ORIGINAL RESEARCH

Spatial Arrangement of *Legionella* Colonies in Intact Biofilms from a Model Cooling Water System

Michael Taylor, Kirstin Ross and Richard Bentham

School of the Environment, Flinders University of South Australia, Adelaide, Australia.
Corresponding author email: michael.taylor@flinders.edu.au

Abstract: There is disagreement among microbiologists about whether *Legionella* requires a protozoan host in order to replicate. This research sought to determine where in biofilm *Legionellae* are found and whether all biofilm associated *Legionella* would be located within protozoan hosts. While it is accepted that *Legionella* colonizes biofilm, its life cycle and nutritional fastidiousness suggest that *Legionella* employs multiple survival strategies to persist within microbial systems. Fluorescent in situ hybridization (FISH) and confocal laser scanning microscopy (CLSM) demonstrated an undulating biofilm surface architecture and a roughly homogenous distribution of heterotrophic bacteria with clusters of protozoa. *Legionella* displayed 3 distinct spatial arrangements either contained within or directly associated with protozoa, or dispersed in loosely associated clusters or in tightly packed aggregations of cells forming dense colonial clusters. The formation of discreet clusters of tightly packed *Legionella* suggests that colony formation is influenced by specific environmental conditions allowing for limited extracellular replication. This work represents the first time that an environmentally representative, multispecies biofilm containing *Legionella* has been fluorescently tagged and *Legionella* colony morphology noted within a complex microbial system.

Keywords: *Legionella*, biofilm, cooling tower, protozoa, FISH, CLSM

This article is available from http://www.la-press.com.

Introduction

The ecological niche of Legionella is often disputed. An ongoing debate exists over the multiplication requirements of *Legionella* within environmental biofilms. Some reports suggest growth of *Legionella* occurs solely via parasitization of amebic hosts, and others that multiple survival strategies exist that exploit other avenues of nutrition and proliferation.[1]

As our understanding of microbial ecology expands, research becomes increasingly directed towards whole system biology rather than individual species interactions. Biofilms represent a pinnacle of microbial ecology, being complex aggregations of bacteria, fungi, viruses and protozoa, and higher eukaryotes that physiologically coordinate to form colonies that efficiently sequester nutrients.[2–4] Biofilms persist under conditions unfavorable to microbial growth, proliferate, and disseminate their component organisms.[3,5–8] *Legionella* bacteria are the cause of Legionnaires' disease, a serious bacterial pneumonia. They are of particular interest due to their survival in and dissemination from environmental biofilms.[9–12]

Biofilms harboring human pathogens, such as *Legionella*, present problems, as their removal from industrial water systems and cooling towers is problematic and the emphasis has been placed upon control rather than removal as the more realistic option.[13–17]

It is inadequate to study mixtures of planktonic bacteria as a measure of microbial community function and ecology. In order to elucidate the range of processes that occur in biofilms, various systems have been designed that provide adequate conditions for biofilm formation.[18–21] Bioreactors and chemostats are common in industrial microbiology, providing optimal growth conditions for biofilm formation. Conventional bioreactors do not resemble industrial water systems, heat exchangers, or cooling towers, and, thus, the resulting films may not be representative of environmental systems. This paper outlines the use of a modified Robbins device, which has been constructed to resemble an industrial heat exchanger/cooling tower in its operation to produce lab-scale analogues of environmental biofilms containing *Legionella*.[22]

Optical methods to observe biofilms have been used to give insight into the architecture of communities and their formation and persistence.[22–24] Visual reconstruction of the components of these films provides a nonmolecular avenue of study into the phenomena occurring within complex microbial systems. Developing tools to study interactions between biofilm associated organisms may eventually lead to an understanding of critical events or stages in biofilm formation and better target our attempts to remove them from water systems.

Methods
Biofilm growth in a modified robbins device/chemostat

To culture *Legionella* and biofilm associated microflora, modified chemostats were constructed that resembled heat exchangers in their function and design (Fig. 1). Each system consisted of a reservoir of 10 L volume containing a sparger to provide adequate aeration and a heater to maintain a temperature of 35 °C. Sparged air was vented into a moisture trap to ensure no aerosols were generated, and collected condensate was returned to reservoirs. Any fluid deficiency was made up using distilled water in order to maintain the homeostasis of the system. Water was circulated at a speed of 1 L per minute through a system at of polypropylene pipes, with an approximate volume of 3 liters, via an adjustable peristaltic pump (Masterflex L/S variable-speed modular drive, Cole-Parmer®, Illinois, USA) before being returned to the reservoir. The flow rate of 1 L per minute was chosen in order to achieve a laminar flow within the pipe systems (Reynold's number of <2000). Twelve sampling points were spaced at equal intervals along the length of the piping at which coupons of various materials may be added and periodically sampled for biofilm deposition and analysis. During the course of the study, 6 stainless steel and 6 polypropylene strips
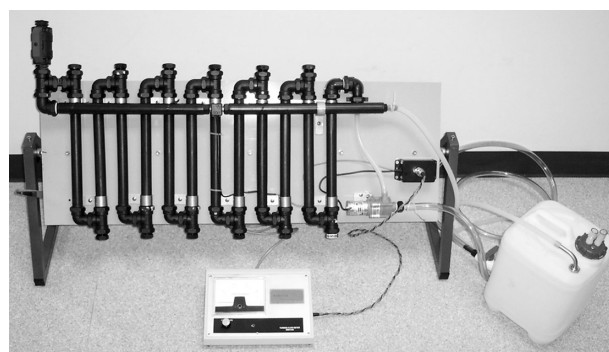

Figure 1. Modified Robbins device consisting of an aerated, heated reservoir connected to a series of opaque pipes.

taken from cooling tower fill were used as biofilm growth substrates. Coupons were cut to provide a final size of 10 cm long and 1 cm wide. Steel coupons were 1 mm thick and polypropylene 0.25 mm thick.

Each system was filled with water sourced from the cooling tower at the Flinders Medical Centre, South Australia, and was provided with 0.05% (final concentration in system) yeast extract to stimulate initial colonization of the system by native microflora. To further ensure that a representative microbial community was present in biofilm, systems were seeded with water derived from a cooling tower associated with a Legionnaires' disease outbreak. This process involved the addition of outbreak associated cooling water to 20% of the final volume of each system (3 L added, final volume ~15 L). Systems were continuously operated without further addition of nutrients or microbial inoculum for a period of 1 month before biofilm was sampled.

Biofilm sampling

Coupons were removed from the system and placed into tubes containing 50 mL Milli-Q water (A10 Academic water purifying system, Millipore, MA, USA). For microscopic visualization, tubes were gently inverted to remove attached biofilm without disrupting film structure. This method was chosen as coupons fit perfectly within 50 mL disposable tubes, with little room for movement by the coupon during inversion, reducing damage to biofilms. As the edges of each biofilm became detached, larger areas of film sloughed from the substrate allowing for the removal of large, mostly intact sections. This method allowed for the harvesting of high quality pieces of biofilm, with minimal damage to microbial structures and overall film architecture.

For culture, tubes were vortexed for 60 seconds, followed by sonication for 60 seconds to break apart biofilm structure.

Microbiological characterization of water systems

The bacterial load of system water was determined using methods described by Chen et al.[25] One cm² sections of coupon surfaces were scraped with cotton swabs to detach biofilm, placed in sterile water, and vortexed for 60 seconds, followed by sonication for 60 seconds to break apart biofilm. Samples were serially diluted to 1×10^{-7} and 10 µL aliquots of 1×10^{-2} to 1×10^{-7} dilutions were carefully transferred onto

R2a agar plates to form a 6×6 grid, with adequate spacing to ensure no drops touched. Plates were left to dry before incubation at 22 °C overnight. The number of colonies in each dilution series were counted and used to calculate the number of colony forming units (CFU) per cm² of coupon surface.

Coupon polysaccharide measurement and comparison

To determine the polysaccharide concentration on coupon surfaces (which can be used as an estimate of the total biofilm present), 1 cm² sections of coupon surfaces were scraped with cotton swabs to detach biofilm and vortexed in 1 mL sterile water to dislodge biomass from the swabs. Coupons were swabbed at the base, center, and tip to determine if sample location had any influence on the polysaccharide concentration. Polysaccharide content of each sample was analyzed using adaptation of methods by Masuko et al.[26]

Isolation and culture of *Legionella* from biofilm

The presence of *Legionella* within system derived biofilm was confirmed by isolation and culture using a modification of methods by Bopp et al.[27] Coupons were placed in 50 mL of sterile water and vortexed for 60 seconds, followed by sonication for 60 seconds to break apart biofilm structure. Settled biofilm was collected and serially diluted into HCl:KCl buffer, adjusted to pH 2.2, and incubated at room temperature for 12 minutes. Then, 100 µL aliquots were plated on selective media consisting of buffered charcoal yeast extract agar (BCYE) supplemented with L-cysteine, α-ketoglutarate and ferric-pyrophosphate, and a selective supplement of glycine, vancomycin, polymixin, and cycloheximide (GVPC) (Oxoid, Adelaide, Australia).

Plates were incubated in candle jars at 37 °C and observed for *Legionella*-like colonies at 3, 5, and 7 days of incubation using a dissecting microscope. Colonies displaying representative *Legionella*-like morphology—circular, off white, with a grainy ground glass appearance and opalescence—were subcultured onto clean BCYE agar and blood agar. Colonies displaying growth on BCYE and not on blood agar were tested using latex agglutination (*Legionella* latex test kit, Oxoid, Adelaide, Australia) for positive identification as *Legionella pneumophila*.

Fluorescent in situ hybridization and mounting

Oligonucleotide probes were selected using Probe-base[28] to select sequences specific to organisms of interest. Three probes were selected to target *Legionella* LEG705 (5′-CTG GTG TTC CTT CCG ATC-3′),[29] prokaryotes EUB338 (5′-GCT GCC TCC CGT AGG AGT-3′),[30] and eukaryotes UNIV1389b (5′-ACG GGC GGT GTG TAC AAA-3′).[31] Probes were tagged with distinct fluorophores with little to no overlap in fluorescence emission, LEG705 with Alexa Fluor 647, EUB338 with Alexa Fluor 546, and UNIV1389b with Alexa Fluor 488 (Invitrogen, Melbourne, Australia).

Sampled biofilm was transferred to tubes containing 4% w/v paraformaldehyde and incubated at 4 °C overnight. Samples were transferred to microscope slides and air dried at 35 °C. Fixed samples were washed once with phosphate buffered saline (PBS) and dehydrated in a series of ethanol baths, of 50%, 80%, and 96% respectively.

Samples were hybridized using an adaptation of methods by Grimm et al.[32] Samples were covered with 200 µL hybridization buffer (0.9 M NaCl, 0.01% sodium dodecyl sulphate, 20 mM tris-HCl [pH 7.6]) containing 100 ng of each probe, covered with hybridization chambers to maintain optimal humidity, and incubated for 90 minutes at 46 °C.

Unbound probes were removed by incubating samples with warmed washing buffer (0.9 M NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl [pH 7.6]) for 10 minutes at 48 °C. Samples were given a final wash with Milli-Q water and air dried in the dark before mounting with Citifluor (Citifluor ltd, Leicester, UK).

Negative and positive controls were generated to ensure probes did not bind to nontarget organisms. Each probe was tested against a culture of *Legionella*, *E. coli* (ATCC 25922) and biofilm derived *Bacillus cereus*, and visualized to ensure the absence or presence of fluorescence.

Confocal laser scanning microscopy

Images were acquired using a Leica SP5 confocal microscope. Bandpath filters were set to encompass the maximal emission of each fluorophore to remove interference from autofluorescence from film components. High resolution image stacks were compiled for regions of interest, encompassing scans through the full thickness of each film sample.

All imaging work was carried out at Flinders Microscopy using Leica LAS AF software and imageJ.[33]

Results

Microbiological content of biofilms

The presence of *Legionella* in biofilm was confirmed by culture and latex agglutination (Oxoid, Adelaide, Australia).

Plate count biofilm organisms

Plate count results generally showed wide variation between replicates. Plate counts showed an average CFU/cm^2 of 9.6×10^5 with a standard deviation of 1.5×10^6. Plates tended to become overgrown rapidly at lower dilutions, making counting impossible, while higher dilutions showed large variability in the number of countable colonies. This is a result of the strongly self-adhesive nature of biofilm, which does not neatly homogenize by agitation or pipetting. This leads to difficulties in sampling as any dilution or attempts to break up samples generally results in an assortment of fragments, which may deposit unevenly leading to high variability in results.

Biofilm deposition on coupons

Biofilm deposition was relatively homogeneous between coupons (Fig. 2) but varied along the length of the coupon, with greater deposition on the proximal end. Sample location showed minimal influence over carbohydrate content of sample except where large sections of biofilm remain attached to coupons, resulting in large spikes in variation. Comparisons of coupon material and related biofilm showed no significant differences ($P > 0.05$) in quantified polysaccharide, with an average polysaccharide concentration of 317 ± 35 mg/cm^2 on stainless steel coupons and 299 ± 48 mg/cm^2 on polypropylene coupons.

Figure 2. Black polypropylene substrate with associated biofilm. Biofilm appears as rusty orange/brown depositions on the surface of the plastic.

Biofilm tended to be a rusty brown/orange color on coupons, which were not exposed to light. Biofilm forming in the semitransparent tubing and reservoir tended to be a dark green color due to the presence of algae. Associated with biofilms were a wide range of grazing organisms, including ciliates and amebae, rotifers, and nematodes. There appeared to be no discernible difference in the presence of *Legionella* or the structure and composition of biofilms derived from either polypropylene or steel coupons.

Probes showed little to no nonspecific binding in controls and fluorescently tagged biofilms. Organisms of interest appeared distinctly against background fluorescence and with identifiable microbial structures appearing within the appropriate fluorescent channels.

Figure 3 is a typical representation of biofilm harvested from this system, with inset image of a transverse section of the film showing undulating surface architecture. Fluorescence has been artificially colored to increase contrast. EUB338 (all bacteria) was colored red, LEG705 (*Legionella*), blue and UNIV1389b (eukaryotes), green. Some localized areas of increased bacterial density were present, which punctuated an otherwise homogeneous distribution. Protozoa generally appeared clustered, that is, appearing as groups of green spheroids visible in the top right and middle left of Figure 3. Samples were previsualized using light microscopy to observe the presence of chlorophyll and to further discriminate between protozoa and algae. *Legionella* appeared as distinct groups of cells (indicated by arrows), often surrounding or associated with protozoa, and occasionally as high density clusters or dispersed groups of cells in localized areas of biofilm. Diffuse, low intensity fluorescence was occasionally seen where the eukaryote probe bound to targets originating from eukaryotic cell remnants or nonspecifically to EPS.

Figure 4 presents a series of common representations of *Legionella* colonies in fluorescently tagged biofilms derived from this system. *Legionella* appeared in 3 conformations within biofilms derived from this system. Figure 3A shows a cluster of protozoa (green) that appear as elliptical/spheroid forms surrounded with *Legionella* (blue). Where *Legionella* was observed interacting with protozoa, typically trophozoites (not shown) and cysts were surrounded or pervaded with substantially higher numbers of *Legionella* bacteria than in other
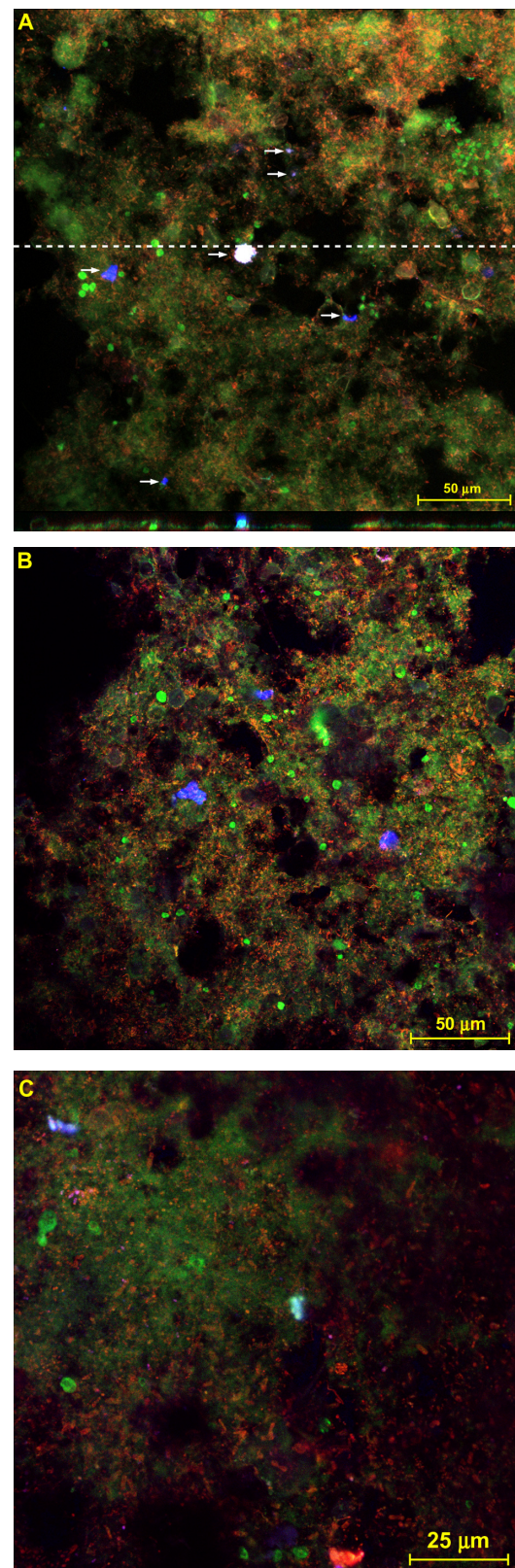

Figure 3. Typical representations of FISH tagged biofilms from this system. (**A**) is annotated to highlight *Legionella* colonies (indicated by arrows), with inset image showing transverse view displaying undulating surface. Fluorescent tags have been artificially colored to increase image contrast. Eukaryotes appear green, Legionella, blue, and all other bacteria, red.
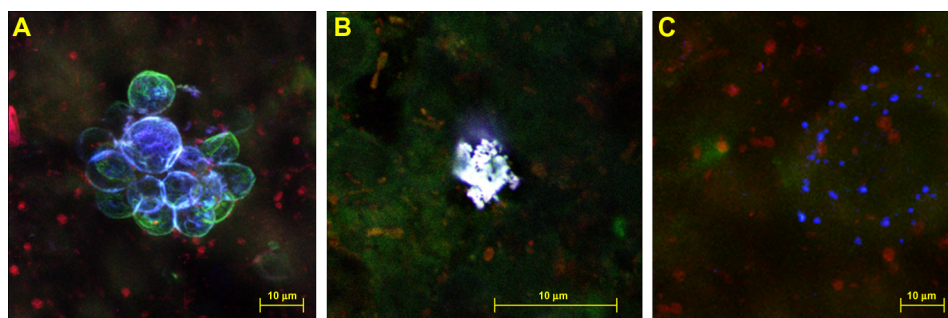
Figure 4. Legionella appeared in 3 distinct colony morphologies, either present associated with or encapsulated within protozoa (**A**), as closely grouped collections of cells (**B**), or as individual cells apparently dispersing from a roughly central origin (**C**).

areas of biofilm. Figure 4B shows a high concentration of clustered *Legionella*. Cells in this conformation were very closely packed with little to no edgewise dispersion indicating a lack of motility. Figure 4C shows *Legionella* in a loosely associated group. Cells are separated by distances ranging from 1 to 10 microns, clustered in a roughly spherical distribution. This suggests cells are motile and are displaced over the remains of a lysed protozoa indicated by weak fluorescence.

Discussion

This study represents the first time that the structure of a complex, multispecies biofilm containing *Legionella* has been visualized and reconstructed using FISH and CLSM. Previously, *Legionella* has been visualized parasitizing amebae using fluorescent in situ hybridization (FISH), confocal laser scanning microscopy (CLSM), and epifluorescence microscopy.[32] To date, studies have not been conducted to visualize *Legionella* in intact environmental biofilms. Although studies of *Legionella*/protozoa interactions in cocultures are valuable for understanding *Legionella* growth, extrapolation of the observed interactions into the context of complex multispecies environmental biofilm processes are inadequate, as they avoid the complexity of these systems. Processes such as nutrient cycling,[34,35] quorum sensing, predation, and cometabolism[36] may significantly modify the growth and interactions that these organisms have with one another.

The modified chemostat developed for use in this study provides a suite of abiotic conditions which may be altered to suit the requirements of the experiment undertaken. Temperature, aeration, and flow rate may be adjusted to mirror the conditions common to many industrial water systems and heat exchangers.

The length of the polypropylene piping housing containing the test surfaces allows for a natural temperature gradient to form, reflecting the conditions within a cooling tower connected to a heat exchanger. Test surfaces are interchangeable, which allows a wide range of materials to be studied for their biofilm forming potential. Small sections of transparent tubing and a transparent reservoir allowed for algal growth in this study; however, all piping can be replaced with opaque or transparent alternatives to provide the required light levels if other photobiological effects are to be studied.

This approach is limited, however, by the inability to fully characterize the microbiological content of the water used to inoculate the system and any biofilm derived from this inoculum. As a concession, light microscopy can be used to determine the presence of larger grazing organisms, and, as presented in this study, a range of relatively simple microbiological techniques can be employed to give an estimate of the bacterial activity/load of the system.

Figures 3 and 4 demonstrate that *Legionella* colonies tend to exist as distinct clusters of cells rather than being homogenously distributed throughout the film, often forming larger colonies around protozoa or protozoan cysts. This dispersion pattern is common is biofilm inhabiting organisms,[24] but *Legionella* presents a unique case, as its ecological niche, multiplication, and survival mechanisms and still in debate.[1,37–39] If *Legionella* survives as an obligate intracellular pathogen with little extracellular survival capability outside of protozoan hosts, then the vast majority of *Legionella* would be visible associated with these hosts. If other survival mechanisms are employed, then groups of cells should exist in a similar fashion to other common biofilm inhabiting organisms, as noted in Figure 4B.

Numerous studies have demonstrated *Legionella*'s capability to multiply intracellularly within suitable amebic and macrophagic hosts.[40–47] Figure 4 shows the localization of *Legionella* to specific regions of the film, which may reflect its lifecycle and ability to act as an intracellular pathogen of amebae.

Legionella multiply within hosts until amino acid levels become too low to facilitate further growth, at which point they become flagellated assisting in host lysis and egress.[48] This process would leave pockets of *Legionella* located around the lysed remains of their host in relatively uniform groupings of dispersed cells originating from the remains of their host (Fig. 4C) or located within amebic hosts during intracellular replication (Fig. 4A). This presents a distinctly different appearance than Legionella in Figure 4B, which are clearly not associated with a protozoan host and are nonmotile as denoted by their tight clustering.

Legionella has been demonstrated to multiply on algal exudates[49,50] and saprotrophically on the remains of other dead organisms if the nutrient content is sufficiently high.[51] The closely grouped cells notable in Figure 4B may reflect the formation of nutrient rich areas within the biofilm that provide the conditions required to form the observed colonies.

Recent biochemical work has shown that *Legionella* produces at least one siderophore, legiobactin,[52] a compound that chelates Fe^{3+} increasing its solubility and bioavailability. Many aquatic bacteria possess siderophores, and their production is a necessity for multiplication in low iron environments such as soil, aquatic and marine sediments, and their associated biofilms.[53] The role of iron in *Legionella* multiplication has been well documented, and it is known to be a crucial nutrient required for the growth of *Legionella*.[54] Cianciotto[52] demonstrated that *Legionella* possess siderophore synthetase and secretion genes, *lbtAB*, with structures homologous to *E. coli*, *Sinorhizobium,* and *Bordetella*. *lbtAB* deficient *Legionella* mutants were still capable of normal infection and multiplication inside human macrophages, hartmanella, and mouse lungs.[52] This strongly suggests that the presence and expression of this siderophore is required for growth and multiplication within aquatic environments rather than during intracellular growth.

The ecological niche of *Legionella* may not be as straightforward as previously assumed. Parasitism appears to be the primary mechanism by which *Legionellae* multiply in environmental sources but may represent only one survival strategy in a relatively complex lifecycle. Work by Garduno et al[55,56] has demonstrated the existence of multiple morphological and life cycle stages incorporating periods of dormancy in preparation for intracellular growth. Although this work is not designed to quantitatively compare the likelihood of specific spatial arrangements of *Legionella* occurring in biofilms, we suggest that *Legionella* persists in a dormant state within a biofilm following periods of intracellular replication; however, periods of limited extracellular growth may occur when localized biofilm conditions are nutritionally favorable to multiplication. The ability to culture *Legionella* on nutritionally supplemented media without the presence of a host attests to this replication mechanism. It is also possible that *Legionella* is able to multiply on the nutrient rich remains of lysed protozoa displaying some chemotaxic or, more accurately, a chemosensing ability. The nonmotile *L. longbeachae* genome displays the presence of a chemotaxic region.[57] This suggests that some *Legionella* species may be able to alter gene expression and cellular activity after existing in a viable, nonproliferative stage in response to the presence of protozoa or nutritionally favorable conditions.

This work provides new information for the ongoing discussion of the ecological niche of Legionella within biofilms. Presently, no clear consensus exists as to whether *Legionella* is capable of nonparasitic multiplication in environmental water sources. This work demonstrates the presence of microcolonies of *Legionella* not associated with protozoa. If samples from multiple disparate sources could be analyzed using similar microscopic methods, a model for *Legionella* colonization of biofilms, independent of the presence of protozoa, could be generated based upon the conditions present in each film, such as temperature, nutrient content, and pH.

As our understanding of microbial ecology, biofilms, and quorum sensing increases, so does our capacity to control microbial colonization of surfaces and reduce their persistence. Visualizing the interactions between microbes in an environmentally relevant setting, when coupled with molecular and culture methods of study, provides a greater depth of understanding of the processes occurring within

these complex microbial systems. By altering the conditions that mediate colony formation and overall biofilm architecture, we may gain insight into their control and removal from constructed water systems.

Author Contributions

Conceived and designed the experiments: MT. Analyzed the data: MT. Wrote the first draft of the manuscript: MT. Contributed to the writing of the manuscript: MT. Agree with manuscript results and conclusions: MT, RB, KR. Jointly developed the structure and arguments for the paper: MT, RB, KR. Made critical revisions and approved final version: MT, RB, KR. All authors reviewed and approved of the final manuscript.

Competing Interests

Author(s) disclose no potential conflicts of interest.

Disclosures and Ethics

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

References

1. Taylor M, Ross K, Bentham R. *Legionella*, protozoa, and biofilms: interactions within complex microbial systems. *Microb Ecol*. 2009;58(3): 538–47.
2. Lazarova V, Manem J. Biofilm characterization and activity analysis in water and wastewater treatment. *Water Res*. 1995;29(10):2227–45.
3. Chandy JP, Angles ML. Determination of nutrients limiting biofilm formation and the subsequent impact on disinfectant decay. *Water Res*. 2001; 35(11):2677–82.
4. Costerton WJ, Wilson M. Introducing biofilms. *Biofilms*. 2004;1:1–4.
5. Bachmann RT, Edyvean RGJ. Biofouling: an historic and contemporary review of its causes, consequences and control in drinking water distribution systems. *Biofilms*. 2005;2:197–227.
6. Långmark J, Storey MV, Ashbolt NJ, Stenström T-A. Accumulation and fate of microorganisms and microspheres in biofilms formed in a pilot-scale water distribution system. *Appl Environ Microbiol*. 2005;71(2): 706–12.
7. Schel AJ, Marsh PD, Bradshaw DJ, et al. Comparison of the efficacies of disinfectants to control microbial contamination in dental unit water systems in general dental practices across the European Union. *Appl Environ Microbiol*. 2006;72(2):1380–7.
8. Walker JT, Bradshaw DJ, Fulford MR, Marsh PD. Microbiological evaluation of a range of disinfectant products to control mixed-species biofilm contamination in a laboratory model of a dental unit water system. *Appl Environ Microbiol*. 2003;69(6):3327–32.
9. Fields BS, Benson RF, Besser RE. *Legionella* and Legionnaires' disease: 25 years of investigation. *Clin Microbiol Rev*. 2002;15(3):506–26.
10. Jones GL, Hébert GA. *"Legionnaires": The Disease, the Bacterium, and the Methodology*. Atlanta, GA: US Deptartment of Health, Education, and Welfare, Public Health Service, Center for Disease Control, Bureau of Laboratories; 1979. HEW publication 79 8375.
11. Bollin GE, Plouffe JF, Para MF, Hackman B. Aerosols containing *Legionella pneumophila* generated by shower heads and hot-water faucets. *Appl Environ Microbiol*. 1985;50(5):1128–31.
12. Tossa P, Deloge-Abarkan M, Zmirou-Navier D, Hartemann P, Mathieu L. Pontiac fever: an operational definition for epidemiological studies. *BMC Public Health*. 2006;6:112–22.
13. Almeida MAN, de França FP. Biofilm formation on brass coupons exposed to a cooling system of an oil refinery. *J Ind Microbiol Biot*. 1998;20(1): 39–44.
14. Cloete TE, Jacobs L, Brözel VS. The chemical control of biofouling in industrial water systems. *Biodegradation*. 1998;9(1):23–37.
15. Storey MV, Långmark J, Ashbolt NJ, Stenström TA. The fate of *legionellae* within distribution pipe biofilms: measurement of their persistence, inactivation and detachment. *Water Sci Technol*. 2004;49(11):269–75.
16. Nhu Nguyen TM, Ilef D, Jarraud S, et al. A community-wide outbreak of legionnaires disease linked to industrial cooling towers—how far can contaminated aerosols spread? *J Infect Dis*. 2006;193(1):102–111.
17. Bartram J, Chartier Y, Lee JV, Pond K, Surman-Lee S, eds. *Legionella and the Prevention of Legionellosis*. Geneva, Switzerland: World Health Organization; 2007.
18. Berg JD, Hoff JC, Roberts PV, Matin A. Growth of *Legionella pneumophila* in continuous culture. *Appl Environ Microbiol*. 1985;49(6):1534–7.
19. Burne RA, Chen Y-YM. The use of continuous flow bioreactors to explore gene expression and physiology of suspended and adherent populations of oral streptococci. *Methods Cell Sci*. 1998;20(1):181–90.
20. Pitts B, Willse A, McFeters GA, Hamilton MA, Zelver N, Stewart PS. A repeatable laboratory method for testing the efficacy of biocides against toilet bowl biofilms. *J Appl Microbiol*. 2001;91:110–7.
21. Parini MR. *Biofilm Removal Using Bubbles and Sound* [Master's thesis]. Brigham Young University; 2005.
22. McCoy WF, Bryers JD, Robbins J, Costerton JW. Observations of fouling biofilm formation. *Can J Microbiol*. 1981;27(9):910–7.
23. Walker JT, Keevil CW. Study of microbial biofilms using light microscope techniques. *Int Biodeter Biodegr*. 1994:223–36.
24. Thurnheer T, Gmür R, Guggenheim B. Multiplex FISH analysis of a six-species bacterial biofilm. *J Microbiol Methods*. 2004;56(1):37–47.
25. Chen C-Y, Nace GW, Irwin PL. A 6×6 drop plate method for simultaneous colony counting and MPN enumeration of *Campylobacter jejuni*, *Listeria monocytogenes*, and *Escherichia coli*. *J Microbiol Methods*. 2003;55(2): 475–9.

26. Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S-I, Lee YC. Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. *Anal Biochem*. 2005;339(1):69–72.

27. Bopp CA, Sumner JW, Morris GK, Wells JG. Isolation of *Legionella spp.* from environmental water samples by low-pH treatment and use of a selective medium. *J Clin Microbiol*. 4/1 1981;13(4):714–9.

28. Loy A, Horn M, Wagner M. probeBase: an online resource for rRNA-targeted oligonucleotide probes. *Nucl Acids Res*. 2003;31(1):514–6.

29. Manz W, Wendt-Potthoff K, Neu TR, Szewzyk U, Lawrence JR. Phylogenetic composition, spatial structure, and dynamics of lotic bacterial biofilms investigated by fluorescent in situ hybridization and confocal laser scanning microscopy. *Microb Ecol*. 1999;37(4):225–37.

30. Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol*. 1990;56(6):1919–25.

31. Loy A, Lehner A, Lee N, et al. Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. *Appl Environ Microbiol*. 2002;68(10):5064–81.

32. Grimm D, Merkert H, Ludwig W, Schleifer K-H, Hacker J, Brand BC. Specific detection of *Legionella pneumophila*: construction of a new 16S rRNA-targeted oligonucleotide probe. *Appl Environ Microbiol*. 1998;64(7):2686–90.

33. Abramoff MD, Magelhaes PJ, Ram SJ. Image processing with ImageJ. *Biophotonics International*. 2004;11(7):36–42.

34. Costerton JW, Cheng KJ, Geesey GG, et al. Bacterial biofilms in nature and disease. *Annu Rev Microbiol*. 1987;41(1):435–64.

35. Battin TJ, Kaplan LA, Denis Newbold J, Hansen CME. Contributions of microbial biofilms to ecosystem processes in stream mesocosms. *Nature*. 2003;426(6965):439–42.

36. Spratt DA, Pratten J. Carbon substrate utilization as a method of studying biofilm development. *Biofilms*. 2005;2:239–43.

37. Declerck P. Biofilms: the environmental playground of *Legionella pneumophila*. *Environ Microbiol*. 2010;12(3):557–66.

38. Paszko-Kolva C, Shahamat M, Yamamoto H, Sawyer T, Vives-Rego J, Colwell R. Survival of *Legionella* pneumophila in the aquatic environment. *Microb Ecol*. 1991;22(1):75–83.

39. Swanson MS, Hammer BK. *Legionella pneumophila* pathogenesis: a fateful journey from amoebae to macrophages. *Ann Rev Microbiol*. 2000;54:567–613.

40. Rowbotham TJ. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *J Clin Pathol*. 1980;33(12):1179–83.

41. Tyndall RL, Domingue EL. Cocultivation of *Legionella* pneumophila and free-living amoebae. *Appl Environ Microbiol*. 1982;44(4):954–9.

42. Fields BS, Shotts JEB, Feeley JC, Gorman GW, Martin WT. Proliferation of *Legionella pneumophila* as an intracellular parasite of the ciliated protozoan Tetrahymena pyriformis. *Appl Environ Microbiol*. 1984;47(3):467–71.

43. Holden EP, Winkler HH, Wood DO, Leinbach ED. Intracellular growth of *Legionella pneumophila* within Acanthamoeba castellanii Neff. *Infect Immun*. 1984;45(1):18–24.

44. Smith-Somerville HE, Huryn VB, Walker C, Winters AL. Survival of *Legionella* pneumophila in the cold-water ciliate Tetrahymena vorax. *Appl Environ Microbiol*. 9/1 1991;57(9):2742–9.

45. Kuchta JM, Navratil JS, Shepherd ME, et al. Impact of chlorine and heat on the survival of Hartmannella vermiformis and subsequent growth of *Legionella pneumophila*. *Appl Environ Microbiol*. 1993;59(12):4096–100.

46. Steele TW. Interactions between soil amoebae and soil *Legionellae*. In: Barbaree AM, Breiman RF, Dufour AP, editors. *Legionella: Current Status and Emerging Perspectives*. Washington, DC: American Society for Microbiology; 1993:140–2.

47. Berk SG, Ting RS, Turner GW, Ashburn RJ. Production of respirable vesicles containing live *Legionella pneumophila* cells by two Acanthamoeba spp. *Appl Environ Microbiol*. 1998;64(1):279–86.

48. Byrne B, Swanson MS. Expression of *Legionella pneumophila* virulence traits in response to growth conditions. *Infect Immun*. 1998;66(7):3029–34.

49. Tison DL, Pope DH, Cherry WB, Fliermans CB. Growth of *Legionella pneumophila* in association with blue-green algae (Cyanobacteria). *Appl Environ Microbiol*. 1980;39(2):456–9.

50. Berendt RF. Influence of blue-green algae (cyanobacteria) on survival of *Legionella pneumophila* in aerosols. *Infect Immun*. 1981;32(2):690–2.

51. Temmerman R, Vervaeren H, Noseda B, Boon N, Verstraete W. Necrotrophic growth of *Legionella pneumophila*. *Appl Environ Microbiol*. 2006;72(6):4323–8.

52. Cianciotto N. Iron Acquisition by *Legionella pneumophila*. *Biometals*. 2007;20(3):323–31.

53. Kraemer S. Iron oxide dissolution and solubility in the presence of siderophores. *Aquat Sci*. 2004;66(1):3–18.

54. Reeves MW, Pine L, Hutner SH, George JR, Harrell WK. Metal requirements of *Legionella pneumophila*. *J Clin Microbiol*. 1981;13(4):688–95.

55. Garduno RA, Garduno E, Hiltz M, Hoffman PS. Intracellular growth of *Legionella pneumophila* gives rise to a differentiated form dissimilar to stationary-phase forms. *Infect Immun*. 2002;70(11):6273–83.

56. Garduno E, Faulkner G, Ortiz-Jimenez MA, Berk SG, Garduno RA. Interaction with the ciliate Tetrahymena may predispose *Legionella pneumophila* to infect human cells. In: Cianciotto NP, editor. *Legionella: State of the Art* 30 *Years after Its Recognition*. Washington, DC: ASM Press; 2006.

57. Cazalet C, Gomez-Valero L, Rusniok C, et al. Analysis of the *Legionella longbeachae* genome and transcriptome uncovers unique strategies to cause Legionnaires' disease. *PLoS Genet*. 2010;6(2):e1000851.