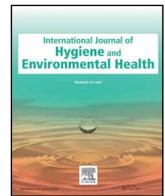




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Long-term persistence of infectious *Legionella* with free-living amoebae in drinking water biofilms



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ABSTRACT

Prolific growth of pathogenic *Legionella pneumophila* within engineered water 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, we studied *in-situ* the colonization of *L. pneumophila* in the presence of two water-related FLA species, *Willertia magna* and *Acanthamoeba polyphaga* in drinking water biofilms. During water flow as well as after periods of long-stagnation, the attachment and colonization of *L. pneumophila* to predeveloped water-biofilm 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 *Legionella* and were observed with internalized *L. pneumophila* cells after 3 weeks from their introduction to 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 2 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 the 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 intracellular growth of *Legionella* is possibly cell-concentration dependent. Observing the preferential feeding behavior, we hypothesize that an initial increase of FLA numbers through feeding on a range of other available bacteria could lead to an enrichment of *L. pneumophila*, and later force predation of *Legionella* by the amoeba trophozoites results in rapid intracellular replication, leading to problematic concentration of *L. pneumophila* in water. In order to find sustainable control options for legionellae and various other saprozoic, amoeba-resisting bacterial pathogens, this work emphasizes the need for better understanding of the FLA feeding behavior and the range of ecological interactions impacting microbial population dynamics within engineered water systems.

1. 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). *L. 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* 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 under-reporting 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* coexists with other bacteria and

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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* interacts with other bacteria and potential eukaryotic hosts (Bryers and Characklis, 1982). Microeukaryotes 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 interaction is thus considered responsible for increased environmental fitness of ARB like *Legionella* (Cavalier-Smith, 2002; Hahn and Höfle, 2001) and for shifting the environmental bacterial community towards higher ARB abundance (Raghupathi et al., 2017). *L. 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 well 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* under different conditions (in the presence of other microorganisms within a water-biofilm environment). The most commonly reported FLA genera in EWS are *Acanthamoeba*, *Vermamoeba* (*Hartmannella*) and *Naegleria* (Coşkun et al., 2013; Delafont et al., 2013), therefore we focused on studying the interaction of *A. polyphaga* and *W. magna* (as a non-pathogenic species closely related to *N. fowleri* (Robinson et al., 1989) which is known to cause fatal meningoencephalitis in young children) with a pathogenic *L. pneumophila* strain, and their persistence within the tap-water biofilm environment. 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 *Legionella* that have been estimated to be about $3.5 \times 10^6\text{--}8$ cells·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 interaction, although the exact mechanisms are unknown (Declerck et al., 2005). *P. 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., 2018; Wang et al., 2012). The last meters of pipe before the point-of-use is considered the most vulnerable regarding problematic growth of *Legionella* 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* 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; Bartram et al., 2007), yet we feel it is fundamental for estimating risk and for advising site-specific strategic management options for EWS. In this study, we used a water-flow system that does not simulate EWS, but rather satisfies some of the conditions like no residual chlorine level, relatively warmer temperature (22–40 °C) that are considered facilitating *Legionella* growth to enable us 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). We used fluorescent microscopy 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 interaction of FLA species with *L. pneumophila* within the natural drinking water biofilm environment.

2. Material and methods

2.1. 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 (S1 A). 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 (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 (monochloraminated) supply at the laboratory tap and stored at room temperature in a 20-L reservoir for 10–12 d 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, Thermo Fisher Scientific) to observe biofilms formation *in-situ*.

2.2. Inoculating water-biofilms with *L. pneumophila*

L. pneumophila (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 room temperature for 7–8 d (to avoid filamentous growth) (Piao et al., 2006). Several colonies were picked, re-suspended in filter-sterilized (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 §2.3) were diluted with aged tap water (having no residual chlorine) in a 2 L-glass reservoir to have a final concentration of 10⁵ 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 (S1 B). The water containing *L. pneumophila* with amoeba was circulated as a closed-loop system at a

rate of $0.5 \text{ mL} \cdot \text{min}^{-1}$ 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 during the entire 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).

2.3. 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 $400 \times g$ for 10 min, washed three times with filter-sterilized tap-water (following the same procedure that was used for bacteria with the exception of centrifugation at $400 \times g$ for 10 min) and was re-suspended in sterile tap-water to a concentration of approximately 10^5 trophozoites $\cdot \text{mL}^{-1}$. An equal number of each amoeba's trophozoites (20 mL of each amoeba suspension with a concentration 10^5 cfu $\cdot \text{mL}^{-1}$) were introduced to 2 L-reservoir at the same time when *L. pneumophila* cells were introduced to make a final amoeba: *Legionella* ratio of about 1:50 (Concentration of *L. pneumophila* = 10^5 cfu $\cdot \text{mL}^{-1}$, *W. magna* = 10^3 trophozoites $\cdot \text{mL}^{-1}$ and *A. polyphaga* = 10^3 trophozoites $\cdot \text{mL}^{-1}$ 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.

2.4. Fluorescent microscopy and image processing

The flow-cell containing biofilms with amoebae and *Legionella* 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 brightfield 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 1.52 g) (National Institutes of Health, US), if required.

2.5. Enumeration of *L. pneumophila*

Water samples were collected from the 2 L-reservoir bottle for three days immediately after introducing the *Legionella*, for three consecutive days after the first month and daily (except the weekend) for the weeks mentioned in [supplementary Table 1](#). Samples were assayed immediately for *L. pneumophila* by culture and for qPCR analysis, 10 mL water samples were filtered through a $0.22 \mu\text{m}$ filter (MicroFunnel™ Filter Funnels with Polycarbonate Membrane, Pall Corporation) and were stored at -80°C . *L. pneumophila* were enumerated by direct plating (spread plate method) of $25 \mu\text{L}$ and $50 \mu\text{L}$ 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 d. Since BCYE may support growth of other bacteria, to confirm legionellae, 60 random colonies of presumptive *Legionella* were picked from 20 different BCYE plates of 10 different water samples (10 d) and checked for the presence of the *mip* (macrophage infectivity potentiator) gene by qPCR according to

[Mentasti et al. \(2015\)](#) (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 GU $\cdot \text{mL}^{-1}$ ([Mentasti et al., 2015](#)). For molecular analysis, DNA was extracted from the filters (stored at -80°C) following EPA Method 1611 (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.

2.6. 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 E. Campbell, University of Alberta). Four 10 mL water samples were withdrawn from the 2 L-reservoir and centrifuged at $400 \times g$. The pellets were 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.

2.7. 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 ($\pm 1^\circ\text{C}$) from room temperature 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 everyday for two weeks ([Supplementary Table 1](#)) to determine the total *L. pneumophila* by culture and qPCR (*mip* gene) and to observe recovery of amoebae as described above. The flow-cell was also directly observed under a microscope (Brightfield and fluorescence) (without disconnecting it from the flow-system) for any changes in interactions of *L. pneumophila* and amoebae.

3. Results

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

Uniform, mature biofilms formation within the water flow system at room temperature was confirmed by observing the biofilms on the glass surfaces of the flow-cell ([Fig. 1](#)). No FLA was observed within the biofilms in the flow-cell during the first six months while running tap water only. Upon connecting the flow-cell to the 2 L-glass reservoir (containing tap water with *Legionella* 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

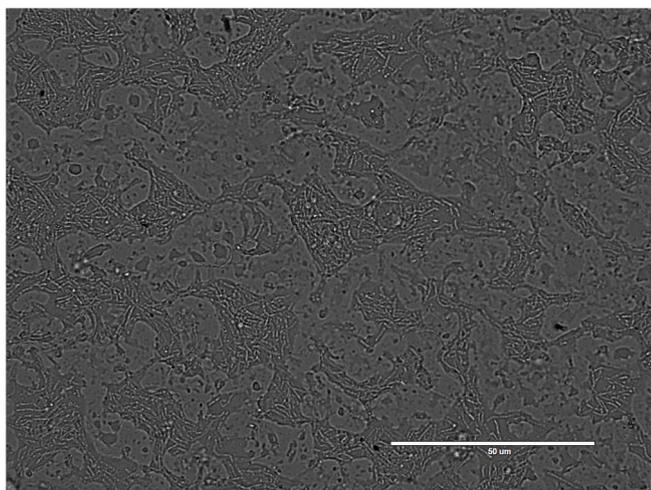


Fig. 1. Near-surface image of the water-biofilms developed after 6 months on a glass surface in the flow-cell at room temperature by running aged tap water through the system.

Legionella cells that were observed within the biofilm during 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 (S2 and S3). No micro-colony formation was observed by *L. pneumophila* during the entire study period (850 d). 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 d average 8.9×10^4 cfu mL⁻¹ that was close to the initial concentration of 1.07×10^5 cfu mL⁻¹).

3.2. *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) (S4 and S5). *W. magna* were frequently observed moving freely on the surface of the biofilms (Fig. 2A and S6). On the other hand, *A. polyphaga* appeared to be stressed and was mostly observed as rounded trophozoites and eventually as cysts within 5–7 d of its introduction. The flow-cell was observed everyday for a month after 16 h of stagnation before resuming the flow, and no engulfment of *Legionella* cells by the trophozoites was observed until the 20th day of their introduction (Fig. 2B and S7). At this point *W. magna* trophozoites were observed to contain

Legionella 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*, grow intracellularly and cause loss of mobility of the amoeba trophozoites under laboratory co-culture conditions in sterile tap water at room temperature (S8 and S9) (Shaheen and Ashbolt, 2018). Putative intracellular growth of *Legionella* 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 *Legionella* (Fig. 3). The green fluorescent patch surrounded with no apparent membrane-like structure (Fig. 3 right) could be a small cluster of *L. pneumophila* cells within a vesicle (or the bacterial cells released in the biofilms from a vesicle) as also seen in pure culture *Legionella*-*Williaertia* interactions (S10) (Shaheen and Ashbolt, 2018). The size and number of *W. magna* trophozoites decreased over time, yet trophozoites containing fluorescent *Legionella* cells were observed in the biofilms throughout the study period at room temperature (S11 and S12) but not when the temperature of the system was increased to 40 °C.

3.3. 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 (Fig. 4). No GFP-*Legionella* 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 amoeba cysts, 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 centre of the flow-cell (Fig. 4. D).

3.4. 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 2L-reservoir and was 8.9×10^4 cfu mL⁻¹ after the first month (3 d average). Interestingly, culturable *L. pneumophila* was observed even after 850 d from the time of introduction, with only about 2.0 log reduction in the total viable count ($\sim 1.5 \times 10^3$ cfu mL⁻¹). Small variations in the culturable cell concentration of *L. pneumophila*, determined continuously after 85 weeks of the study period indicated that the studied flow-system was in an ecological balance (Fig. 5). However, incubation at elevated temperature (40 °C) disturbed the system and produced a significantly higher (about 4 times higher; $P < 0.001$) *Legionella* culturable *Legionella* cell count compared to that at room temperature 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

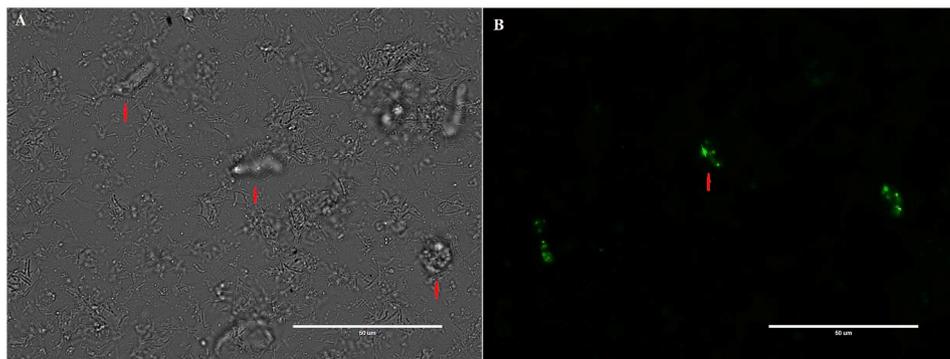


Fig. 2. (A) *W. magna* trophozoites (Red arrows) within the water-biofilm environment at room temperature. (B) *W. magna* trophozoites containing *L. pneumophila* (Green) at room temperature after 20 d in the system. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

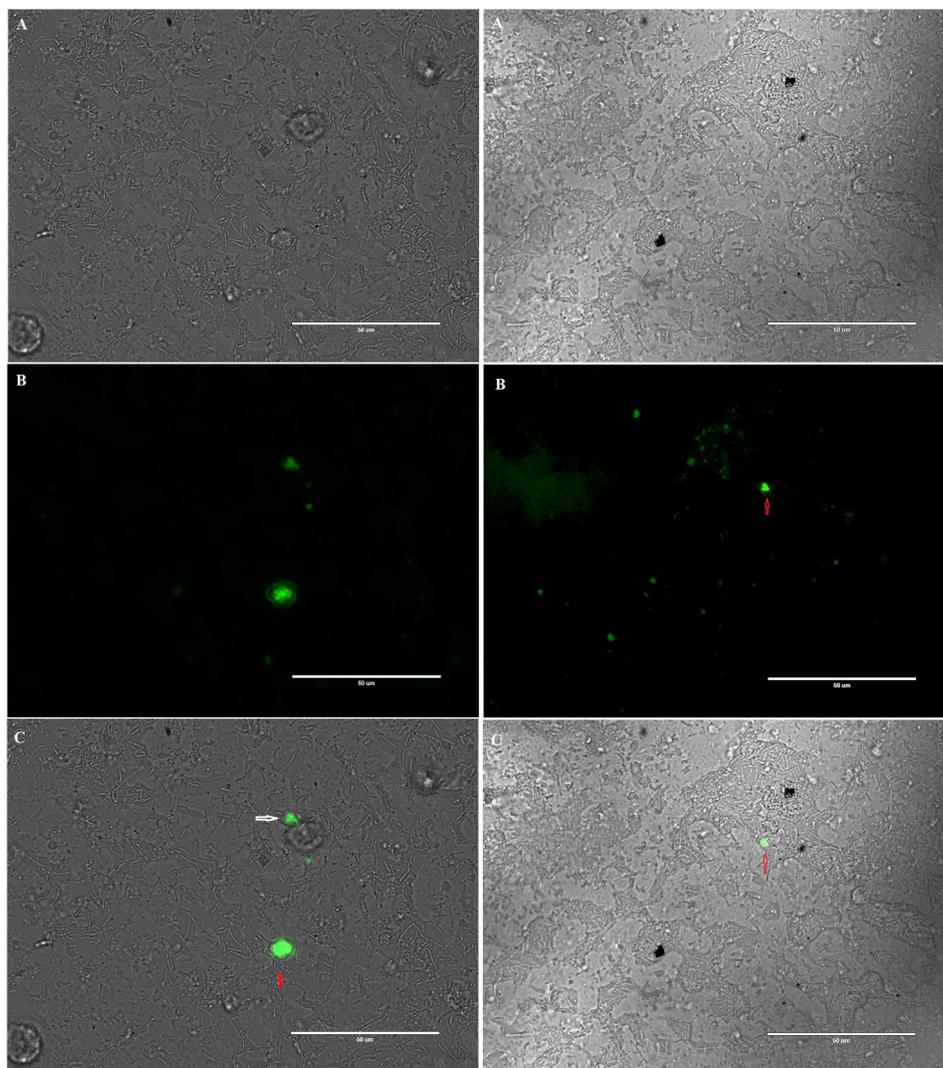


Fig. 3. (Left) Intracellular growth of *L. pneumophila* within amoeba trophozoites (red arrow) and formation of vesicle containing *L. pneumophila* from an amoeba trophozoites within tap water biofilms (white arrow). (Right) Cluster of newly replicated *L. pneumophila* cells possibly released from amoeba or vesicles (Red Arrow). (A) Brightfield mono color image (B) Image under green fluorescent channel and (C) composite image of the two channels. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

was lowered again to room temperature, the culturable legionellae count was reduced by 30% ($P < 0.001$) and remained in the range ($\sim 4.0 \times 10^3$ cfu mL $^{-1}$) for the remaining 12 weeks of the study period. The randomly selected presumptive *Legionella* colonies (non-fluorescent 60 colonies from 20 different BCYE plates representing 10 different water sample days) 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 *Legionella* 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 *Legionella* on the BCYE-PCV agar plates. Even during the incubation at 40 °C for two weeks the *Methylorubrum* sp. survived and was recovered by culture on BCYE-PCV agar plates till the end of the study period.

4. Discussions

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 *Legionella* 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 *Legionella* persisted as culturable cells in tap water/biofilms and within FLA trophozoites (intracellular) at room temperature for 850 d, which is the longest period ever reported for *Legionella* to survive in drinking water. Pathogenic *Legionella* 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* 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* to different plumbing materials exposed to sterile tap water inoculated with natural sludge containing an indigenous population of *Legionella*, other bacteria and amoebae (from a Legionnaires' disease outbreak source) has been reported, however what was striking was that the 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* within known multi-species bacterial biofilms has also been reported in potable water, but the bacterial biofilm was developed with rich

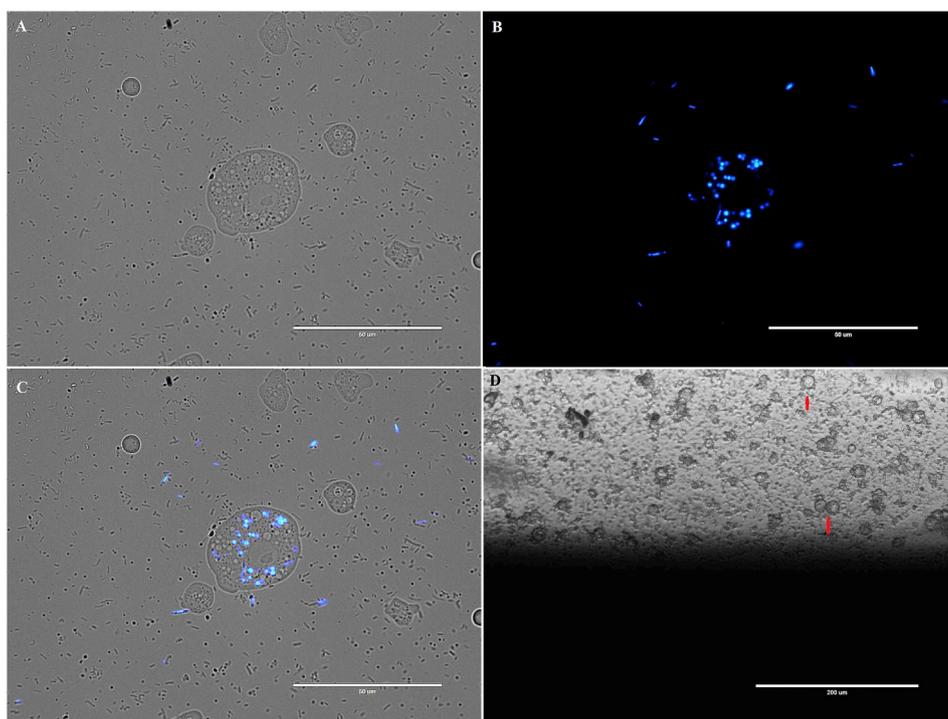


Fig. 4. (A) Isolation of amoebae from the water samples of the flow-cell system on non-nutrient agar plate with a lawn of *E. coli* (Mono color brightfield image). (B) The amoeba (*W. magna*) trophozoites containing *E. coli* (blue fluorescent) demonstrating active feeding of bacteria by trophozoites (Image under blue channel). (C) Composite image of A and B. (D) Cysts (Red Arrow) accumulate near the edge of the flow-cell flow-cell where the velocity of the water could be lower than at the centre of the flow-cell. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

microbiological media (Murga et al., 2001). In contrast, we did not observe any strong adherence of *Legionella* to predeveloped natural drinking water-biofilms.

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*-amoeba interactions may not be the same in a multi-species water biofilm environment (containing both prokaryotes and eukaryotes). The time delay in (phagocytosis) feeding of *Legionella* by *W. magna* provided an impression that given a choice, FLA may not prefer to feed on *L. pneumophila* in water-biofilms. It raises a question, however, if common water associated FLA are reluctant to feed on *L. pneumophila* when given a choice, how does intracellular growth get initiated to reach the critical concentration 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 complex in real biofilm environment and we propose that understanding the FLA initial food selection process could help to predict prolific growth conditions for *Legionella* and its control. Many regulatory guidelines for building water system describe common water associated FLA such as *Acanthamoeba* spp. and *N. fowleri* as a human health risk due to their inherent pathogenicity and to a lesser extent as host for intracellular growth of opportunistic bacterial pathogens like *Legionella* (Cope et al., 2015), but none of the guidelines have specified monitoring protocol or standard for amoebae. Non-*Legionella* bacteria has 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 FLA-bacteria interactions could be affected by different bacterial biofilm community members (could be general or FLA species-specific) and the selective feeding behavior of FLA may lead to the natural selection of amoeba-resisting human

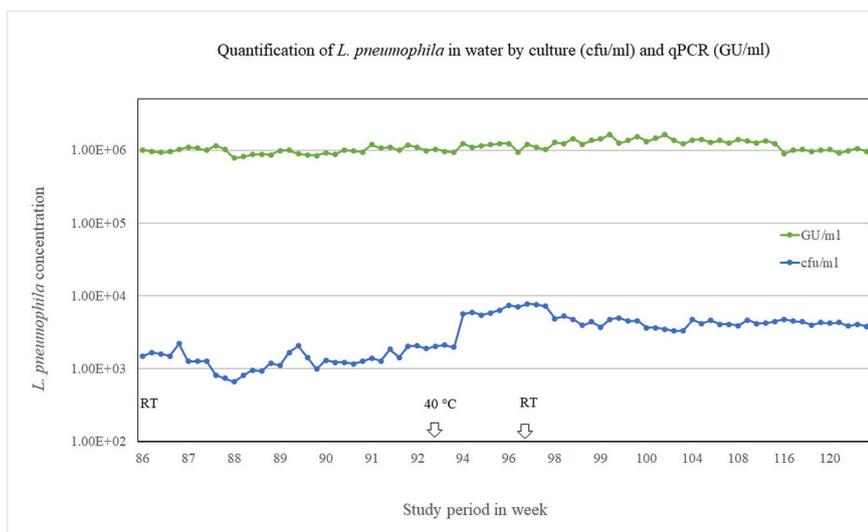


Fig. 5. Concentrations of *L. pneumophila* in the flow-cell drinking water determined by culture and qPCR (*mip* gene) over the last 35 weeks of the study period. This long-term *L. pneumophila* cell count demonstrated a stable ecosystem with minimum changes in cell numbers. Sudden changes on the incubation temperature by increasing to 40 °C from room temperature (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 indicate that lower temperature induce VBNC forms of the bacteria.

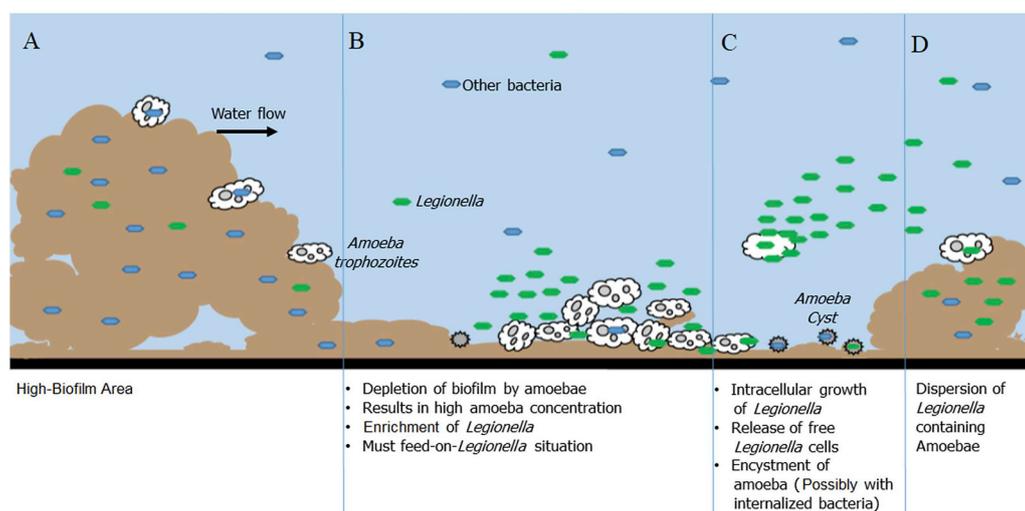


Fig. 6. Hypothetical model for FLA-bacteria interactions in drinking water pipe environment. Zone A: High bio-film formation (due to nutrients inflow or other reasons) encouraging FLA grazing and multiplication. Zone B: Depletion (diversity of food source) of biofilm by FLA grazing, increase of FLA numbers and selective grazing leaving a must-feed-on-*Legionella* condition for FLA. Zone C: Force-feeding on pathogenic *Legionella* may cause high concentration of *Legionella* cells through intracellular growth. Due to 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.

pathogens, thus changes the dynamics of drinking water-biofilm bacterial community composition in water-biofilms. 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* feeding – the initial step of the intracellular growth.

The presence of viable, pathogenic *Legionella* 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 harbour bacterial symbionts to resist the intracellular growth of *Legionella* (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*) (Schell et al., 2014; Simon et al., 2015; Tieden 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; Tieden et al., 2007) and to 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 *Legionella* within amoeba trophozoites indicate a common environmental pressure that forced the bacteria to maintain the GFP containing 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, but 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 room temperature suggested that low temperature induces 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 higher temperature (40 °C) also seemed to improve the overall fitness and viability of *L. pneumophila* cells, as lowering the temperature back to the room temperature 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* after more than 850 d from their introduction emphasizes a nutrient recycling within the system and a balanced relationship between FLA and *Legionella* at room temperature.

Further research is still required to better resolve FLA-*L. pneumophila* interactions in more controlled environments with multiple known bacterial species to characterize the differential feeding behavior of different 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* (Shaheen and Ashbolt, 2018).

Considering all the above, we propose a new model for *Legionella* growth within the water-biofilm environment (Fig. 6). We hypothesize that the conditions like high carbon concentration or sudden nutrient inflow, optimum growth temperature and low or no residual disinfectants increases biofilm formation that encourages FLA growth from selective feeding on non-pathogenic, preferred bacteria of the biofilm community. This differential feeding by FLA could cause a localized enrichment of water-based amoeba-resisting pathogens, which increases the likelihood for a must-feed-on-*L. pneumophila* condition (by increasing the amoeba to *Legionella* ratio). The amoeba-*Legionella* ratio can also be increased during stagnation periods due to the settling of free-floating planktonic *Legionella* cells on biofilms (S1). Under this condition of ‘force-feeding’, high number of intracellular *L. pneumophila* accumulated within amoeba trophozoites may initiate explosive growth/lysis cycles leading to problematic concentrations of *L. pneumophila* released into bulk water.

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 composition in the drinking water system (Lu et al., 2014; Pinto et al., 2012). Thus, understanding the ecology of *Legionella* and its relationship with FLA could be key to ensuring public health safety and to provide an effective monitoring target for predicting near future

growth-potential of *Legionella* in order to develop proactive control strategies. Overall, this study provided insight into how pathogenic *Legionella* possibly behave in a multispecies water-biofilm environment and their growth dynamics.

5. Conclusions

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

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijheh.2019.04.007>.

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