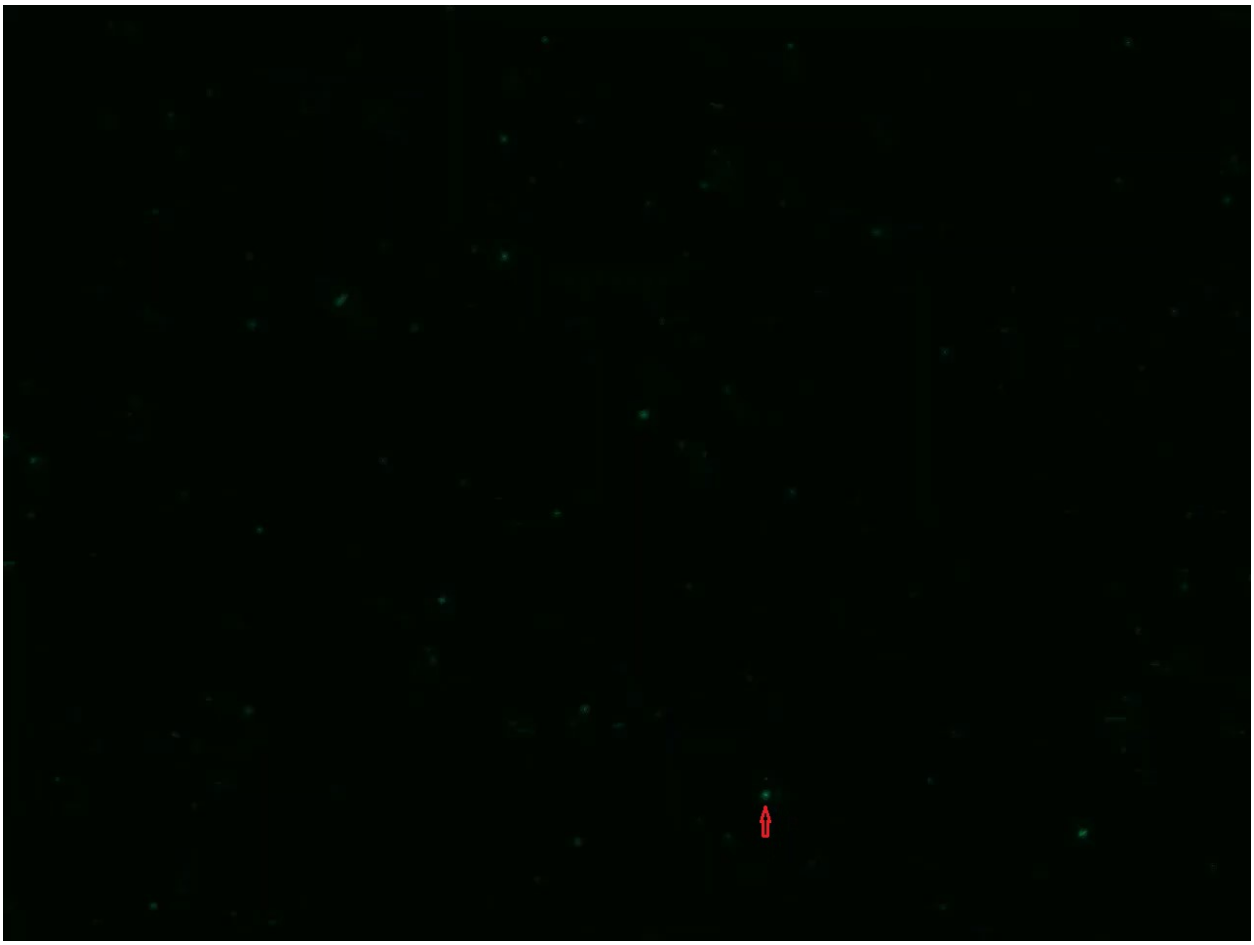

Figure S3.3: *L. pneumophila* cells (green) distributed evenly on the predeveloped tap-water biofilms on standard microscope glass-coverslips of the flow-cell system after 10 d of stagnation were swept away after resuming water flow. This indicated poor attachment and initial colonization of *L. pneumophila* cells to pre-developed natural biofilms. Only few cells showed relative resistance to the water flow (indicated by red arrow).

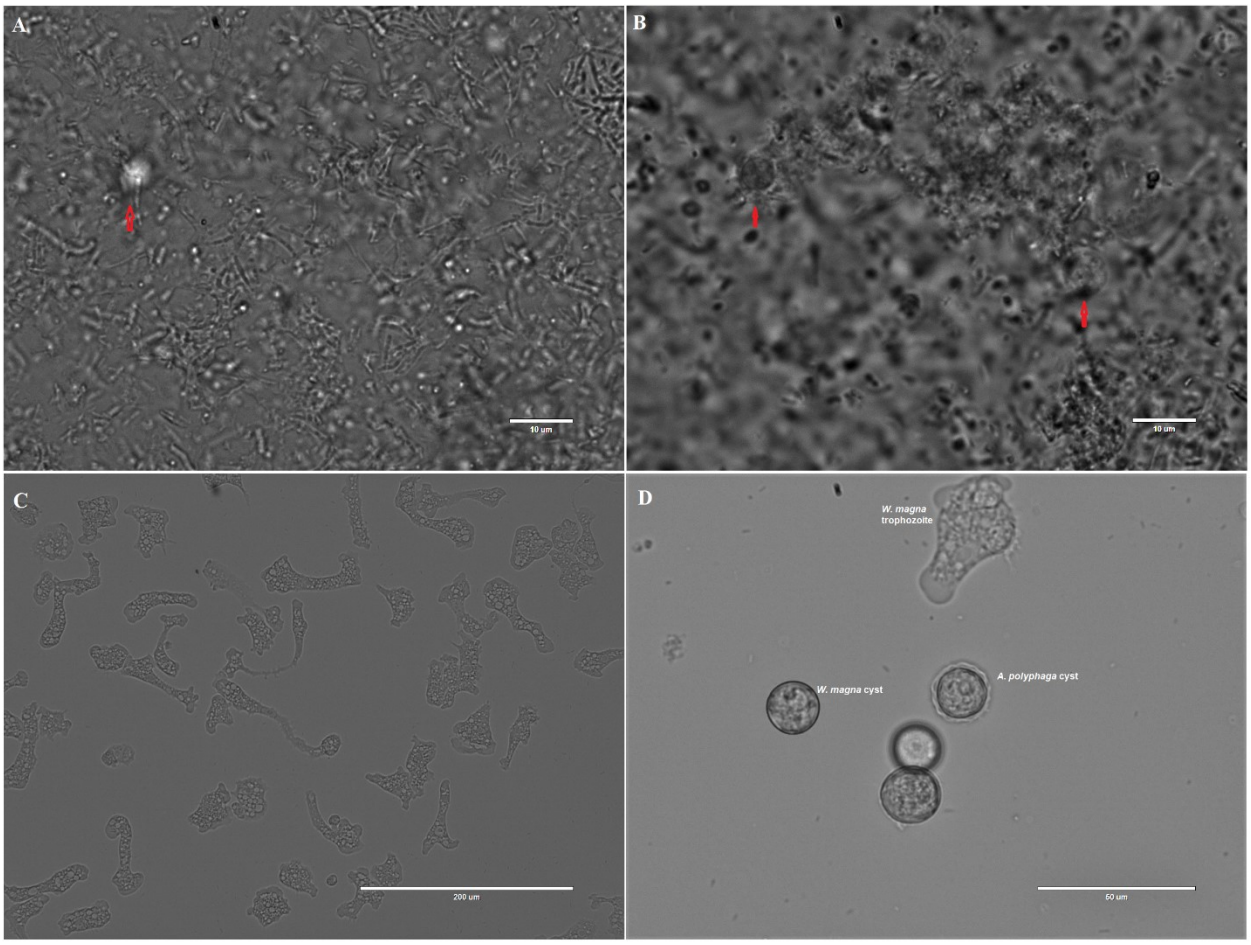

Figure S3.4: (A) Near surface image of natural tap water biofilm with amoeba cysts. (B) Image of the floating biofilm attached to the glass surface with amoeba cysts (same field of view of image A). (C) *W. magna* trophozoites (grown in SCGYEM) in pure culture in tap water. (D) Cysts of the two studied amoeba species in tap water.

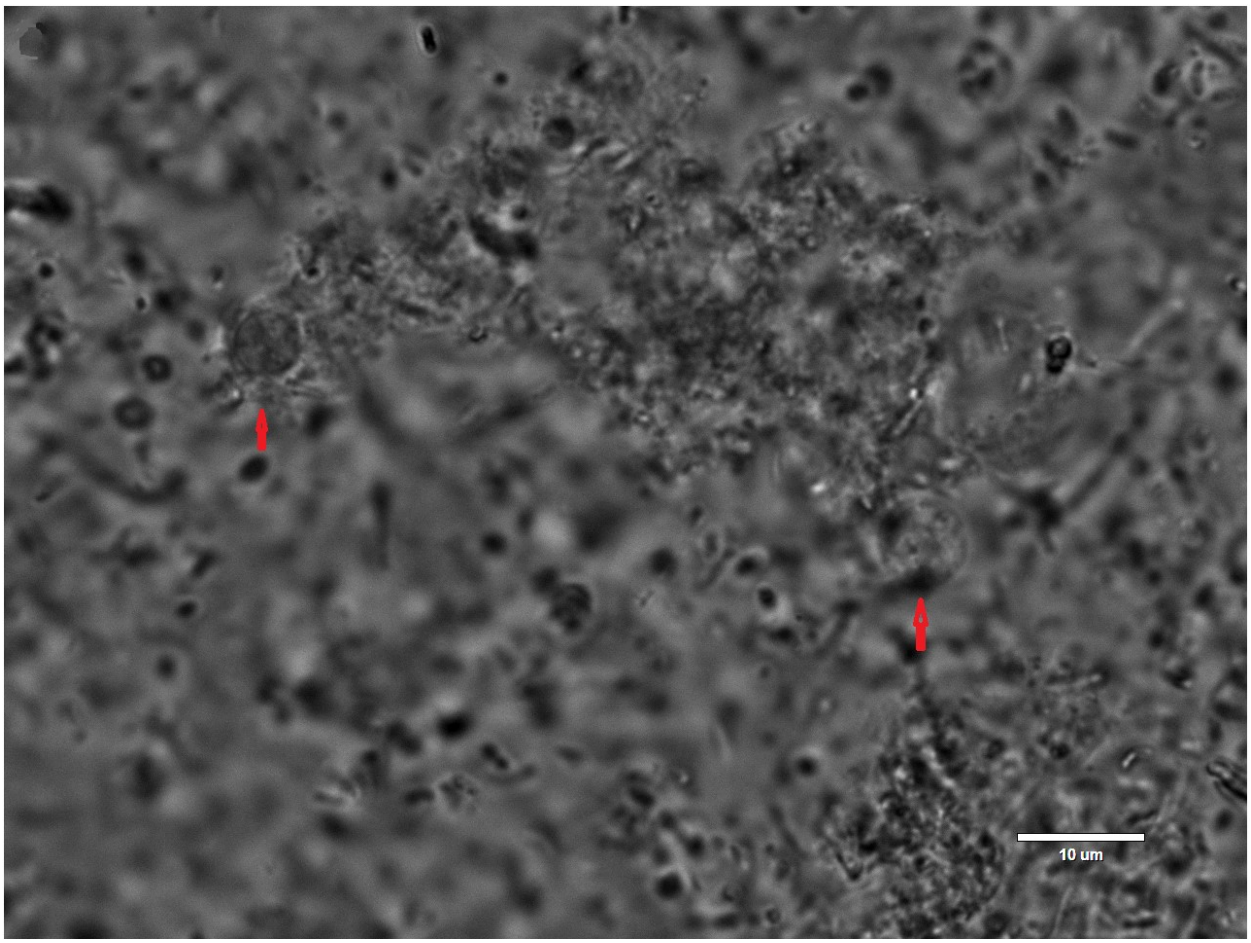

Figure S3.5: Z-stack of 39 images (taken with 0.903 µm Z- axis resolution) of the same filed-of-view illustrating a three-dimensional structure of the tap water biofilm with impregnated amoeba cysts.

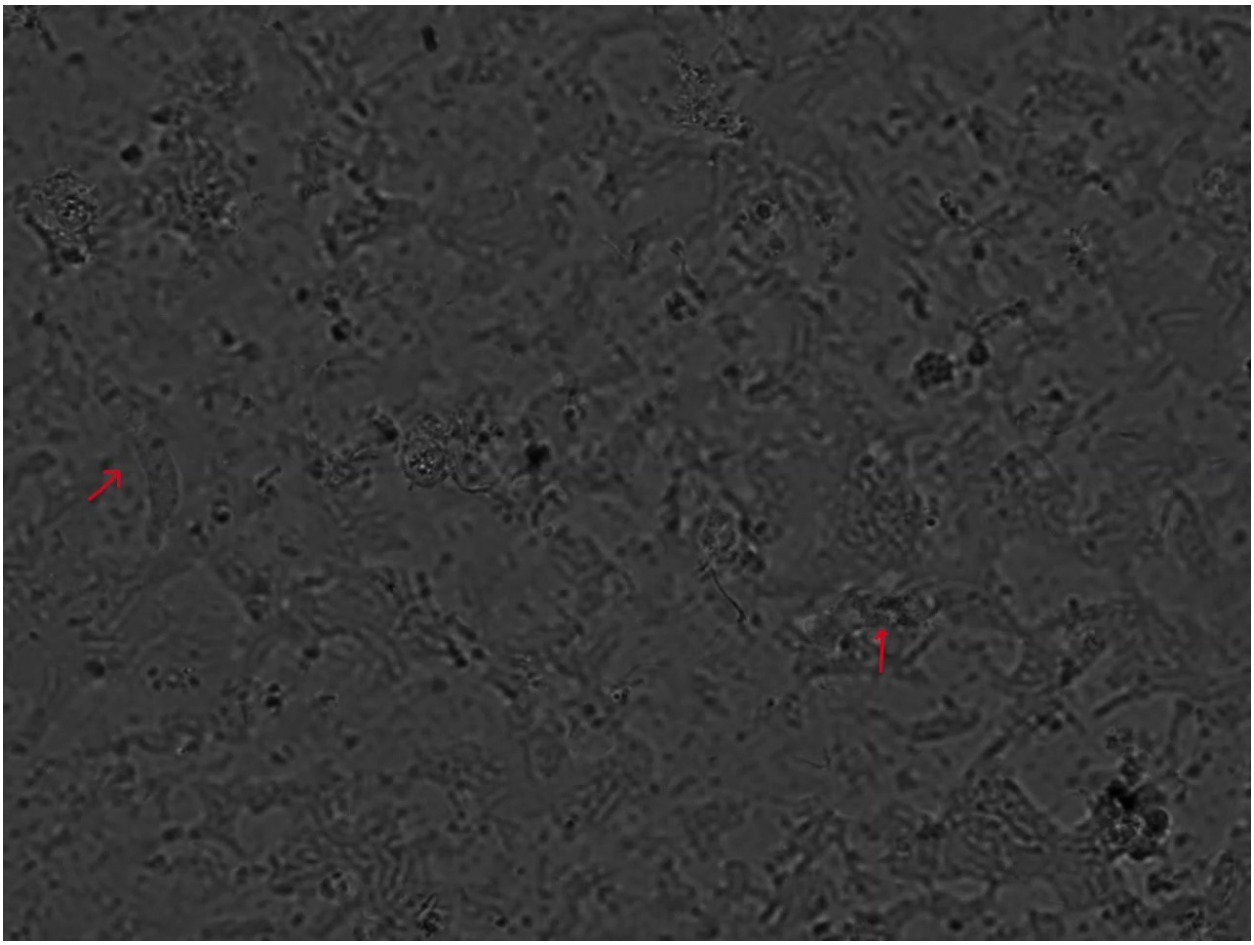

Figure S3.6: Freely moving *W. magna* trophozoites on pre-developed water-biofilms after a month of their introduction to the flow-cells system.

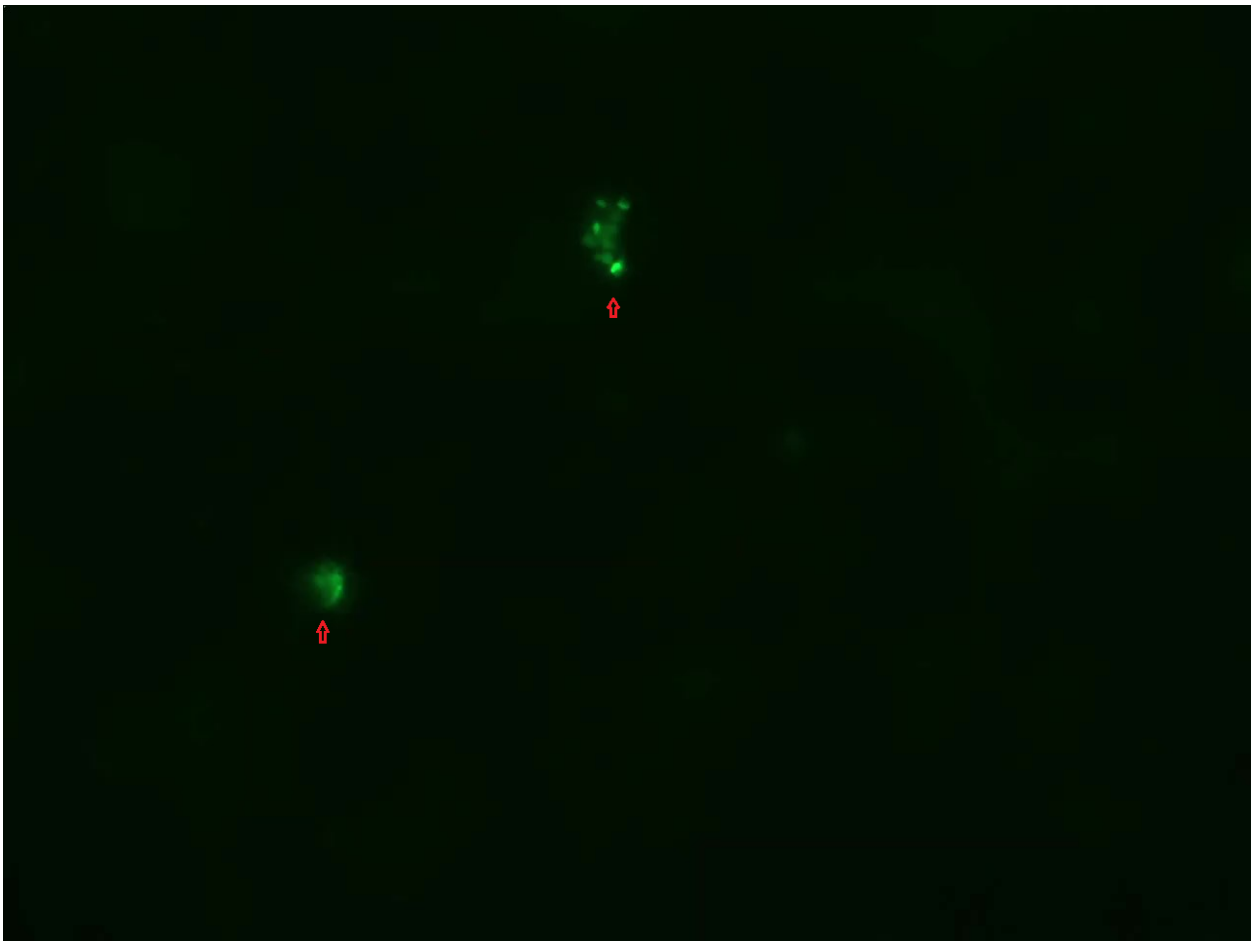

Figure S3.7: *W. magna* trophozoites with Fluorescent *L. pneumophila* freely roaming on pre-developed water-biofilms after a month of their introduction to the flow-cell system.

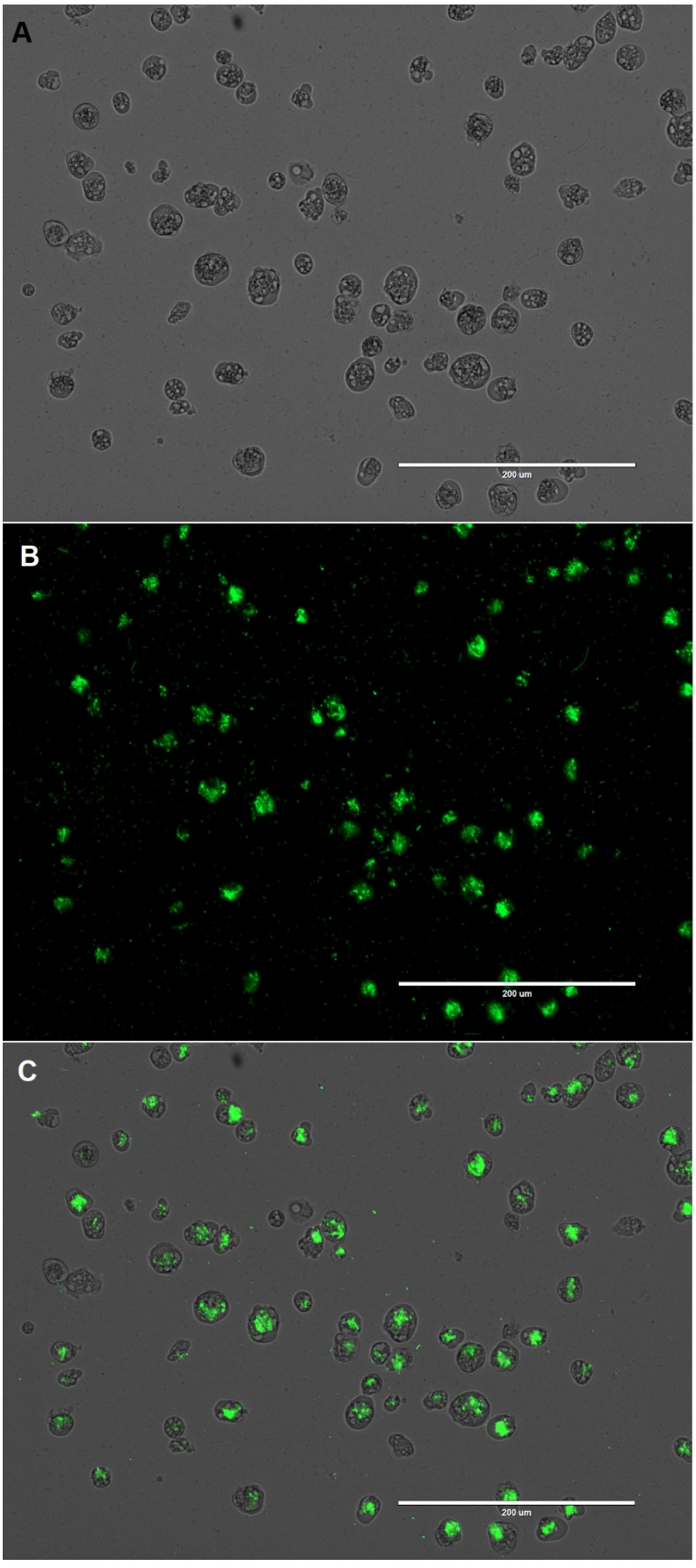
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Figure S3.8: *W. magna* - *L. pneumophila* interactions in pure culture in filter-sterilized tap water at RT. *W. magna* trophozoites readily phagocytosed *L. pneumophila* cells and become round shaped (trophozoites no longer exhibited amoeboid movement and *L. pneumophila* initiated intracellular growth). (A) Transmission light mono color image (B) Image under green fluorescent channel and (C) composite image of the two channels.

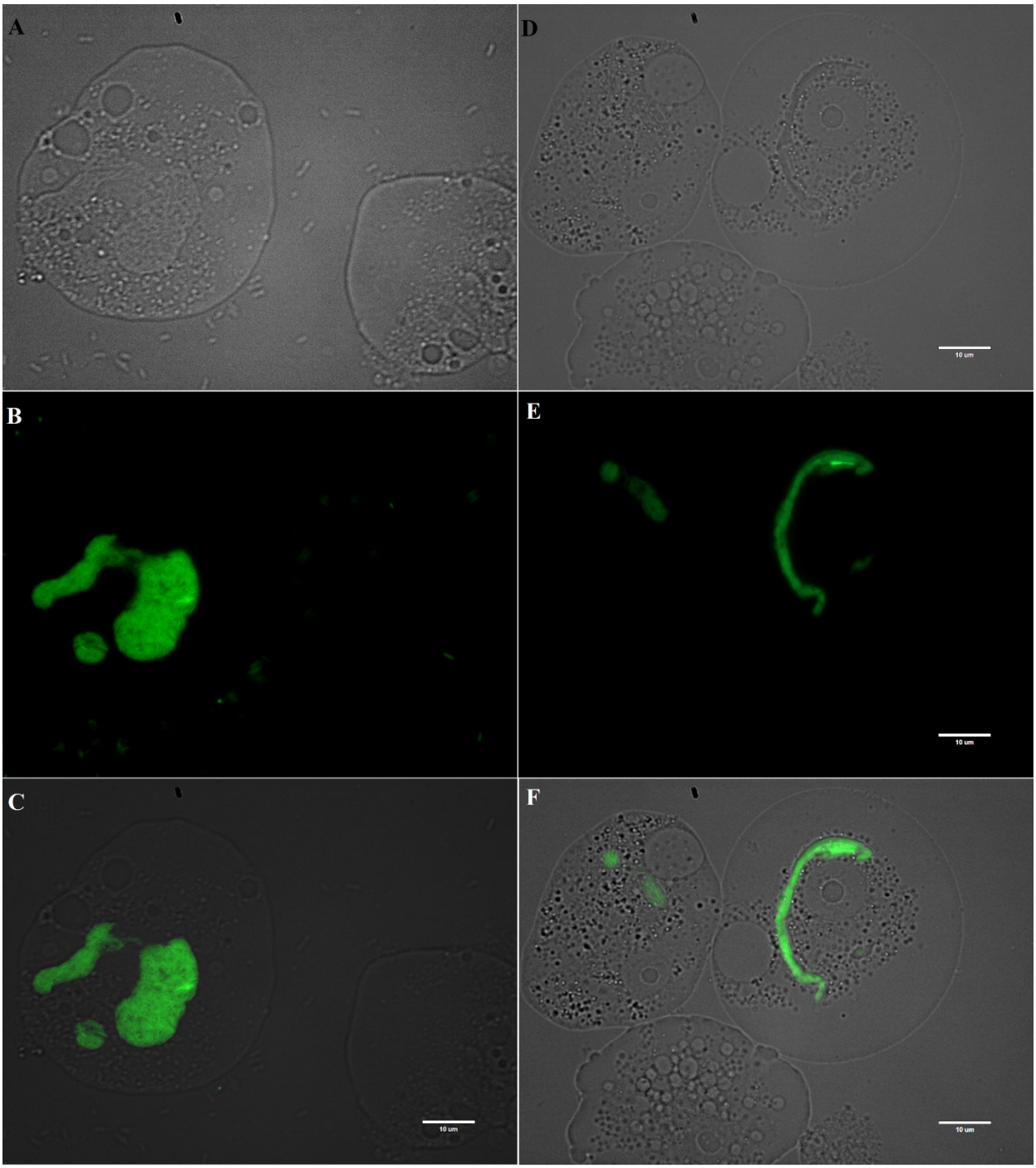

Figure S3.9: Different stages of Intracellular growth of *L. pneumophila* within *W. magna* trophozoites in filter-sterilized tap water. The cluster of individual green fluorescent *Legionella* cells were visible within the trophozoites. (A, D) Transmission light mono color image (B, E) Image under green fluorescent channel and (C, F) composite image of the two channels.

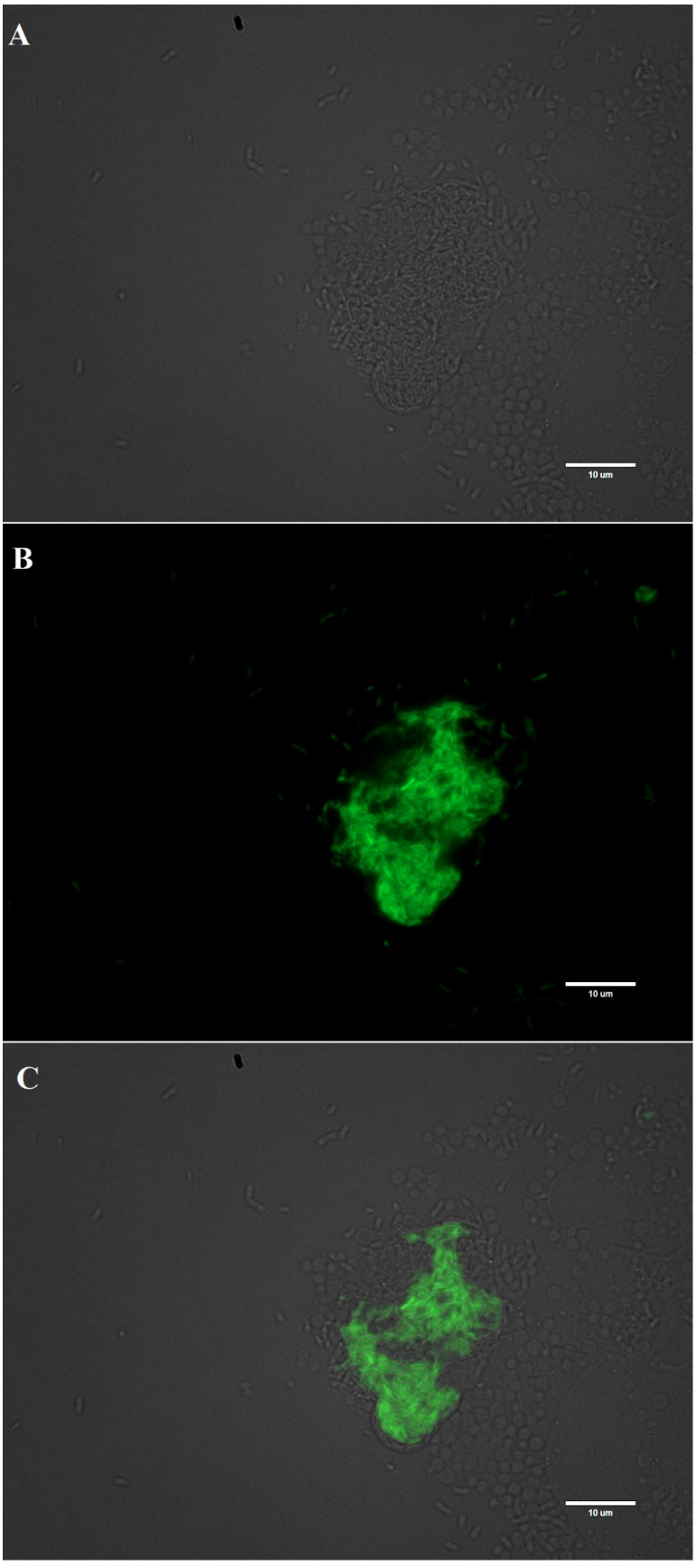
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Figure S3.10: A vesicle produced from *W. magna* during intracellular growth of *L. pneumophila* in filter-sterilized tap water at RT. The green fluorescent *Legionella* cells were visible within the vesicle. (A) Brightfield mono color image (B) Image under green fluorescent channel and (C) composite image of the two channels.

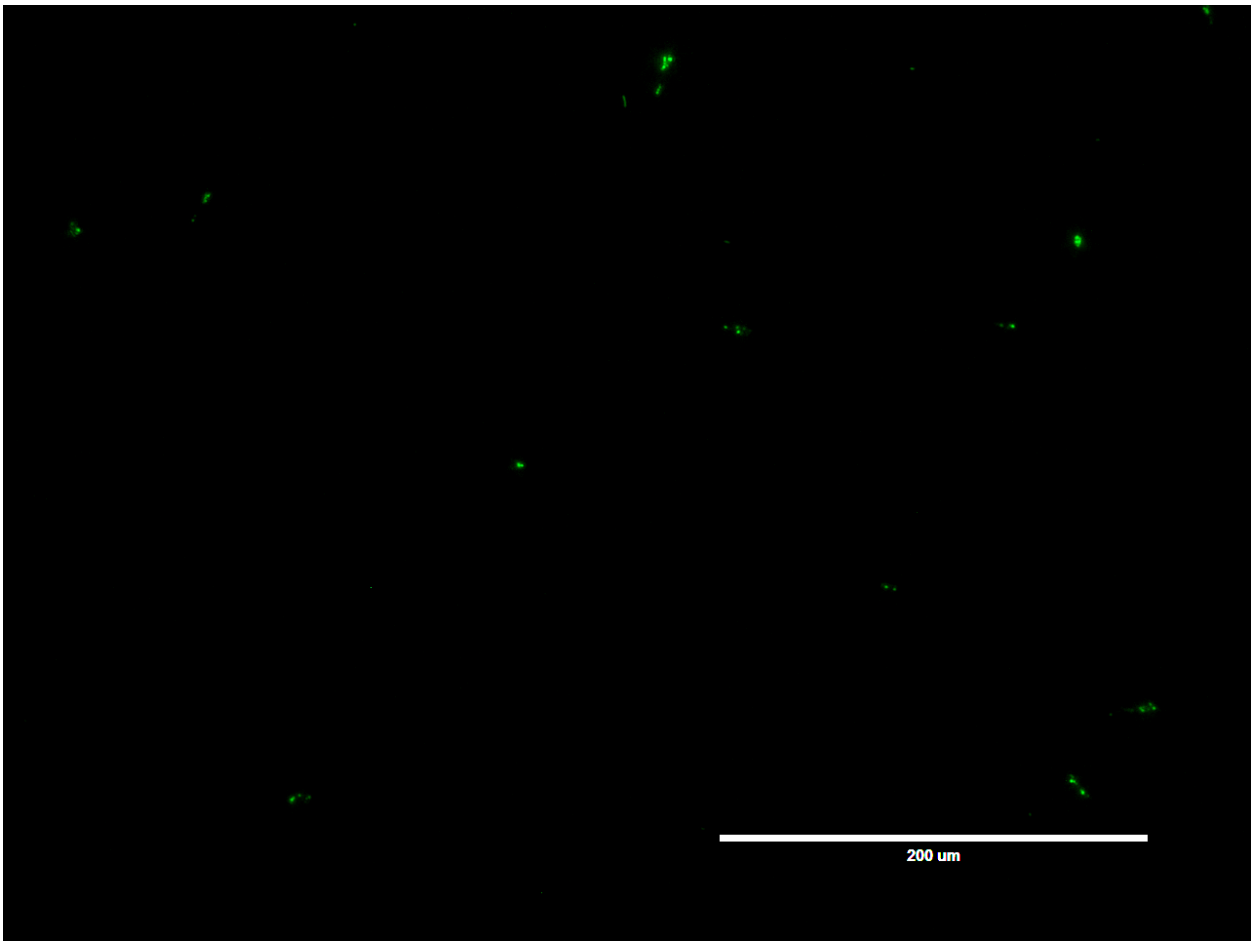


Figure S3.11: *W. magna* trophozoites containing green-fluorescent *L. pneumophila* cells within the food vacuoles on pre-developed water-biofilms after 6 months of their introduction to the flow-cells system at RT.

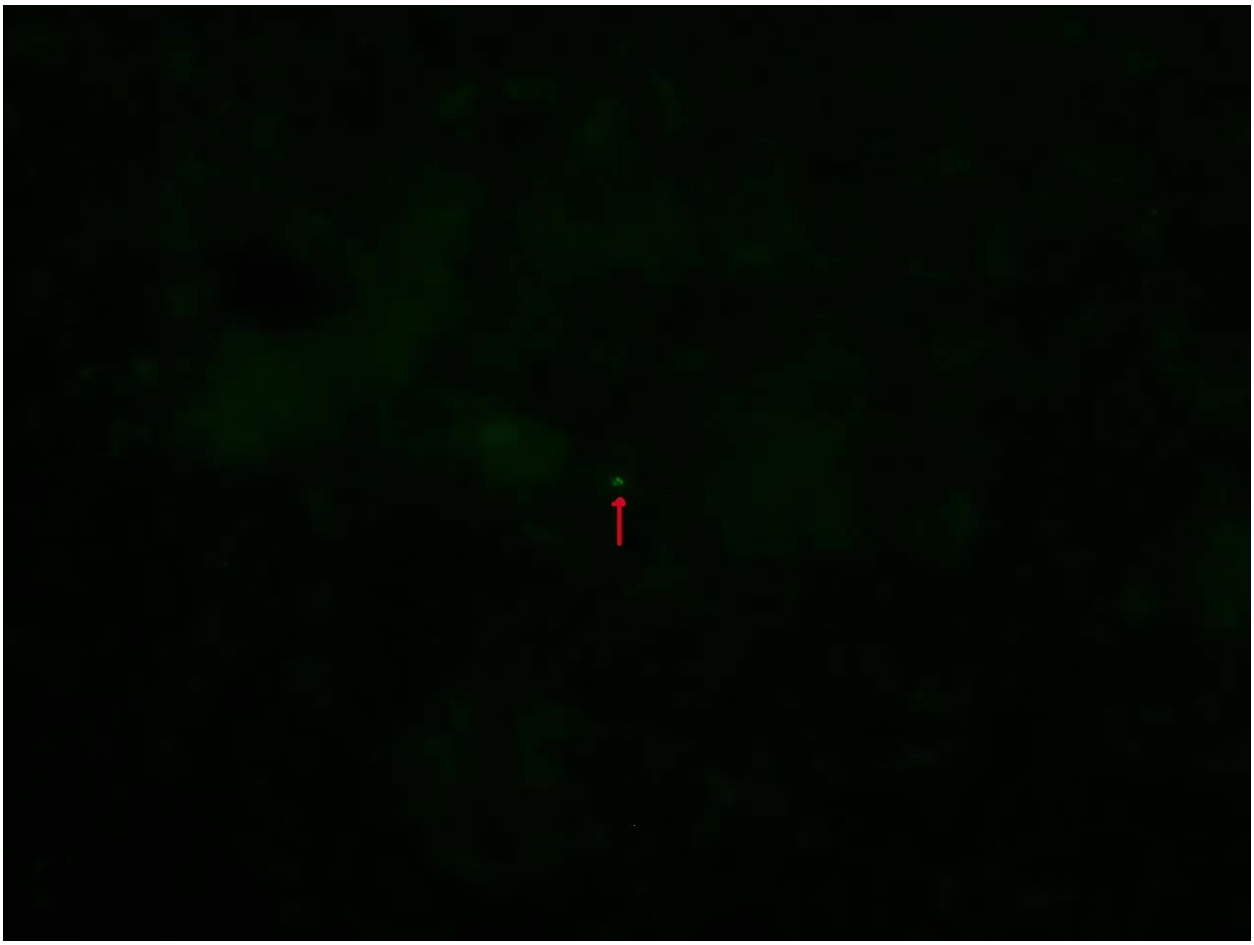


Figure S3.12: Freely moving *W. magna* trophozoites containing green-fluorescent *L. pneumophila* cells in the food vacuoles within pre-developed water-biofilms after 2 years of their introduction to the flow-cells system.

Appendix B: Supplementary information for chapter 4

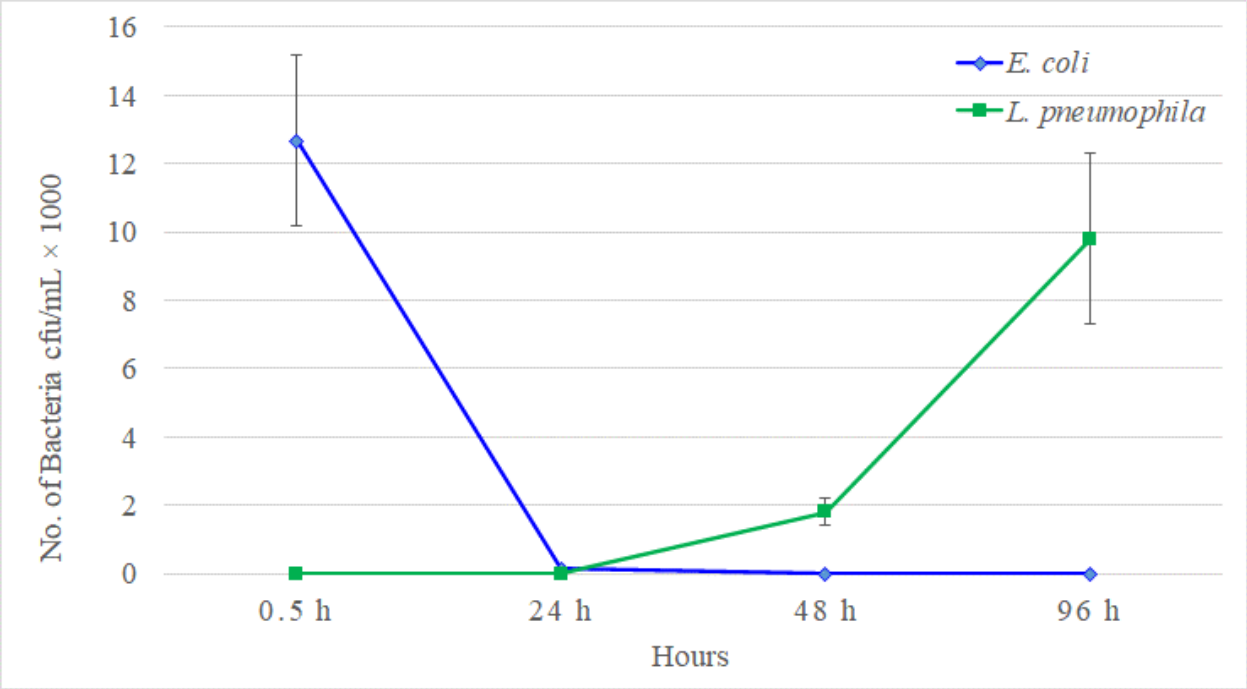


Figure S4.1: Intracellular concentrations of *L. pneumophila* and *E. coli* TOP10 cells within *W. magna* trophozoites at different time points. At 0.5 h the intracellular bacteria are mostly *E. coli* but after 24 h it is almost absent on the other hand at 0.5 h there is hardly any intracellular *L. pneumophila* but after 48 h it becomes the main intracellular bacterium in *W. magna* trophozoites.

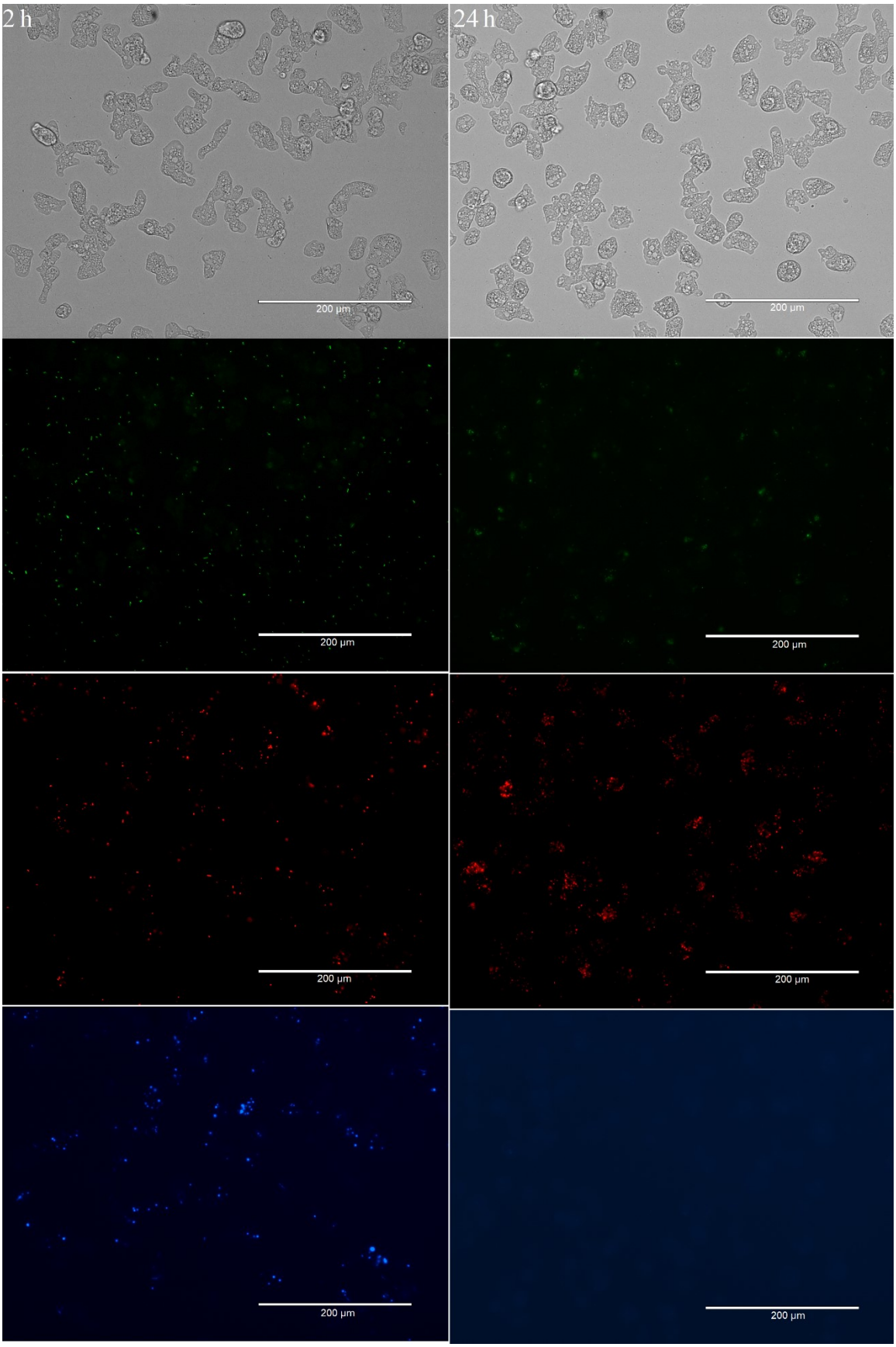

2 h
24 h
200 µm
200 µm
200 µm
200 µm
200 µm
200 µm
200 µm
200 µm

Figure S4.2: Co-culture of *W. magna* at RT with heat killed *L. pneumophila* in presence of the *E. coli* strains at different time points (2 and 24 h). The four images (from top to bottom) represent the same field of view under different fluorescent light channels, Mono-color transmission light channel, Green fluorescent channel to observe heat killed GFP-*L. pneumophila*, Texas-Red channel for mCherry-*E. coli* MG1655, and DAPI channel for BFP-*E. coli* TOP10 respectively.

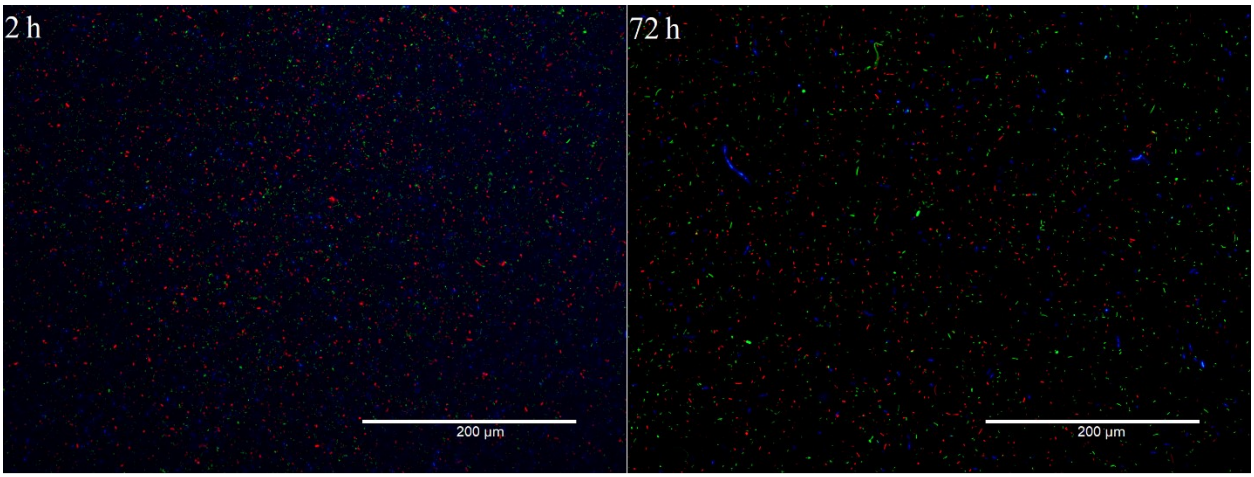
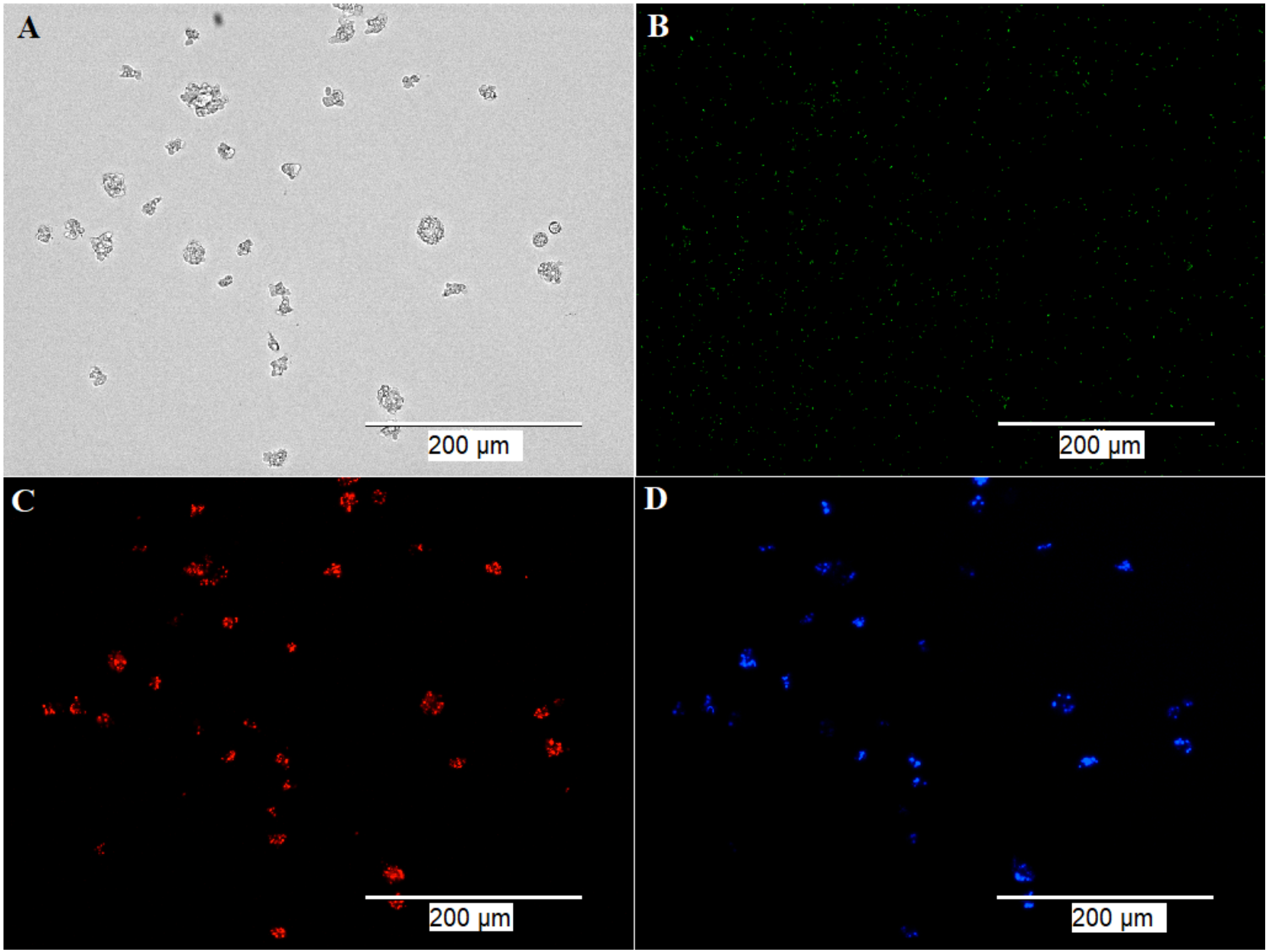


Figure S4.3: Co-incubation of *L. pneumophila*, *E. coli* MG1655 and *E. coli* TOP10 cells at RT in sterile tap water after 2h (left) and 72 h (right). No adverse effects were observed on any bacterium due their co-presence.


A
200 µm
B
200 µm
C
200 µm
D
200 µm

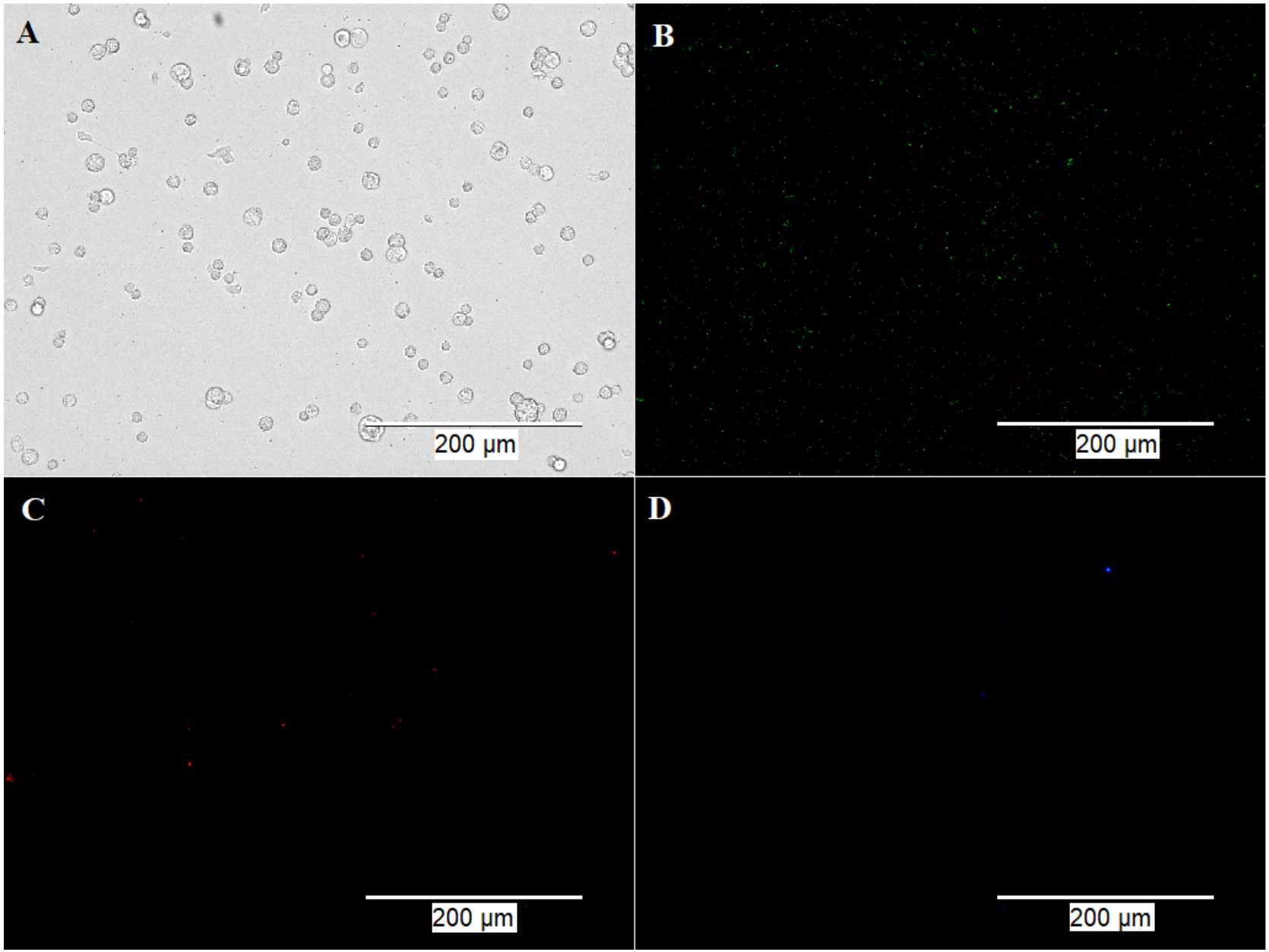
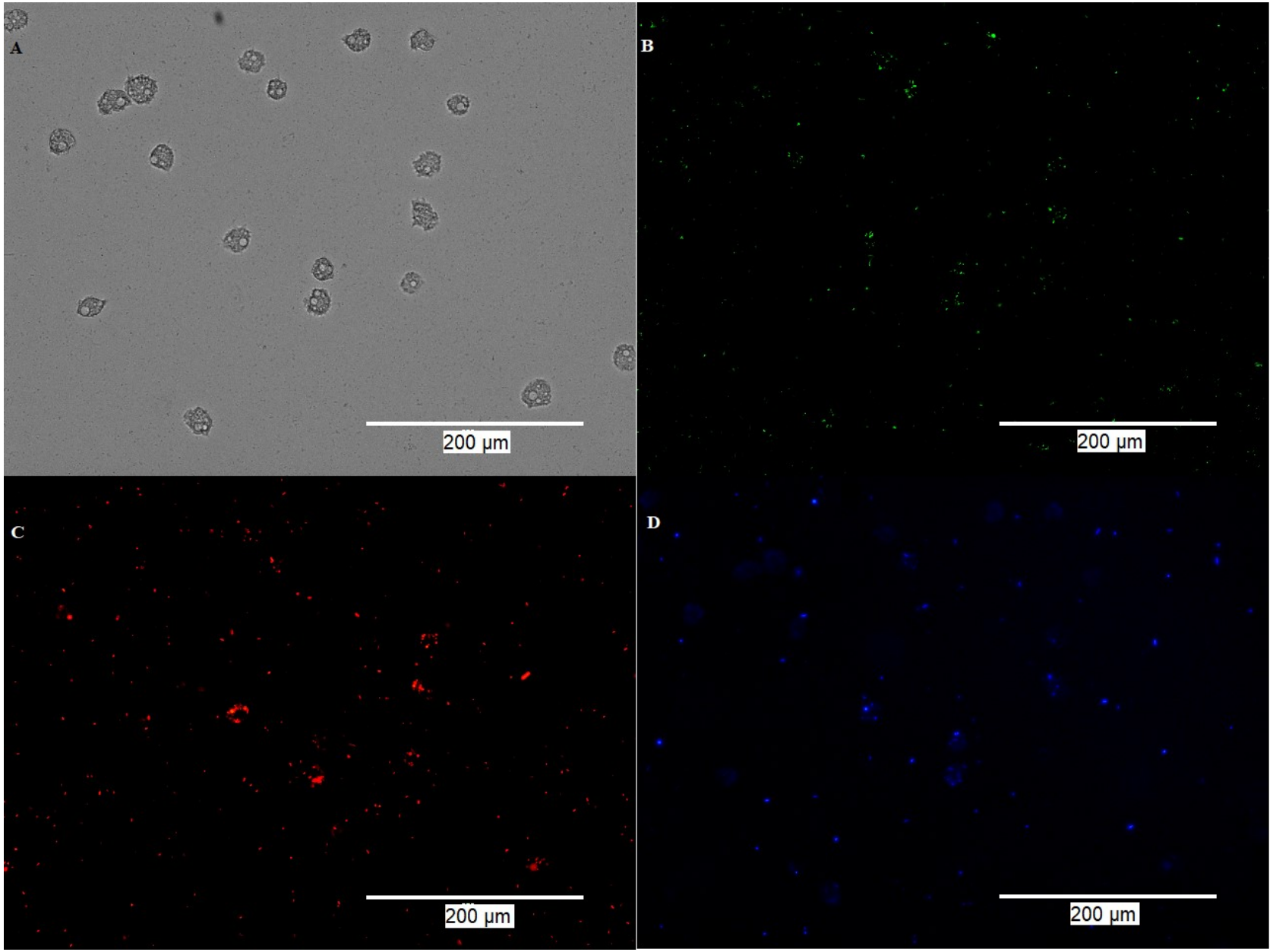

A
200 µm
B
200 µm
C
200 µm
D
200 µm

Figure S4.4: Preferential feeding on bacteria by *V. vermiformis* at RT at 2 h (Top) and 24 h (Bottom) of co-culture. The four images represent the same field of view under different fluorescent light channels. A. Mono-color transmission light channel, B. Green fluorescent channel to observe GFP-*L. pneumophila* C. Texas-Red channel mCherry-*E. coli* MG1655 D. DAPI channel for BFP-*E. coli* TOP10. The cluster of Red cells in image C and Blue cells in image D indicate the presence of *E. coli* MG1655 and *E. coli* TOP10 in the food vacuoles of *V. vermiformis* trophozoites. The scattered green cells represent planktonic *L. pneumophila* cells in the medium.


A
200 µm
B
200 µm
C
200 µm
D
200 µm

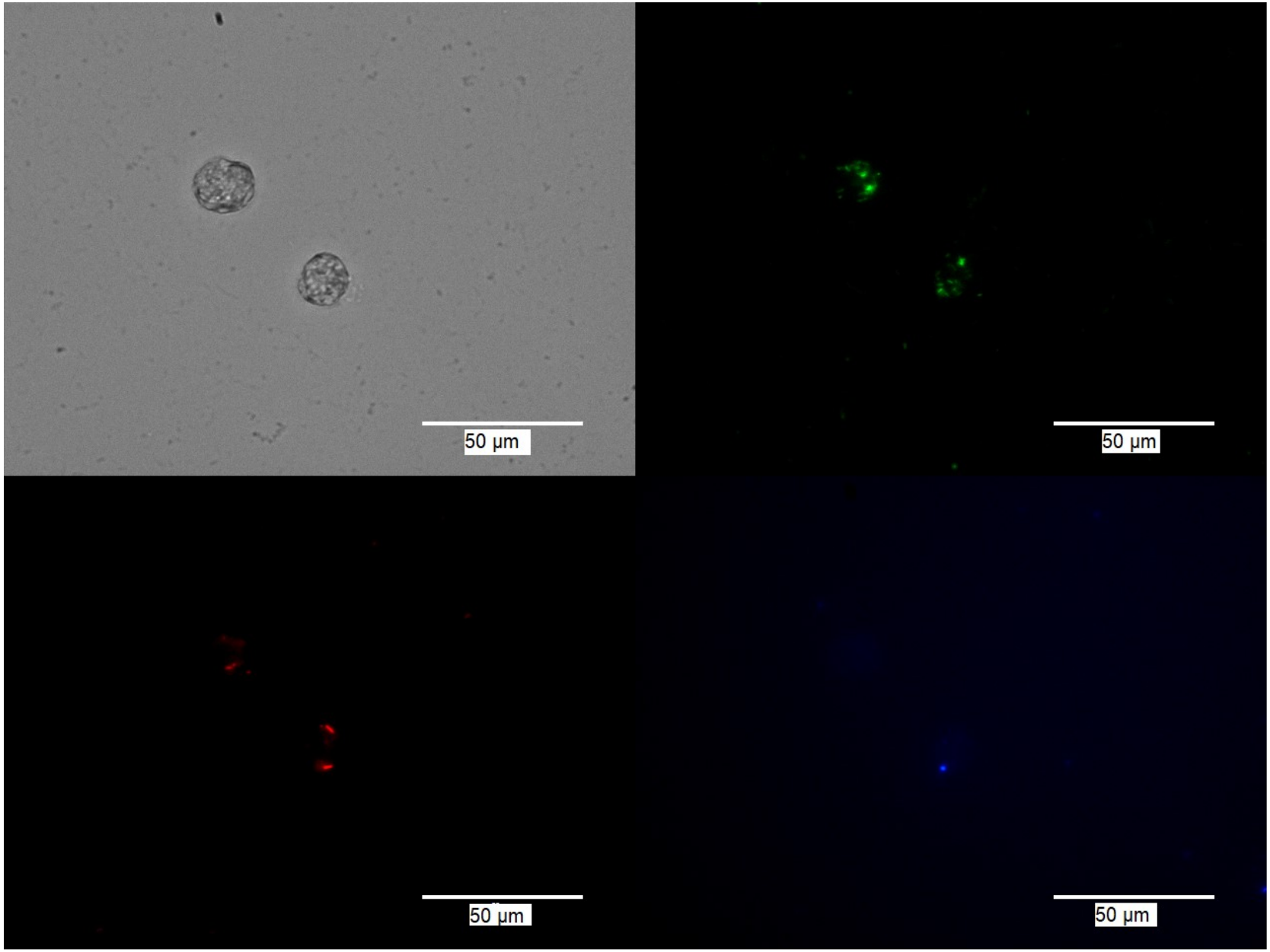

50 µm
50 µm
50 µm
50 µm

Figure S4.5: Preferential feeding on bacteria by *A. polyphaga* at RT at 2 h (Top) and 24 h (Bottom) of co-culture. The four images represent the same field of view under different fluorescent light channels to observe different bacteria. A. Mono-color transmission light channel for *A. polyphaga*, B. Green fluorescent *L. pneumophila* C. Texas-Red *E. coli* MG1655 D. DAPI channel for *E. coli* TOP10. The composite image (bottom) shows that *A. polyphaga* trophozoites phagocytose all the bacteria with equal preference for *L. pneumophila* and *E. coli*.

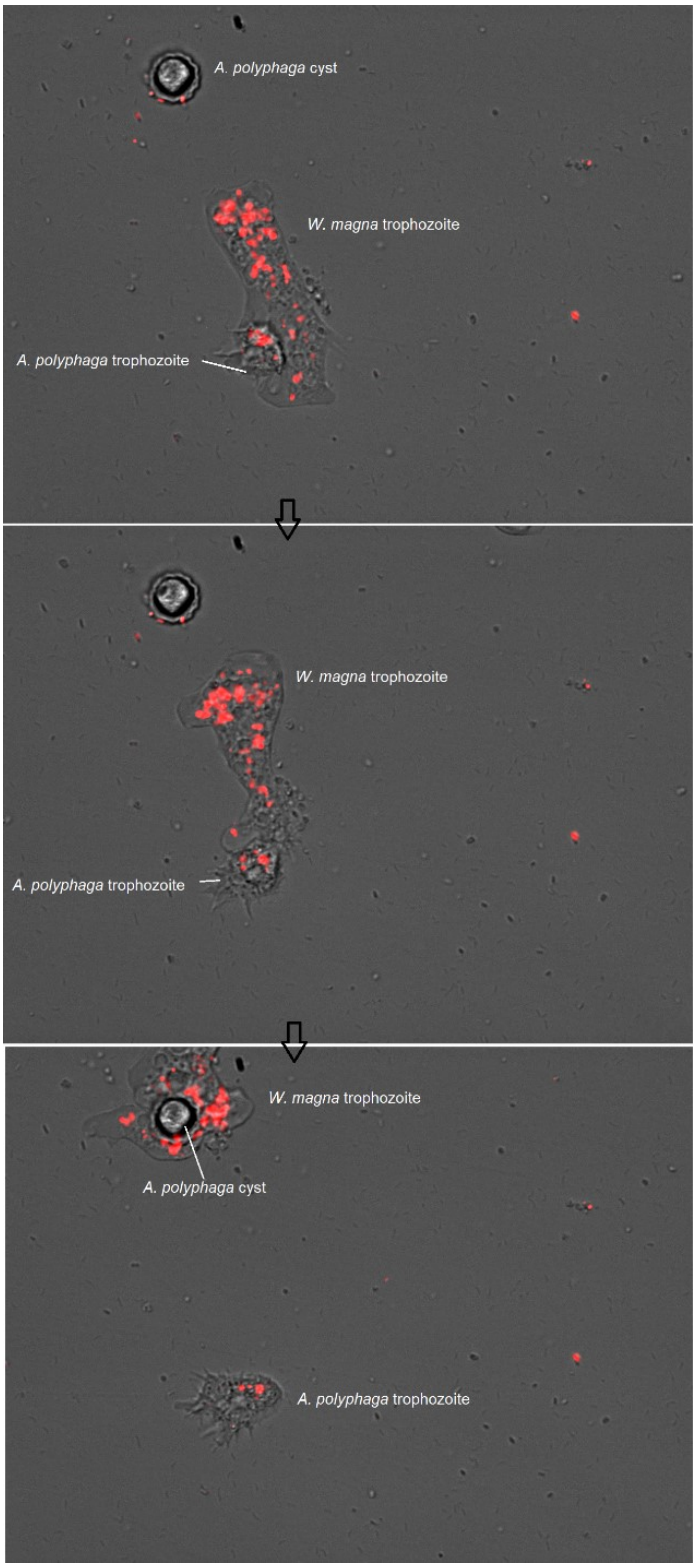


Figure S4.6. Intracellular locations of *E. coli* MG1655 (red) in amoeba trophozoites (*W. magna* and *A. polyphaga*) and cyst (*A. polyphaga*). *W. magna* trophozoites was moving closely around the *A. polyphaga* trophozoites and cyst in water.

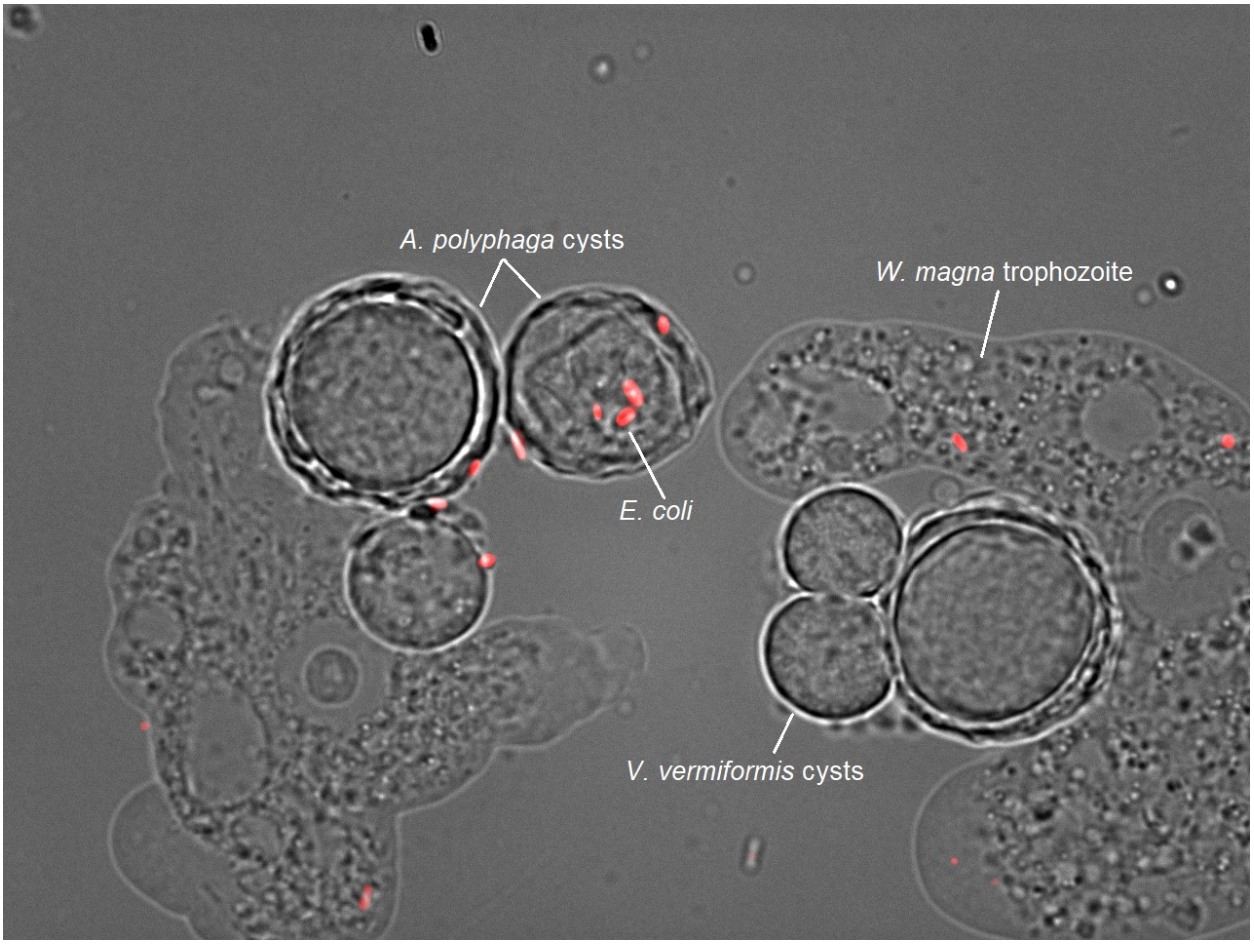


Figure S4.7: *E. coli* MG1655 (red) in the amoeba cysts at 96 h of co-culture. In *W. magna* trophozoites still some *E. coli* MG1655 cells left inside one of the food vacuoles.

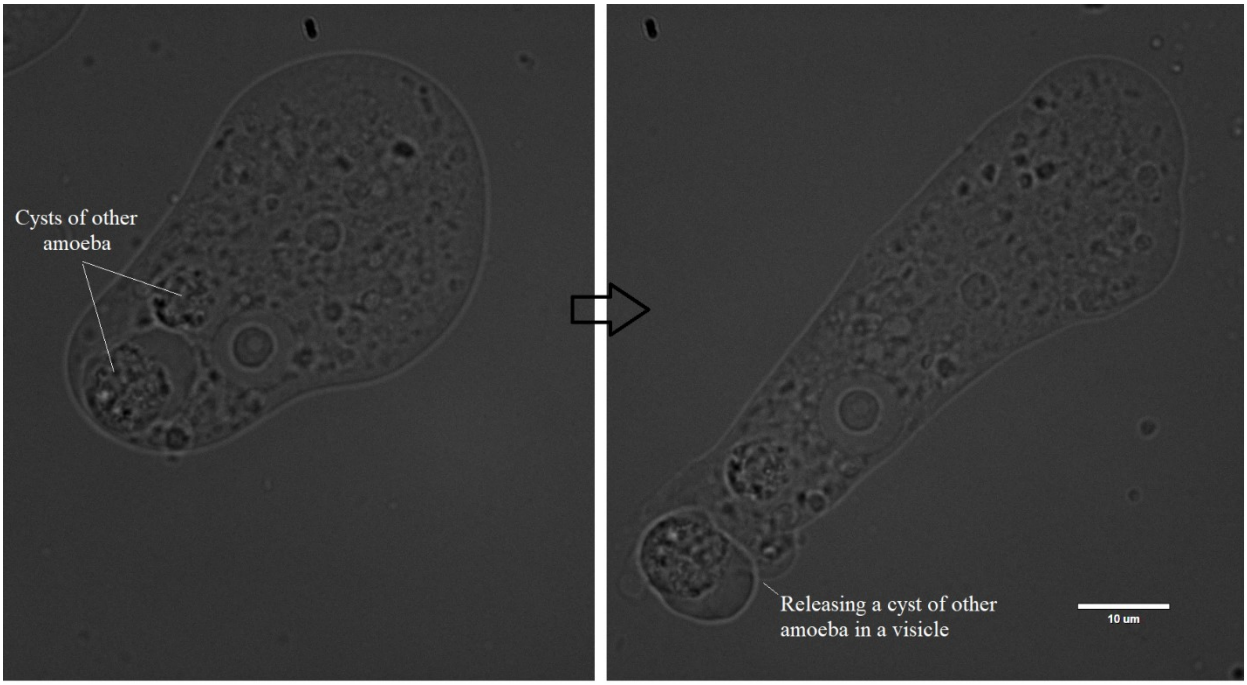


Figure S4.8: *W. magna* with cysts of other amoeba (*A. polyphaga* and *V. vermiformis* were present in the same culture) in a co-culture with *E. coli* (MG1655) in water (Left). Release of a cyst (possibly in a vesicle) of another amoeba from *W. magna* trophozoites (right).

Appendix C: Supplementary information for chapter 5

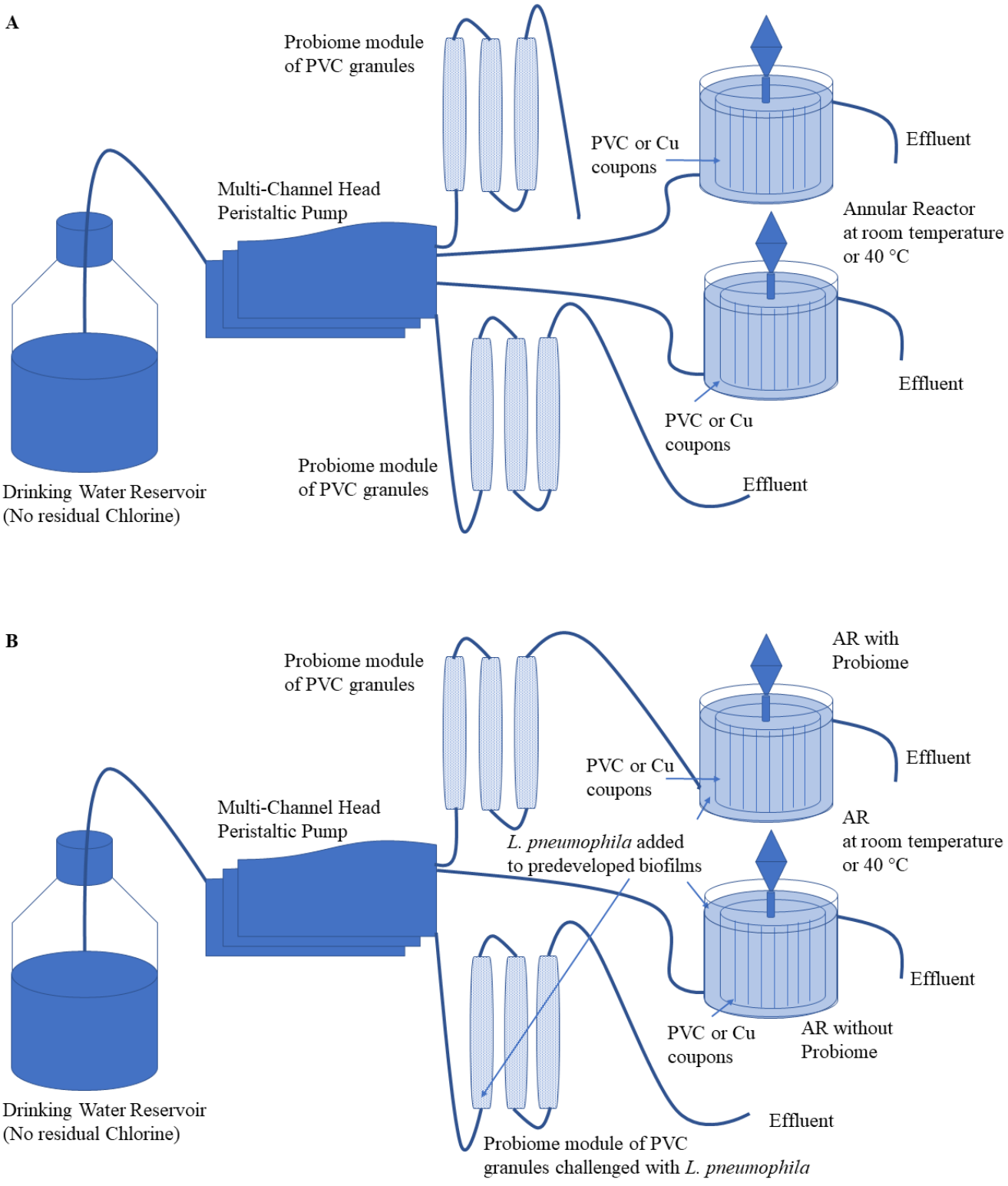
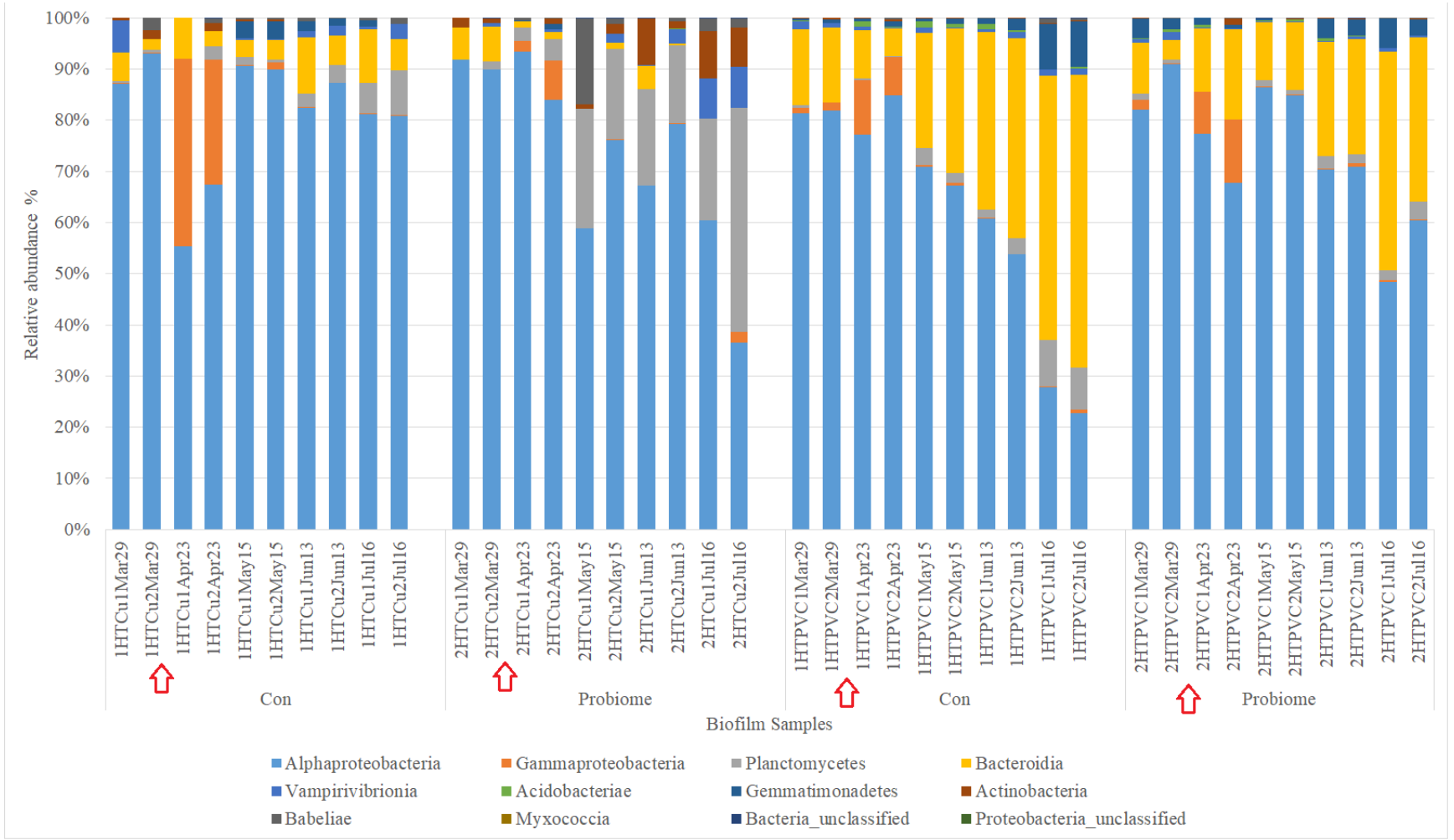


Figure S5.1: A schematic diagram of tap water biofilm developed in annular reactors (ARs) with Cu or PVC coupons under RT and 40 °C (HT) to determine the bacterial community compositions. **A.** Tap-water biofilms development within the probiome modules (PVC pipe sections filled with PVC grannules), and in ARs containing PVC or Cu coupons. **B.** Introduction of pathogenic *L. pneumophila* to all the ARs after collecting the first month biofilm samples (in duplicate) from the coupons. Follow up of the ARs and the probiome modules for another four months and collecting the coupon-biofilm samples periodically from the ARs and at the end of the experimental period from the probiome modules.


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abundance %
1HTCu1Mar29
1HTCu2Mar29
1HTCu1Apr23
1HTCu2Apr23
1HTCu1May15
1HTCu2May15
1HTCu1Jun13
1HTCu2Jun13
1HTCu1Jul16
1HTCu2Jul16
2HTCu1Mar29
2HTCu2Mar29
2HTCu1Apr23
2HTCu2Apr23
2HTCu1May15
2HTCu2May15
2HTCu1Jun13
2HTCu2Jun13
2HTCu1Jul16
2HTCu2Jul16
1HTPVC1Mar29
1HTPVC2Mar29
1HTPVC1Apr23
1HTPVC2Apr23
1HTPVC1May15
1HTPVC2May15
1HTPVC1Jun13
1HTPVC2Jun13
1HTPVC1Jul16
1HTPVC2Jul16
2HTPVC1Mar29
2HTPVC2Mar29
2HTPVC1Apr23
2HTPVC2Apr23
2HTPVC1May15
2HTPVC2May15
2HTPVC1Jun13
2HTPVC2Jun13
2HTPVC1Jul16
2HTPVC2Jul16
Con
Probiome
Con
Probiome
Biofilm Samples
Alphaproteobacteria
Gammaproteobacteria
Planctomycetes
Bacteroidia
Vampirivibrionia
Acidobacteriae
Gemmatimonadetes
Actinobacteria
Babeliae
Myxococcia
Bacteria_unclassified
Proteobacteria_unclassified

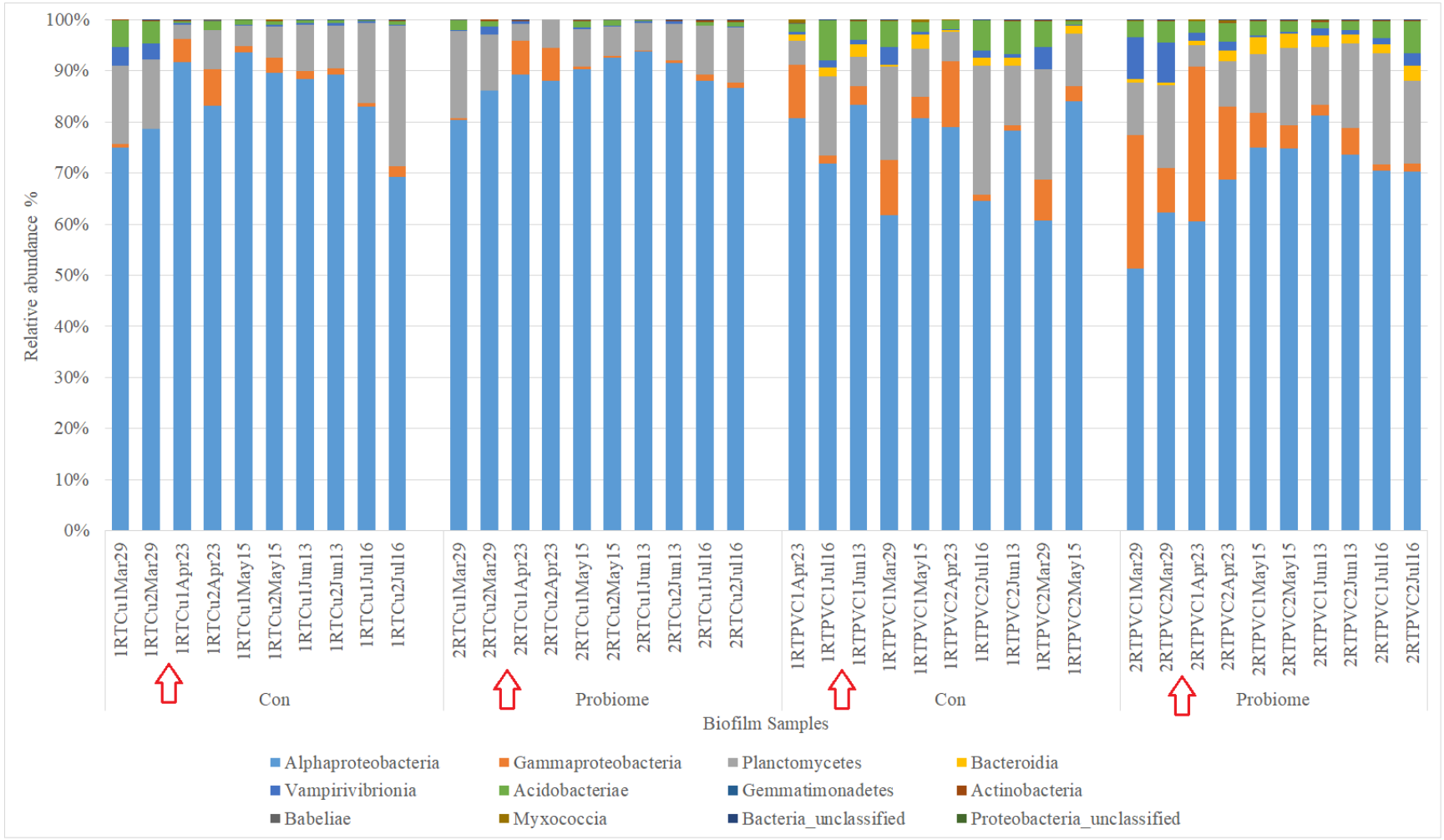


Figure S5.2: RA of different bacterial taxa in class level in biofilms on Cu and PVC coupons under HT (Top) and RT (Bottom) with and without probiome. *L. pneumophila* and upstream probiome were introduced after Mar29 samples (Red Arrow).

Table 5.3: Sample coverage

Sample	No. of singleton	No. of sequences	Sample Coverage
1HTCu1Apr23	4	27320	99.98536
1HTCu1Jul16	10	31602	99.96836
1HTCu1Jun13	13	36149	99.96404
1HTCu1Mar29	7	33474	99.97909
1HTCu1May15	8	33420	99.97606
1HTCu2Apr23	10	33169	99.96985
1HTCu2Jul16	3	36368	99.99175
1HTCu2Jun13	10	34567	99.97107
1HTCu2Mar29	6	30083	99.98006
1HTCu2May15	4	27874	99.98565
1HTCuMar29H1	8	34184	99.9766
1HTCuMar29H2	8	33466	99.9761
1HTPVC1Apr23	8	26507	99.96982
1HTPVC1Jul16	5	25010	99.98001
1HTPVC1Jun13	12	29781	99.95971
1HTPVC1Mar29	8	33565	99.97617
1HTPVC1May15	5	41367	99.98791
1HTPVC2Apr23	10	32266	99.96901
1HTPVC2Jul16	8	28208	99.97164
1HTPVC2Jun13	6	27717	99.97835
1HTPVC2Mar29	13	40688	99.96805
1HTPVC2May15	7	27033	99.97411
1HTPVCMar29H1	10	24268	99.95879
1HTPVCMar29H2	9	29759	99.96976
1RTCu1Apr23	16	31952	99.94992
1RTCu1Jul16	6	34385	99.98255
1RTCu1Jun13	13	35974	99.96386
1RTCu1Mar29	13	33073	99.96069
1RTCu1May15	11	38881	99.97171
1RTCu2Apr23	12	35386	99.96609
1RTCu2Jul16	11	42932	99.97438
1RTCu2Jun13	13	41666	99.9688
1RTCu2Mar29	7	28118	99.9751
1RTCu2May15	8	32082	99.97506
1RTCuMar29H1	5	28150	99.98224
1RTCuMar29H2	11	29369	99.96255
1RTPVC1Apr23	11	28786	99.96179
1RTPVC1Jul16	10	32971	99.96967
1RTPVC1Jun13	10	23808	99.958
1RTPVC1Mar29	7	31406	99.97771

1RTPVC1May15	6	31405	99.98089
1RTPVC2Apr23	7	28229	99.9752
1RTPVC2Jul16	6	31958	99.98123
1RTPVC2Jun13	10	29910	99.96657
1RTPVC2Mar29	1	35681	99.9972
1RTPVC2May15	6	35239	99.98297
1RTPVCMar29H1	10	31023	99.96777
1RTPVCMar29H2	14	29392	99.95237
2HTCu1Apr23	6	28710	99.9791
2HTCu1Jul16	7	25922	99.973
2HTCu1Jun13	8	22774	99.96487
2HTCu1Mar29	9	31792	99.97169
2HTCu1May15	7	22478	99.96886
2HTCu2Apr23	9	28464	99.96838
2HTCu2Jul16	9	30297	99.97029
2HTCu2Jun13	6	36800	99.9837
2HTCu2Mar29	10	39073	99.97441
2HTCu2May15	10	33538	99.97018
2HTCuMar29H1	9	28685	99.96862
2HTCuMar29H2	17	27942	99.93916
2HTPVC1Apr23	12	34561	99.96528
2HTPVC1Jul16	10	28203	99.96454
2HTPVC1Jun13	15	26877	99.94419
2HTPVC1Mar29	7	29622	99.97637
2HTPVC1May15	9	37905	99.97626
2HTPVC2Apr23	7	38005	99.98158
2HTPVC2Jul16	7	28230	99.9752
2HTPVC2Jun13	15	33326	99.95499
2HTPVC2Mar29	6	31544	99.98098
2HTPVC2May15	7	26409	99.97349
2HTPVCMar29H1	9	28087	99.96796
2HTPVCMar29H2	9	29142	99.96912
2RTCu1Apr23	11	36848	99.97015
2RTCu1Jul16	10	33997	99.97059
2RTCu1Jun13	7	32267	99.97831
2RTCu1Mar29	8	27660	99.97108
2RTCu1May15	6	40116	99.98504
2RTCu2Apr23	11	42716	99.97425
2RTCu2Jul16	14	31747	99.9559
2RTCu2Jun13	11	30964	99.96447
2RTCu2Mar29	8	30067	99.97339
2RTCu2May15	13	32900	99.96049
2RTCuMar29H1	10	28341	99.96472

2RTCuMar29H2	8	28505	99.97193
2RTPVC1Apr23	8	29593	99.97297
2RTPVC1Jul16	7	29067	99.97592
2RTPVC1Jun13	10	27126	99.96313
2RTPVC1Mar29	15	31429	99.95227
2RTPVC1May15	10	30236	99.96693
2RTPVC2Apr23	6	26466	99.97733
2RTPVC2Jul16	9	28788	99.96874
2RTPVC2Jun13	4	32355	99.98764
2RTPVC2Mar29	5	29092	99.98281
2RTPVC2May15	9	35152	99.9744
2RTPVCMar29H1	12	30549	99.96072
2RTPVCMar29H2	14	32248	99.95659
CNTLFC2Y1	3	33021	99.99091
CNTLFC2Y2	12	32491	99.96307
DW1JUN12	14	40131	99.96511
DW2JUN12	6	34941	99.98283
LP1B1	7	32371	99.97838
LP1B2	14	35470	99.96053
LP1T1	8	36077	99.97783
LP1T2	14	38706	99.96383
LP2B1	7	23955	99.97078
LP2B2	8	28428	99.97186
LP2T1	6	32150	99.98134
LP2T2	5	26245	99.98095
LP3B1	9	33210	99.9729
LP3B2	8	36219	99.97791
LP3T1	11	32293	99.96594
LP3T2	5	26399	99.98106
NLP4B1	9	33846	99.97341
NLP4B2	11	33762	99.96742
NLP4T1	6	29915	99.97994
NLP4T2	6	31692	99.98107
NLP5B1	12	33504	99.96418
NLP5B2	11	31975	99.9656
NLP5T1	12	28049	99.95722
NLP5T2	6	30539	99.98035
NLP6B1	10	28028	99.96432
NLP6B2	9	33798	99.97337
NLP6T1	14	37122	99.96229
NLP6T2	4	31238	99.9872
WMLPFC2Y1	21	34843	99.93973
WMLPFC2Y2	5	30176	99.98343

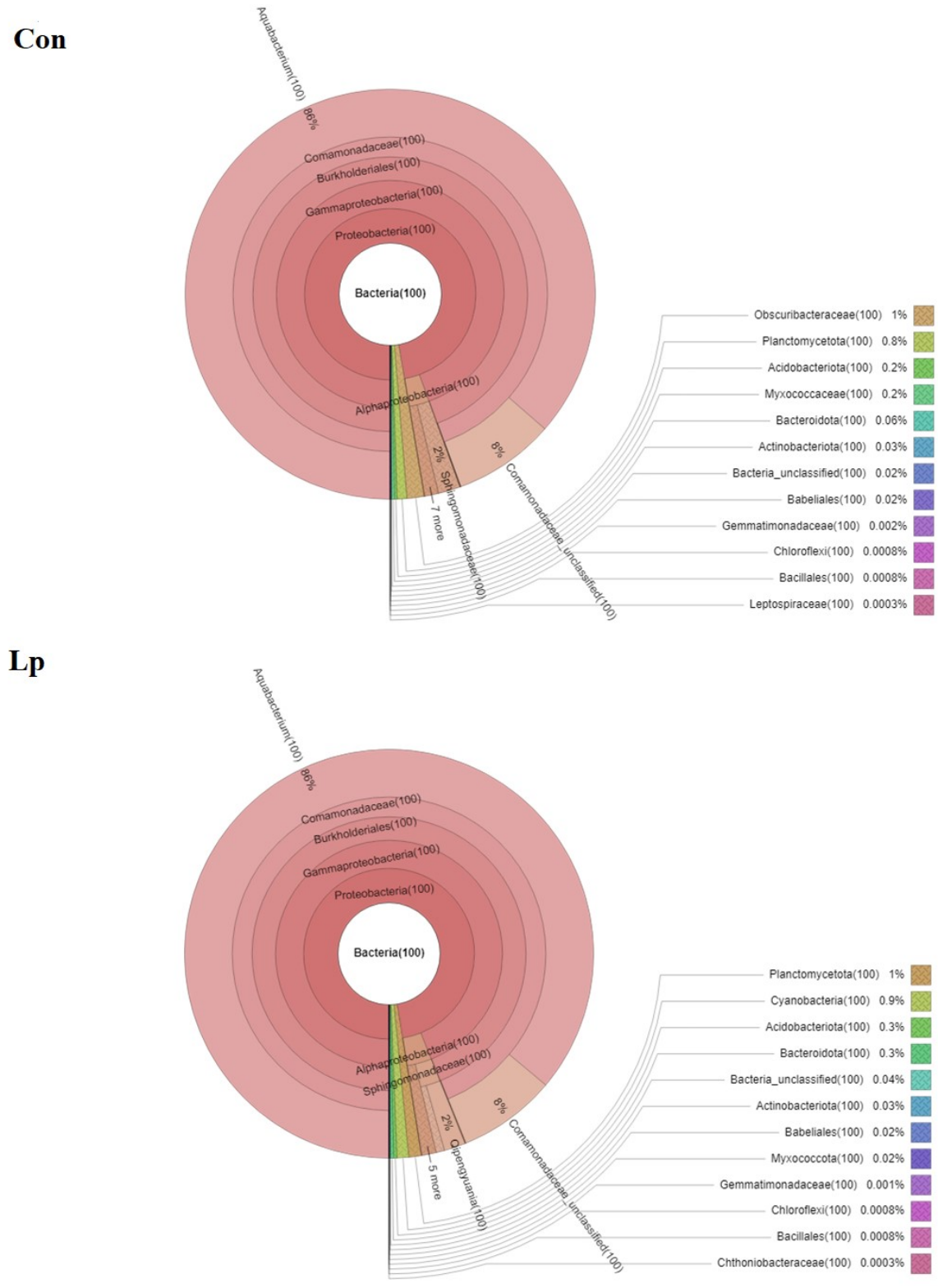


Figure S5.4: Bacterial taxa of probiome biofilms (Con, Control probiome and Lp, probiome primed with *L. pneumophila* once) developed at RT.

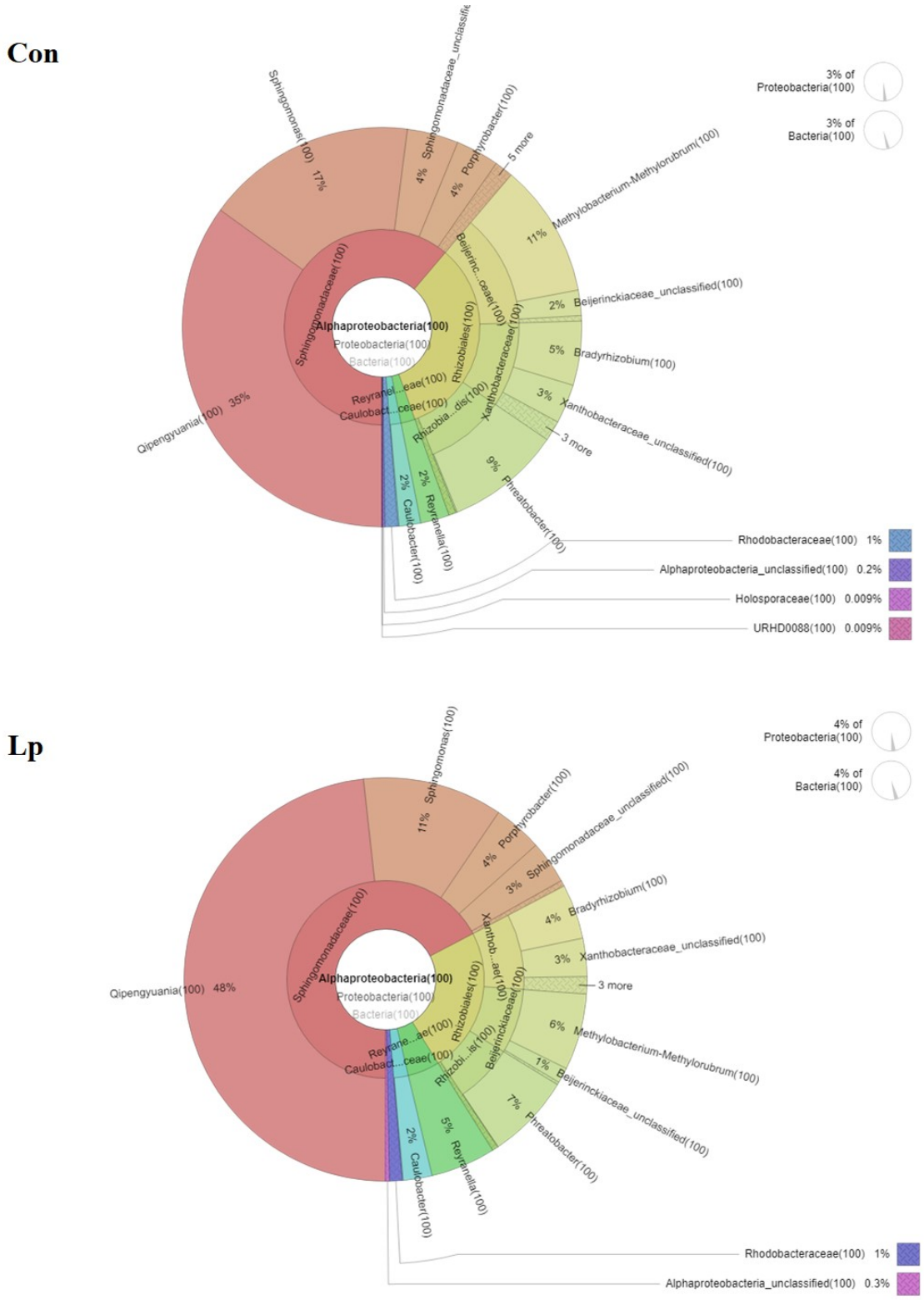
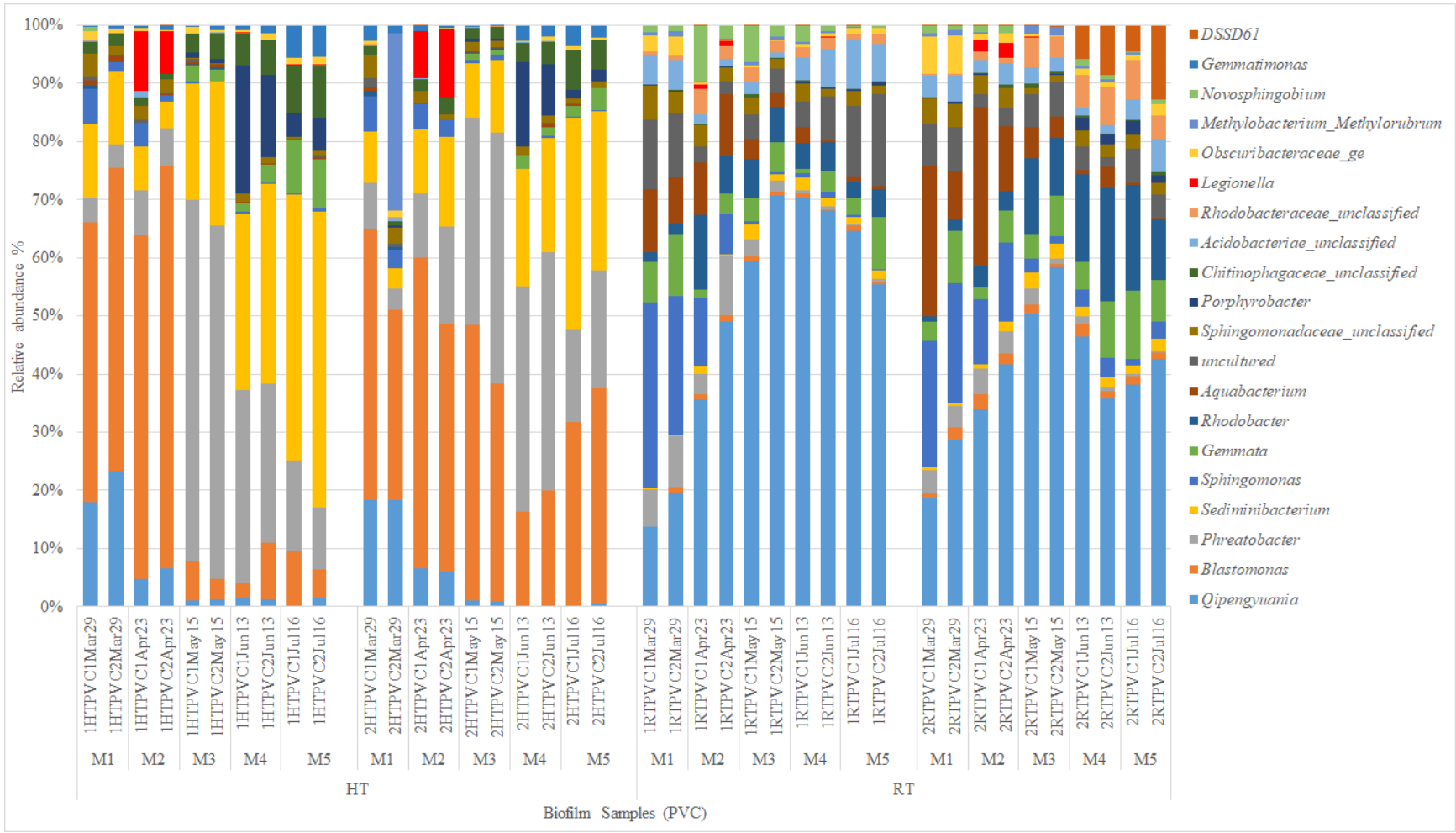


Figure S5.5: Bacterial genera within the Alphaproteobacteria of probiome (Con, Control probiome and Lp, probiome primed with *L. pneumophila* once) developed at RT.

Table S5.6: RA of Bacterial genera of the Alphaproteobacteria in probiome with and without spiking with *L. pneumophila*

Genus	Con (% of total Alphaproteobacteria)	Lp (% of total Alphaproteobacteria)
Qipengyuania	35	48
Sphingomonas	17	11
Methylobacterium_Methylorubrum	11	6
Phreatobacter	9	7
Bradyrhizobium	5	4
Porphyrobacter	4	4
Reyranella	2	5
Caulobacter	2	2


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abundance %
DSSD61
Gemmatimonas
Novosphingobium
Methylobacterium_Methylorubrum
Obscuribacteraceae_ge
Legionella
Rhodobacteraceae_unclassified
Acidobacteriae_unclassified
Chitinophagaceae_unclassified
Porphyrobacter
Sphingomonadaceae_unclassified
uncultured
Aquabacterium
Rhodobacter
Gemmata
Sphingomonas
Sediminibacterium
Phreatobacter
Blastomonas
Qipengyuania
1HTPVC1Mar29
1HTPVC2Mar29
1HTPVC1Apr23
1HTPVC2Apr23
1HTPVC1May15
1HTPVC2May15
1HTPVC1Jun13
1HTPVC2Jun13
1HTPVC1Jul16
1HTPVC2Jul16
2HTPVC1Mar29
2HTPVC2Mar29
2HTPVC1Apr23
2HTPVC2Apr23
2HTPVC1May15
2HTPVC2May15
2HTPVC1Jun13
2HTPVC2Jun13
2HTPVC1Jul16
2HTPVC2Jul16
1RTPVC1Mar29
1RTPVC2Mar29
1RTPVC1Apr23
1RTPVC2Apr23
1RTPVC1May15
1RTPVC2May15
1RTPVC1Jun13
1RTPVC2Jun13
1RTPVC1Jul16
1RTPVC2Jul16
2RTPVC1Mar29
2RTPVC2Mar29
2RTPVC1Apr23
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2RTPVC1Jun13
2RTPVC2Jun13
2RTPVC1Jul16
2RTPVC2Jul16
M1
M2
M3
M4
M5
HT
RT
Biofilm Samples (PVC)

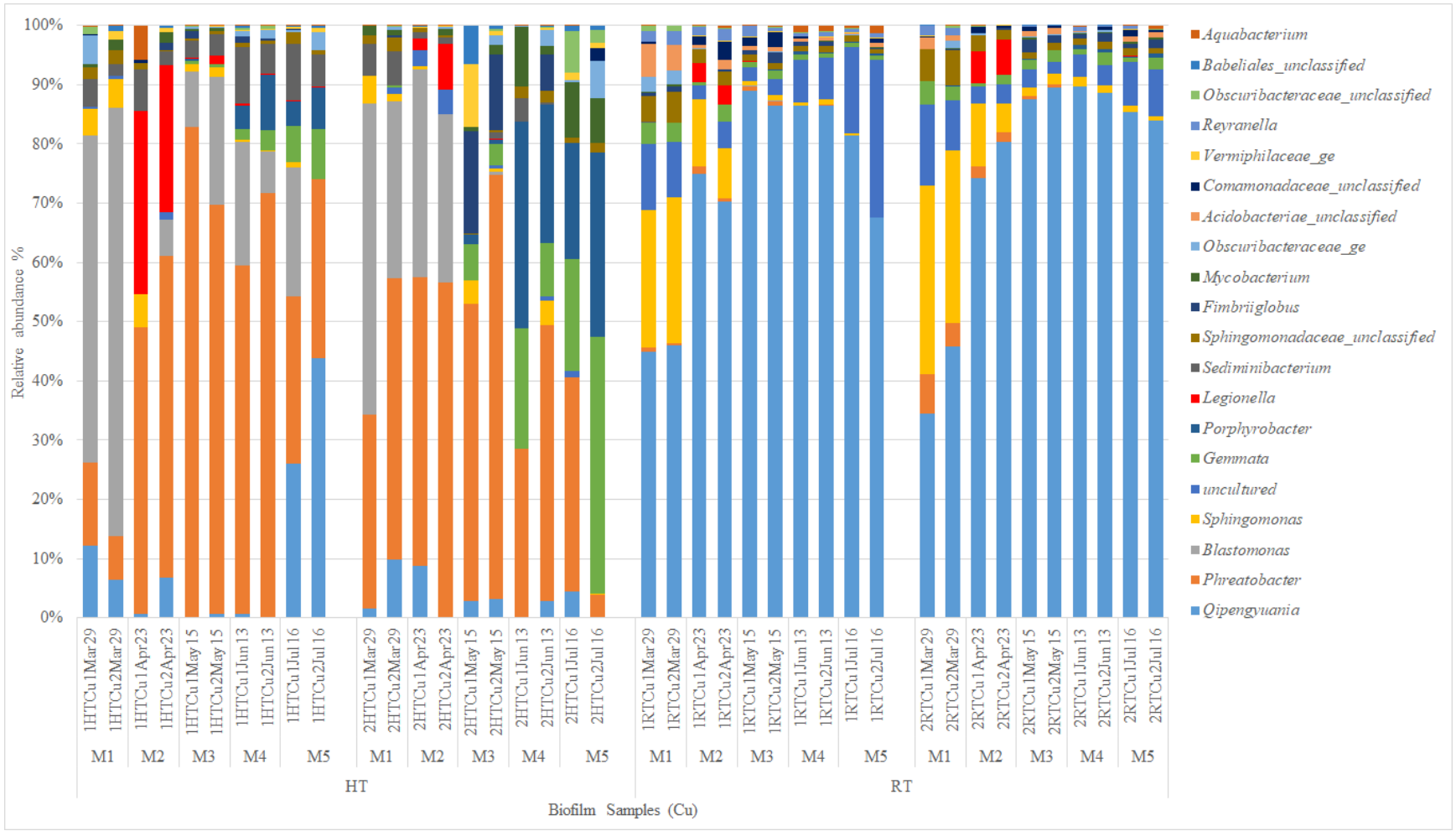


Figure S5.7: RA of the 20 most dominant genera present in PVC (Top) Cu (Bottom) biofilms at RT and HT. M1-M5 indicates the number of months (M1 is the 1st month, Mar 29 samples). *L. pneumophila* and probiome were introduced to the AR after M1 sampling.

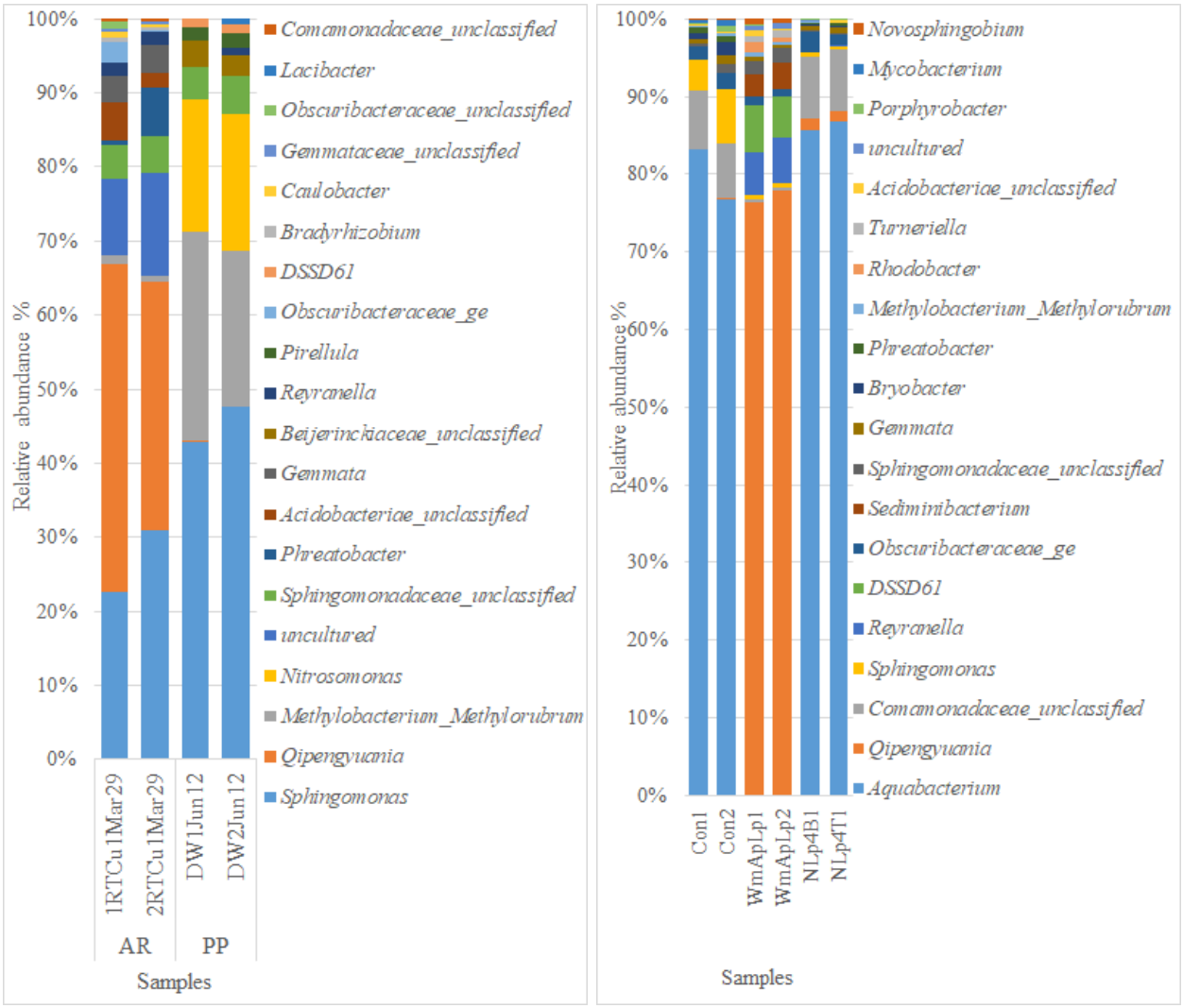


Figure S5.8: RA of the 20 most dominant genera present in biofilms of building Cu-pipe (PP) and on Cu-coupons of AR (Left) and biofilms on plastic surfaces (Norprene™ tubing control, Norprene™ tubing developed at under the influence of *L. pneumophila* and amoebae (*Willaertia magna,* Wm and *Acanthamoeba polyphaga,* Ap) and two probiome samples (NLP4T1 and NLP4B1) at RT (Right).

Table S5.9: Alpha diversity (Chao1 and Shannon index)

	Samples	Alpha diversity	Value	SE	Alpha diversity	Value
1	1HTCu1Apr23	Chao1	23	4.150694	Shannon	1.424224
2	1HTCu1Jul16	Chao1	55.25	9.544004	Shannon	2.041593
3	1HTCu1Jun13	Chao1	62.6	11.6394	Shannon	1.545235
4	1HTCu1Mar29	Chao1	37	7.100628	Shannon	1.559769
5	1HTCu1May15	Chao1	48.6	5.342448	Shannon	1.019027
6	1HTCu2Apr23	Chao1	60.5	19.27596	Shannon	1.605345
7	1HTCu2Jul16	Chao1	30.5	1.025628	Shannon	1.577103
8	1HTCu2Jun13	Chao1	64.5	19.28098	Shannon	1.343427
9	1HTCu2Mar29	Chao1	38.75	4.202021	Shannon	1.214933
10	1HTCu2May15	Chao1	40.5	2.228334	Shannon	1.26114
11	1HTCuMar29H1	Chao1	54.5	3.443104	Shannon	1.633308
12	1HTCuMar29H2	Chao1	56	3.888384	Shannon	1.496087
13	1HTPVC1Apr23	Chao1	48.5	3.442815	Shannon	1.576579
14	1HTPVC1Jul16	Chao1	46.5	3.15696	Shannon	1.933051
15	1HTPVC1Jun13	Chao1	64.5	12.89356	Shannon	1.760163
16	1HTPVC1Mar29	Chao1	46.66667	4.488502	Shannon	1.75924
17	1HTPVC1May15	Chao1	46.33333	4.119571	Shannon	1.428281
18	1HTPVC2Apr23	Chao1	61	12.82767	Shannon	1.316978
19	1HTPVC2Jul16	Chao1	53.33333	8.849915	Shannon	1.900259
20	1HTPVC2Jun13	Chao1	44	3.415739	Shannon	1.882995
21	1HTPVC2Mar29	Chao1	87	30.34985	Shannon	1.520937
22	1HTPVC2May15	Chao1	48	7.105969	Shannon	1.340749
23	1HTPVCMar29H1	Chao1	64.5	19.28098	Shannon	1.90323
24	1HTPVCMar29H2	Chao1	51.2	6.434085	Shannon	1.854661
25	1RTCu1Apr23	Chao1	75	16.41261	Shannon	1.051342
26	1RTCu1Jul16	Chao1	39.75	4.202241	Shannon	0.721964
27	1RTCu1Jun13	Chao1	55.75	7.198037	Shannon	0.700325
28	1RTCu1Mar29	Chao1	72.14286	8.228764	Shannon	1.894519
29	1RTCu1May15	Chao1	63.16667	7.377675	Shannon	0.660291
30	1RTCu2Apr23	Chao1	58.25	6.358989	Shannon	1.332183
31	1RTCu2Jul16	Chao1	66.5	22.70806	Shannon	0.972958
32	1RTCu2Jun13	Chao1	67.5	14.73513	Shannon	0.701968
33	1RTCu2Mar29	Chao1	47.2	4.336145	Shannon	1.821557
34	1RTCu2May15	Chao1	55	3.88833	Shannon	0.817527
35	1RTCuMar29H1	Chao1	55.42857	1.933678	Shannon	1.588705
36	1RTCuMar29H2	Chao1	64.875	5.563903	Shannon	1.5501
37	1RTPVC1Apr23	Chao1	78.33333	15.06549	Shannon	2.32698
38	1RTPVC1Jul16	Chao1	61.625	4.812342	Shannon	1.497567
39	1RTPVC1Jun13	Chao1	64.25	9.546196	Shannon	1.487136
40	1RTPVC1Mar29	Chao1	63.2	4.337612	Shannon	2.455518

41	1RTPVC1May15	Chao1	60.75	4.205187	Shannon	1.861394
42	1RTPVC2Apr23	Chao1	60	7.109559	Shannon	2.037827
43	1RTPVC2Jul16	Chao1	50.14286	2.529514	Shannon	1.696462
44	1RTPVC2Jun13	Chao1	61.5	6.349124	Shannon	1.487814
45	1RTPVC2Mar29	Chao1	54	0.09907	Shannon	2.570366
46	1RTPVC2May15	Chao1	61.5	1.874622	Shannon	1.48283
47	1RTPVCMar29H1	Chao1	70	4.312015	Shannon	1.905596
48	1RTPVCMar29H2	Chao1	78.375	8.08251	Shannon	1.900982
49	2HTCu1Apr23	Chao1	36	5.524916	Shannon	1.423611
50	2HTCu1Jul16	Chao1	42.25	5.365546	Shannon	2.142626
51	2HTCu1Jun13	Chao1	47.33333	8.847262	Shannon	2.006605
52	2HTCu1Mar29	Chao1	40.2	6.431811	Shannon	1.321491
53	2HTCu1May15	Chao1	50	17.45695	Shannon	1.710032
54	2HTCu2Apr23	Chao1	73	25.76394	Shannon	1.327149
55	2HTCu2Jul16	Chao1	49	10.75326	Shannon	1.720664
56	2HTCu2Jun13	Chao1	59.14286	2.529838	Shannon	1.857671
57	2HTCu2Mar29	Chao1	64	12.829	Shannon	1.619854
58	2HTCu2May15	Chao1	61.625	4.812342	Shannon	1.304916
59	2HTCuMar29H1	Chao1	59	8.039593	Shannon	1.557616
60	2HTCuMar29H2	Chao1	100.4615	6.7526	Shannon	1.787697
61	2HTPVC1Apr23	Chao1	55.5	12.89047	Shannon	1.688345
62	2HTPVC1Jul16	Chao1	53.25	9.543416	Shannon	1.707898
63	2HTPVC1Jun13	Chao1	80	25.59551	Shannon	1.814783
64	2HTPVC1Mar29	Chao1	46	7.105188	Shannon	1.874912
65	2HTPVC1May15	Chao1	57.2	6.434957	Shannon	1.421779
66	2HTPVC2Apr23	Chao1	43.2	4.335609	Shannon	1.778143
67	2HTPVC2Jul16	Chao1	46.2	4.33602	Shannon	1.700773
68	2HTPVC2Jun13	Chao1	73	14.73991	Shannon	1.782087
69	2HTPVC2Mar29	Chao1	58	5.531692	Shannon	2.018879
70	2HTPVC2May15	Chao1	48.25	5.366858	Shannon	1.437578
71	2HTPVCMar29H1	Chao1	51.5	4.104927	Shannon	1.810689
72	2HTPVCMar29H2	Chao1	53.2	6.434398	Shannon	1.811575
73	2RTCu1Apr23	Chao1	57.75	11.16222	Shannon	1.052263
74	2RTCu1Jul16	Chao1	55	7.610352	Shannon	0.734319
75	2RTCu1Jun13	Chao1	45.5	10.532	Shannon	0.568956
76	2RTCu1Mar29	Chao1	55.33333	8.850671	Shannon	1.846894
77	2RTCu1May15	Chao1	44	3.415739	Shannon	0.727829
78	2RTCu2Apr23	Chao1	60.5	22.69912	Shannon	0.882152
79	2RTCu2Jul16	Chao1	61.2	13.14699	Shannon	0.777522
80	2RTCu2Jun13	Chao1	60.33333	15.05751	Shannon	0.674753
81	2RTCu2Mar29	Chao1	52	3.888154	Shannon	1.731262
82	2RTCu2May15	Chao1	57	9.628631	Shannon	0.615061
83	2RTCuMar29H1	Chao1	66	12.82982	Shannon	1.688068

84	2RTCuMar29H2	Chao1	55.5	3.443146	Shannon	1.332489
85	2RTPVC1Apr23	Chao1	64.66667	4.489888	Shannon	2.226676
86	2RTPVC1Jul16	Chao1	61.2	4.33747	Shannon	2.241248
87	2RTPVC1Jun13	Chao1	79.5	19.29532	Shannon	2.201469
88	2RTPVC1Mar29	Chao1	88	14.74248	Shannon	2.435269
89	2RTPVC1May15	Chao1	73.5	6.350031	Shannon	2.101371
90	2RTPVC2Apr23	Chao1	63.5	8.154964	Shannon	2.268675
91	2RTPVC2Jul16	Chao1	93	25.85755	Shannon	2.259071
92	2RTPVC2Jun13	Chao1	59.5	2.229688	Shannon	2.357665
93	2RTPVC2Mar29	Chao1	59.11111	1.574903	Shannon	2.494809
94	2RTPVC2May15	Chao1	96	25.86821	Shannon	1.851543
95	2RTPVCMar29H1	Chao1	77	8.472581	Shannon	2.179937
96	2RTPVCMar29H2	Chao1	84.16667	10.85485	Shannon	2.291413
97	CNTLFC2Y1	Chao1	43.75	1.421306	Shannon	0.777241
98	CNTLFC2Y2	Chao1	63.2	10.21397	Shannon	1.05091
99	DW1JUN12	Chao1	63.75	16.68514	Shannon	1.528161
100	DW2JUN12	Chao1	39.875	2.255249	Shannon	1.570471
101	LP1B1	Chao1	54	7.107964	Shannon	0.626862
102	LP1B2	Chao1	73.16667	10.85373	Shannon	0.66213
103	LP1T1	Chao1	53.6	5.34311	Shannon	0.601767
104	LP1T2	Chao1	75.2	13.14993	Shannon	0.925595
105	LP2B1	Chao1	54.25	5.36788	Shannon	0.692235
106	LP2B2	Chao1	66	13.20124	Shannon	0.840346
107	LP2T1	Chao1	45	3.415845	Shannon	0.51898
108	LP2T2	Chao1	47	2.58317	Shannon	0.577376
109	LP3B1	Chao1	54.5	4.105092	Shannon	0.517194
110	LP3B2	Chao1	51.66667	4.488984	Shannon	0.598354
111	LP3T1	Chao1	60.16667	7.377356	Shannon	0.492555
112	LP3T2	Chao1	50	2.58337	Shannon	0.813075
113	NLP4B1	Chao1	57.5	4.105239	Shannon	0.700534
114	NLP4B2	Chao1	61.16667	7.377466	Shannon	0.81724
115	NLP4T1	Chao1	52.5	2.897191	Shannon	0.687641
116	NLP4T2	Chao1	50.5	2.897082	Shannon	0.886958
117	NLP5B1	Chao1	64.5	12.89356	Shannon	0.49084
118	NLP5B2	Chao1	67.75	11.16487	Shannon	0.819901
119	NLP5T1	Chao1	61	8.470967	Shannon	0.531301
120	NLP5T2	Chao1	49.66667	2.04361	Shannon	0.535061
121	NLP6B1	Chao1	57.25	9.54455	Shannon	0.535763
122	NLP6B2	Chao1	59.14286	4.649713	Shannon	0.574149
123	NLP6T1	Chao1	81.33333	22.7244	Shannon	0.481169
124	NLP6T2	Chao1	42.75	1.254745	Shannon	0.461999
125	WMLPFC2Y1	Chao1	109.125	7.594231	Shannon	1.372846
126	WMLPFC2Y2	Chao1	62.33333	4.122113	Shannon	1.24773

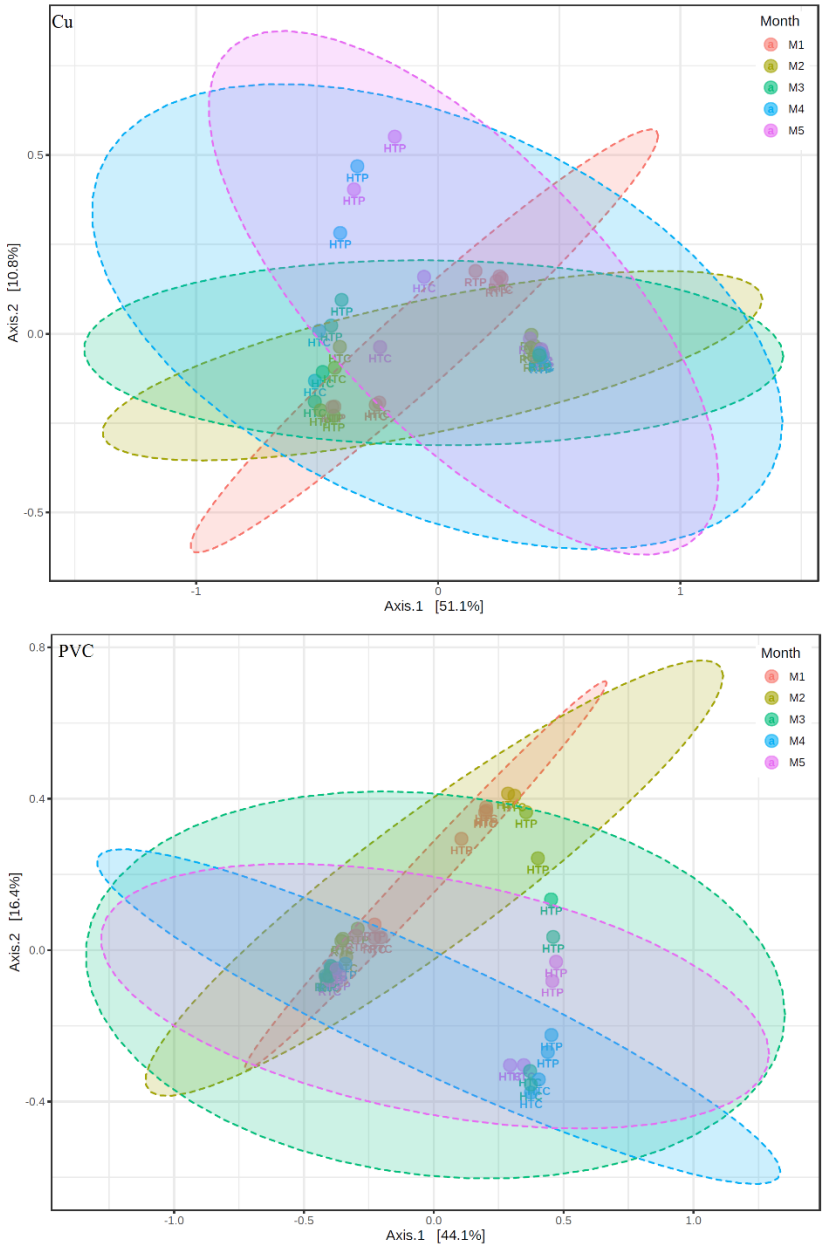
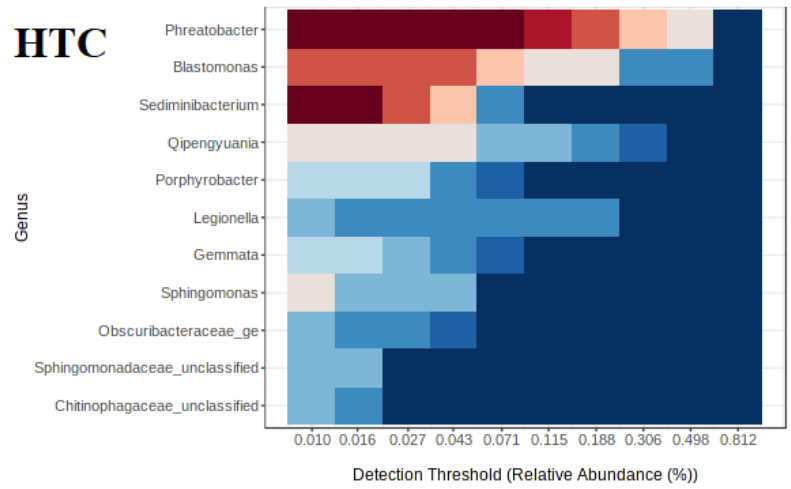
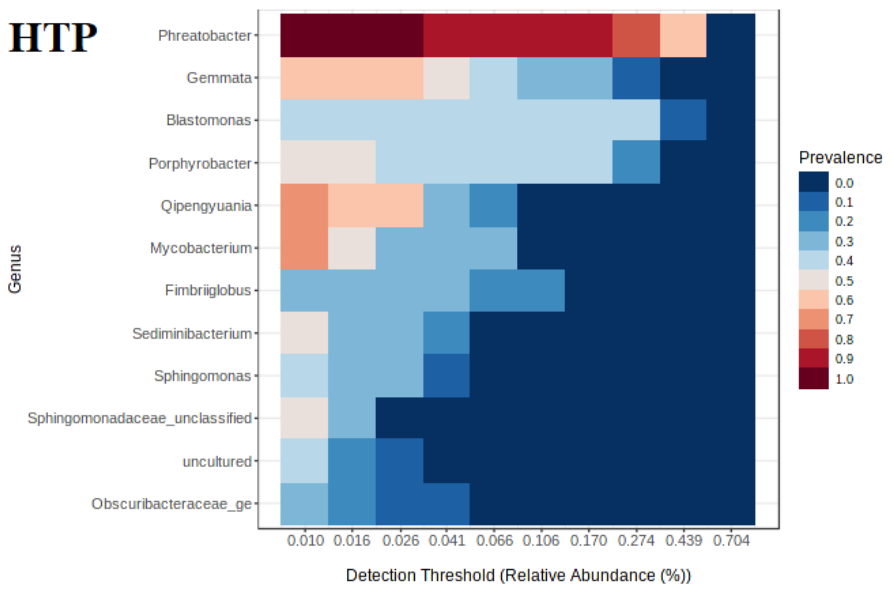
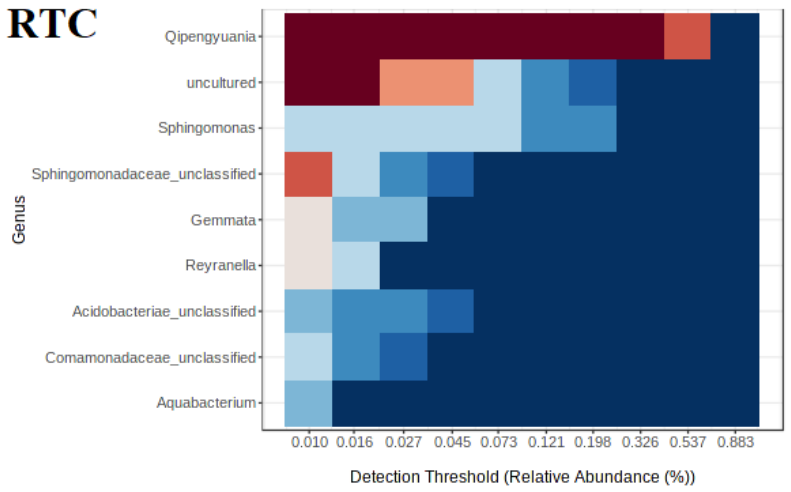
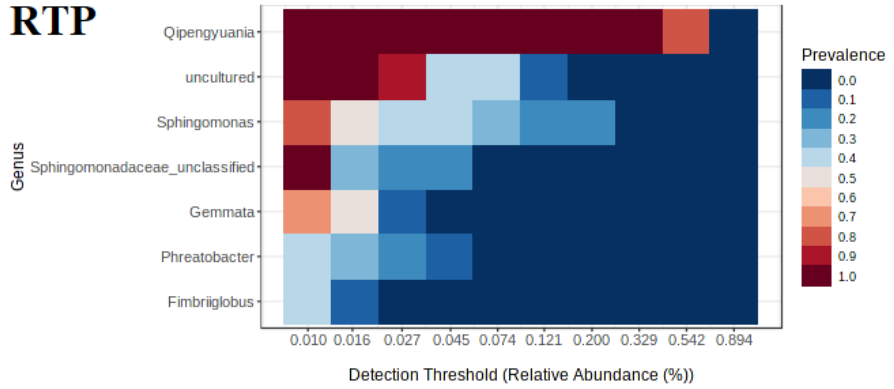


Figure S5.10: Beta diversity (PCoA plot of Jaccard index) of Cu and PVC biofilms with/without upstream probiome at HT (Control=HTC, probiome=HTP) and RT (RTC and RTP) on different months.


HTC
Phreatobacter
Blastomonas
Sediminibacterium
Qipengyuania
Porphyrobacter
Legionella
Gemmata
Sphingomonas
Obscuribacteraceae_ge
Sphingomonadaceae_unclassified
Chitinophagaceae_unclassified
Genus
0.010 0.016 0.027 0.043 0.071 0.115 0.188 0.306 0.498 0.812
Detection Threshold (Relative Abundance (%))


HTP
Phreatobacter
Gemmata
Blastomonas
Porphyrobacter
Qipengyuania
Mycobacterium
Fimbriiglobus
Sediminibacterium
Sphingomonas
Sphingomonadaceae_unclassified
uncultured
Obscuribacteraceae_ge
Genus
0.010 0.016 0.026 0.041 0.066 0.106 0.170 0.274 0.439 0.704
Detection Threshold (Relative Abundance (%))
Prevalence
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0


RTC
Qipengyuania
uncultured
Sphingomonas
Sphingomonadaceae_unclassified
Gemmata
Reyranella
Acidobacteriae_unclassified
Comamonadaceae_unclassified
Aquabacterium
Genus
0.010 0.016 0.027 0.045 0.073 0.121 0.198 0.326 0.537 0.883
Detection Threshold (Relative Abundance (%))


RTP
Qipengyuania
uncultured
Sphingomonas
Sphingomonadaceae_unclassified
Gemmata
Phreatobacter
Fimbriiglobus
Genus
0.010 0.016 0.027 0.045 0.074 0.121 0.200 0.329 0.542 0.894
Detection Threshold (Relative Abundance (%))
Prevalence
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

Cu

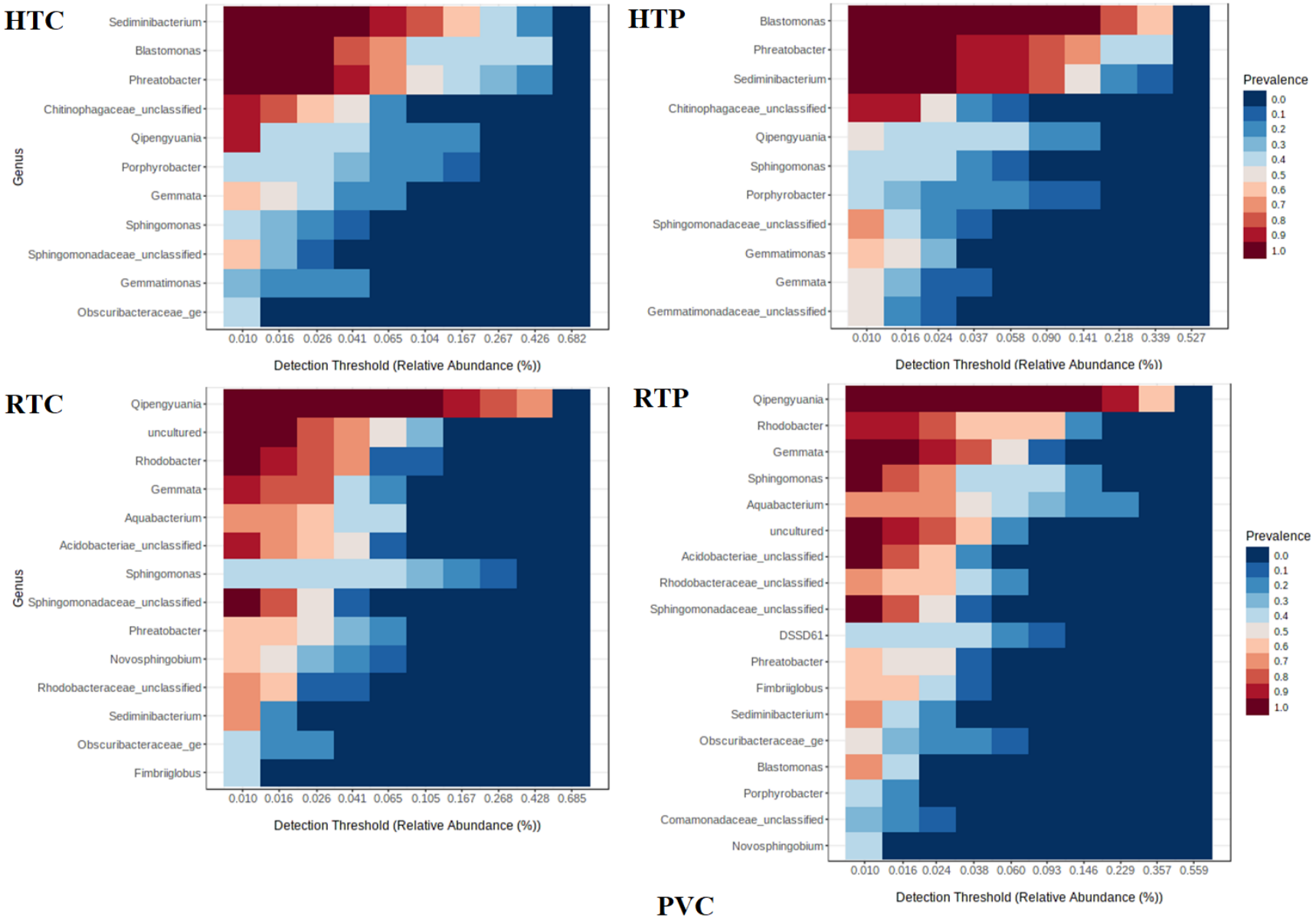
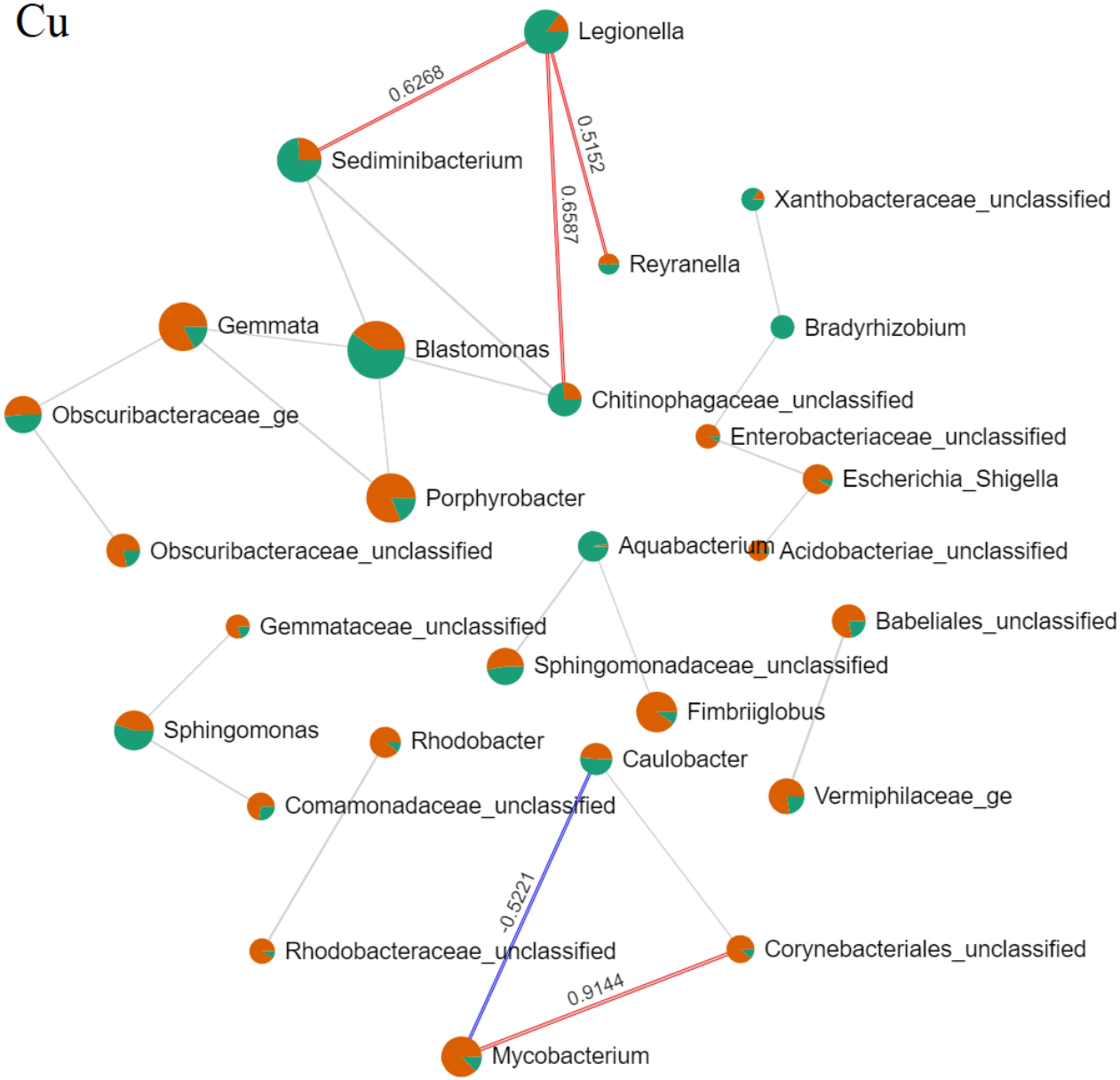
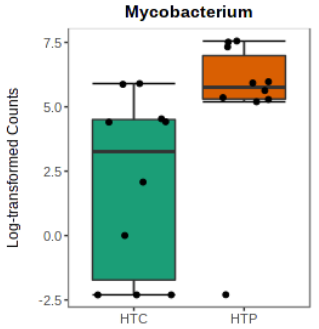
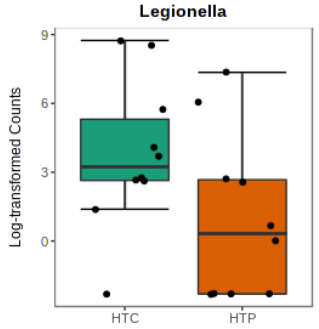

HTC
Sediminibacterium
Blastomonas
Phreatobacter
Chitinophagaceae_unclassified
Qipengyuania
Porphyrobacter
Gemmata
Sphingomonas
Sphingomonadaceae_unclassified
Gemmatimonas
Obscuribacteraceae_ge
Genus
0.010 0.016 0.026 0.041 0.065 0.104 0.167 0.267 0.426 0.682
Detection Threshold (Relative Abundance (%))
HTP
Blastomonas
Phreatobacter
Sediminibacterium
Chitinophagaceae_unclassified
Qipengyuania
Sphingomonas
Porphyrobacter
Sphingomonadaceae_unclassified
Gemmatimonas
Gemmata
Gemmatimonadaceae_unclassified
0.010 0.016 0.024 0.037 0.058 0.090 0.141 0.218 0.339 0.527
Detection Threshold (Relative Abundance (%))
Prevalence
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
RTC
Qipengyuania
uncultured
Rhodobacter
Gemmata
Aquabacterium
Acidobacteriae_unclassified
Sphingomonas
Sphingomonadaceae_unclassified
Phreatobacter
Novosphingobium
Rhodobacteraceae_unclassified
Sediminibacterium
Obscuribacteraceae_ge
Fimbriiglobus
Genus
0.010 0.016 0.026 0.041 0.065 0.105 0.167 0.268 0.428 0.685
Detection Threshold (Relative Abundance (%))
RTP
Qipengyuania
Rhodobacter
Gemmata
Sphingomonas
Aquabacterium
uncultured
Acidobacteriae_unclassified
Rhodobacteraceae_unclassified
Sphingomonadaceae_unclassified
DSSD61
Phreatobacter
Fimbriiglobus
Sediminibacterium
Obscuribacteraceae_ge
Blastomonas
Porphyrobacter
Comamonadaceae_unclassified
Novosphingobium
0.010 0.016 0.024 0.038 0.060 0.093 0.146 0.229 0.357 0.559
Detection Threshold (Relative Abundance (%))
Prevalence
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
PVC

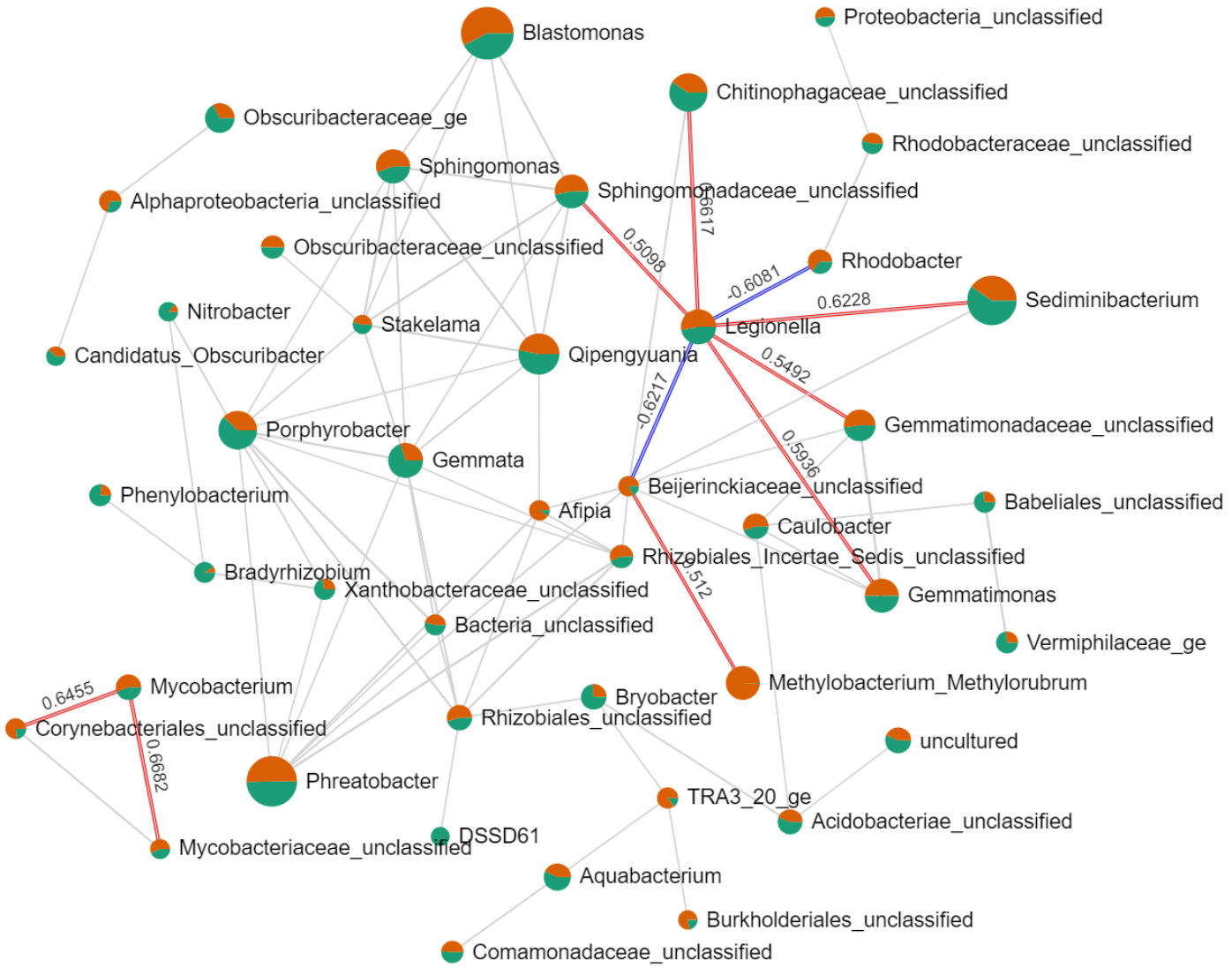
Figure S5.11: Core microbiome (Genus level) on Cu (Top) and PVC biofilms (bottom) at HT and RT with and without probiome.

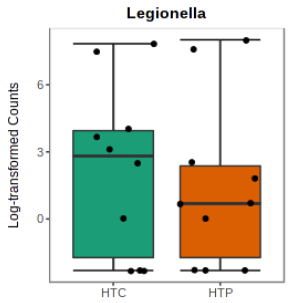

Cu
Legionella
0.6268
Sediminibacterium
0.5152
0.6587
Xanthobacteraceae_unclassified
Reyranella
Gemmata
Blastomonas
Bradyrhizobium
Chitinophagaceae_unclassified
Obscuribacteraceae_ge
Enterobacteriaceae_unclassified
Escherichia_Shigella
Porphyrobacter
Obscuribacteraceae_unclassified
Aquabacterium
Acidobacteriae_unclassified
Gemmataceae_unclassified
Babeliales_unclassified
Sphingomonadaceae_unclassified
Fimbriiglobus
Sphingomonas
Rhodobacter
Caulobacter
Comamonadaceae_unclassified
Vermiphilaceae_ge
-0.5221
Rhodobacteraceae_unclassified
Corynebacteriales_unclassified
0.9144
Mycobacterium

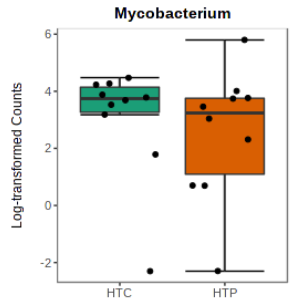

Mycobacterium
Log-transformed Counts
7.5
5.0
2.5
0.0
-2.5
HTC
HTP


Legionella
Log-transformed Counts
9
6
3
0
HTC
HTP

PVC


Blastomonas
Proteobacteria_unclassified
Chitinophagaceae_unclassified
Obscuribacteraceae_ge
Rhodobacteraceae_unclassified
Sphingomonas
Sphingomonadaceae_unclassified
Alphaproteobacteria_unclassified
Obscuribacteraceae_unclassified
Rhodobacter
Nitrobacter
Stakelama
Sediminibacterium
Legionella
Candidatus_Obscuribacter
Qipengyuania
Porphyrobacter
Gemmatimonadaceae_unclassified
Gemmata
Phenylobacterium
Beijerinckiaceae_unclassified
Babeliales_unclassified
Afipia
Caulobacter
Rhizobiales_Incertae_Sedis_unclassified
Bradyrhizobium
Xanthobacteraceae_unclassified
Gemmatimonas
Bacteria_unclassified
Vermiphilaceae_ge
Mycobacterium
Methylobacterium_Methylorubrum
Bryobacter
Corynebacteriales_unclassified
Rhizobiales_unclassified
uncultured
Phreatobacter
TRA3_20_ge
Acidobacteriae_unclassified
DSSD61
Mycobacteriaceae_unclassified
Aquabacterium
Burkholderiales_unclassified
Comamonadaceae_unclassified
0.6617
0.5098
-0.6081
0.6228
0.5492
-0.6217
0.5936
0.512
0.6455
0.6682


Legionella
Log-transformed Counts
HTC
HTP


Mycobacterium
Log-transformed Counts
HTC
HTP

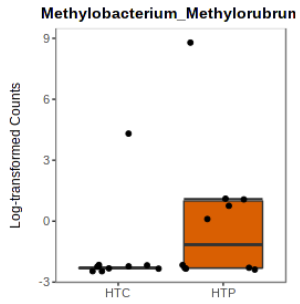

Methylobacterium_Methylorubrun
Log-transformed Counts
HTC
HTP

Figure S5.12: Co-occurrence correlation **n**etwork analysis of Cu (Top) and PVC biofilms (bottom) at HT with and without probiome.