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Minireview

# Biofilms: the environmental playground of *Legionella pneumophila*

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## Summary

***Legionella pneumophila*, the aetiological agent of 90% of legionellosis cases, is a common inhabitant of natural and anthropogenic freshwater environments, where it resides in biofilms. Biofilms are defined as complex, natural assemblages of microorganisms that involve a multitude of trophic interactions. A thorough knowledge and understanding of *Legionella* ecology in relation to biofilm communities is of primary importance in the search for innovative and effective control strategies to prevent the occurrence of disease cases. This review provides a critical update on the state-of-the-art progress in understanding the mechanisms and factors affecting the biofilm life cycle of *L. pneumophila*. Particular emphasis is given to discussing the different strategies this human pathogen uses to grow and retain itself in biofilm communities. Biofilms develop not only at solid-water interfaces (substrate-associated biofilms), but also at the water-air interface (floating biofilms). Disturbance of the water surface can lead to liberation of aerosols derived from the floating biofilm into the atmosphere that allow transmission of biofilm-associated pathogens over considerable distances. Recent data concerning the occurrence and replication of *L. pneumophila* in floating biofilms are also elaborated and discussed.**

## Introduction

*Legionellaceae* are Gram-negative bacilli that belong to the gamma-2 subdivision of the *Proteobacteria* and comprise *Legionella* as sole genus (Fry *et al*., 1991; Benson and Fields, 1998). To date, more than 50 different *Legionella* species have been described (Diederen, 2008) and approximately half of them are associated with human illness, better known as legionellosis (Muder and Yu, 2002). Legionellosis comprises two distinct entities, namely: Legionnaires' disease, an often fatal pneumonia, if not promptly and correctly diagnosed, and Pontiac fever, a milder, flu-like disease (reviewed by Diederen, 2008). *Legionella pneumophila* accounts for 90% of reported disease cases (Yu *et al*., 2002) and was recognized as being pathogenic to humans for the first time after an outbreak of acute pneumonia at a convention of the American Legion in Philadelphia, USA in July 1976 (Fraser *et al*., 1977).

Legionellae are ubiquitously present in both natural and anthropogenic freshwater environments, where they can withstand temperatures of 5.0°C-63°C and a pH range of 5.0–9.2 (Fliermans *et al*., 1981; Atlas, 1999). This shows that the conditions under which *Legionella* spp. occur in the environment are not stringent. Using PCR-based techniques, Sheehan and colleagues (2005) recently detected at least four *Legionella* species in an extremely acidic (pH 2.7), predominately eukaryotic algal biofilm community in Yellowstone National Park. Natural environments are rarely related to legionellosis because habitat conditions do not support extensive *Legionella* spp. growth. The only natural waters considered a source of legionellosis are hot springs, where temperatures generally range between 35°C and 40°C (Mashiba *et al*., 1993). Upon transfer from natural freshwater habitats into anthropogenic systems, where temperatures are generally higher than ambient temperature, *L. pneumophila* colonizes existing biofilms and proliferates to high numbers (Rogers *et al*., 1994). Also, it has the ability to parasitize protozoans, which commonly graze on biofilm communities (Greub and Raoult, 2004; Kuiper *et al*., 2004; Declerck *et al*., 2005; 2007a). As legionellosis is generally considered a preventable illness, efficient control measures of the bacterium in the water concerned will lead to a significant decrease in disease cases. In this context, exhaustive effort is directed towards uncovering the ecology of biofilm-associated *L. pneumophila*. To date, two reviews have been published on this topic (Keevil *et al*., 1993; Wright, 2000). Due to recent

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important findings, e.g., necrotrophic growth of *L. pneumophila* (Temmerman *et al*., 2006), the research on the mechanisms and factors affecting the ecology of *L. pneumophila* in relation to biofilm communities is increasing rapidly. Therefore, a critical and comprehensive update on the progress in the field is timely.

## Biofilms: a safe harbour for *L. pneumophila* in the cruel oligotrophic aqueous environment

In order to grow as a pure culture under lab conditions, *L. pneumophila* is remarkably fastidious and requires several different nutrients including iron salts and a number of amino acids such as *L*-cysteine, which are used as carbon, nitrogen and energy sources (George *et al*., 1980; Edelstein, 1982; Keen and Hoffman, 1984). The fact that *L. pneumophila* is commonly detected in oligotrophic aquatic environments (low nutrient content), despite its fastidious nature implies that the bacterium is able to obtain its necessary supply of amino acids and organic carbon from that same environment, more specifically from the microbial consortium located in biofilms.

Biofilms are defined as complex microbial communities characterized by cells that are attached to a substratum or phase boundary and to each other by means of a matrix of self-produced extracellular polymeric substances (EPS) (Donlan and Costerton, 2002). Microcolonies of bacterial cells encased in the EPS matrix are separated from each other by interstitial water channels, allowing transport of nutrients, oxygen, genes and even antimicrobial agents (Prakash *et al*., 2003). Because of their dynamic character, biofilm communities can continuously change in time and space, providing better survival and growth of the associated microorganisms. For this reason, it is easy to understand that in most natural environments biofilms are the prevailing microbial lifestyle (Watnick and Kolter, 2000). Generally, there are three distinct phases in the 'biofilm life cycle' of bacteria: (i) bacterial attachment to a substratum, (ii) biofilm maturation and (iii) detachment from the biofilm and subsequent dispersal in the environment (reviewed by Donlan, 2002). What is currently known about *L. pneumophila*, in relation to microbial biofilm communities, will be discussed using each of these three phases.

### *Attachment*

Numerous human pathogens such as *Pseudomonas aeruginosa* (Whiteley *et al*., 2001), *Staphylococcus epidermidis* (Mack *et al*., 2000), *Salmonella enteritidis* (Solano *et al*., 2002), *Vibrio cholerae* (Nesper *et al*., 2001), *Streptococcus gordonii* (Loo *et al*., 2000) and *Burkholderia cepacia* (Huber *et al*., 2001) are able to form a monospecies biofilm. In the case of *L. pneumophila*, pure cultures have been reported to form biofilms only under well-defined experimental conditions using the nutrient-rich *Legionella* medium (buffered yeast extract medium, BYE) (Piao *et al*., 2005; Mampel *et al*., 2006). Piao and colleagues (2005) demonstrated the ability of *L. pneumophila* to form biofilms on glass, polystyrene and polypropylene under static conditions in BYE medium at 25°C, 37°C and 42°C. A clear, significant temperature influence on biofilm thickness and structure was detected as seen in Fig. 1. Biofilms grown at 25°C consisted of rod-shaped *L. pneumophila* cells and possessed structural features typical of biofilms reported to date, i.e. pillar- and mushroom-like structures and what appeared to be water channels within (Fig. 1A). However, biofilms formed at 37°C and 42°C showed an even, thicker and more extensive mat of considerably greater cell density without the commonly observed water channels (Fig. 1B). Also, at higher temperatures the morphology of biofilm-associated *L. pneumophila* appeared to be filamentous and filaments were multinucleate but non-septate. The study performed by Mampel and colleagues (2006) reported that biofilm formation of *L. pneumophila* in rich medium at 30°C mostly relies on the adhesion of bacteria grown in the planktonic phase, rather than by clonal replication of sessile cells. Also, biofilms only developed under static conditions and were characterized by rather delicate, fluffy extensions from the surface.

Taking into account the above-mentioned two studies and the fact that *L. pneumophila* requires specific and fastidious growth requirements in order to be cultured in the lab, it is logical that under environmental circumstances the bacterium incorporates in pre-established biofilms as a secondary colonizer. This means that instead of docking to a surface and developing a biofilm, the bacterium will form a transient association with other microbes, previously attached to that surface and which have already initiated or formed a biofilm (Watnick and Kolter, 2000). This transient association allows *L. pneumophila* to search for a place to settle in the community. The concept of one species acting as a primary colonizer and promoting the adhesion and subsequent biofilm formation of another species has been shown for *P. aeruginosa* promoting the biofilm formation of *Escherichia coli* O157:H7 (Klayman *et al*., 2007). Mampel and colleagues (2006) observed adherence of *L. pneumophila* to monospecies biofilms formed by *Empedobacter breve*, *Microbacterium* sp. and *Acinetobacter baumanii* in BYE medium. In contrast, *L. pneumophila* did not attach to *Pseudomonas* spp., *Corynebacterium glutamicum* or *Klebsiella pneumoniae* biofilms, which suggests the existence of bacteria that oppose *L. pneumophila* adherence. Under natural conditions, biofilms occur mostly as complex multispecies communities. Using a more realistic experimental set-up, consisting of multispecies biofilms

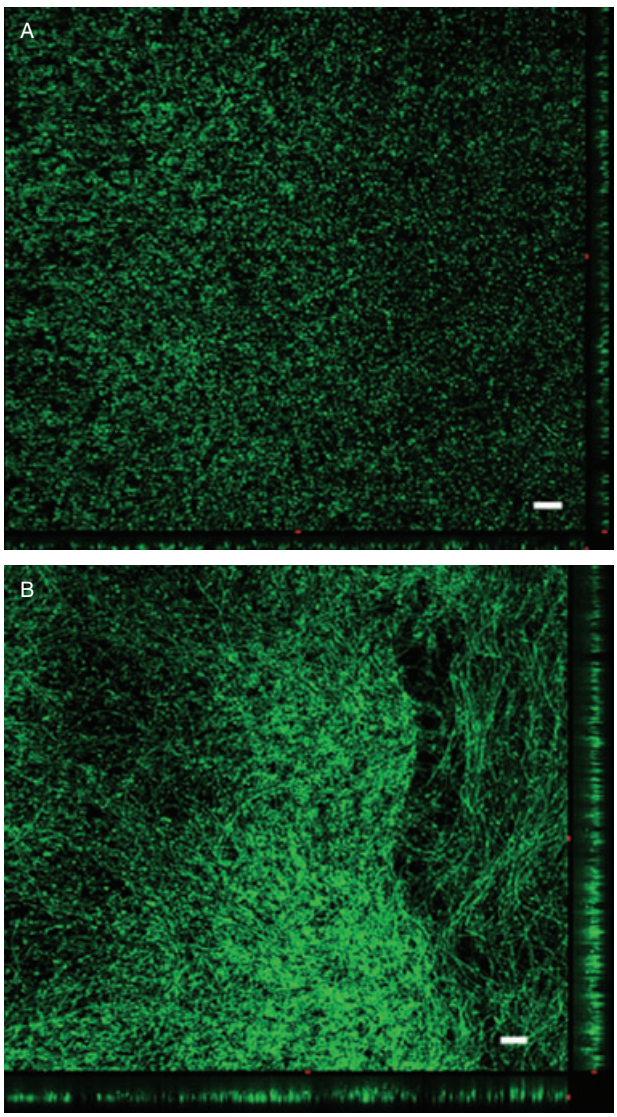


**Fig. 1.** Confocal laser scanning micrographs of GFP-expressing *L. pneumophila* Philadelphia-1 biofilms formed under static conditions in BYE medium at 25°C and 37°C.
A. A 6-day-old *L. pneumophila* biofilm at 25°C, consisting of rod-shaped cells and structural features typical for biofilms reported to date.
B. A 3-day-old *L. pneumophila* biofilm at 37°C, consisting of filamentous cells embedded in an even, thicker and more extensive structure.
Size bar: 10 μm. Reproduced with the permission of Dr Sze Chun Chau.

formed under oligotrophic conditions, it has been demonstrated that *L. pneumophila* rapidly colonizes present biofilm communities (Murga *et al*., 2001; Vervaeren *et al*., 2006; Declerck *et al*., 2007b). Time periods of less than 2 h were detected (Declerck *et al*., 2007b).

Whether the interaction between *L. pneumophila* and a pre-established biofilm is physical or involves cell signalling, better known as quorum sensing, remains unknown at this moment. Previous research has shown that adhesion in biofilms between primary and secondary colonizers may be facilitated by interspecies cell signalling using autoinducer-2 (AI-2) (Rickard *et al*., 2006). *L. pneumophila* lacks the widespread AI-2 signalling system, as well as an *N*-acyl-homoserine lactone (AHL)-based quorum sensing circuit. However, recently Spirig and colleagues (2008) discovered a 3-hydroxypentadecan-4-one signalling system in *L. pneumophila* designated as *Legionella* autoinducer-1 (LAI-1). Together with the *cholerae* autoinducer-1 of *Vibrio cholerae* (Higgins *et al*., 2007), LAI-1 forms a family of α-hydroxyketone autoinducer signalling molecules. Intercellular signalling involving α-hydroxyketones might be common among different bacterial species and genera and it warrants further research to investigate if LAI-1 allows interspecies communication between *L. pneumophila* and other bacteria.

All bacteria produce multiple adhesins permitting organisms to switch from planktonic to sessile forms (or the other way around) triggered by different environmental conditions (An *et al*., 2000). However, the success of bacterial attachment to an available surface is dictated by a number of variables, including environmental factors (e.g., the flow regime or hydrodynamics) and various properties of the cell surface (Donlan, 2002; Prakash *et al*., 2003). An increase in flow velocity may equate to increased attachment and it is known from the medical world that bacteria form biofilms preferentially in very high shear environments [Reynolds number (Re) > 10 000], like catheters. Liu and colleagues (2006) used a plumbing model, consisting of three parallel pipes with either a turbulent (Re > 10 000), laminar (Re < 1000) or stagnant (Re = 0) flow regime, to demonstrate a significantly higher biofilm colonization rate of *Legionella* spp. under turbulent flow conditions compared with laminar conditions as shown in Fig. 2. The lowest biofilm-associated *Legionella* spp. counts were detected under stagnant flow conditions. As mentioned above, the success rate of surface colonization also depends on various properties of the cell surface. Specific structural components like pili, fimbriae, flagella and surface-associated polysaccharides or proteins have been shown to play a critical role in facilitating bacterial interaction with surfaces (Donlan, 2002). Also, type II secretion, the mechanism by which most Gram-negative organisms export pilus machinery for assembly, translocates virulence factors that may contribute to biofilm maturation (Liles *et al*., 1999). In contrast to *L. pneumophila*, many Gram-negative bacterial pathogens have an absolute requirement for type IV pili and the type II secretion system in order to establish a biofilm and allow for maturation (Moorthy and Watnick, 2004; Giltner *et al*., 2006). Lucas and colleagues (2006) showed that *L. pneumophila* does not seem to have an absolute

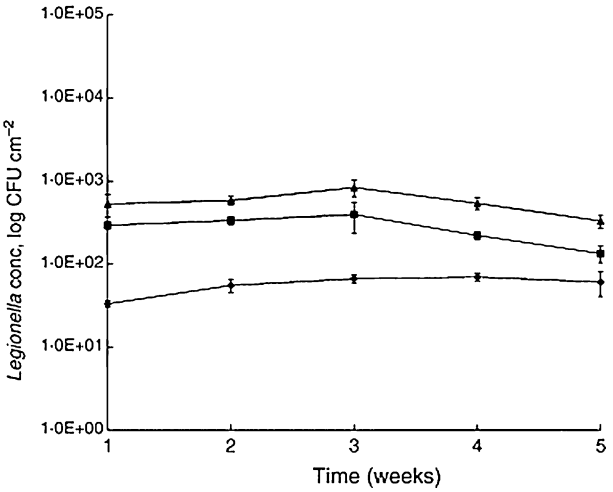


**Fig. 2.** Comparison of the *Legionella* spp. concentration in inlet biofilm samples. A significantly higher number of *Legionella* counts were recovered from the biofilm of the turbulent flow pipe than from the laminar flow pipe, while the lowest counts were recovered from the stagnant flow pipe ($P = 0.01$). Legends: ▲, turbulent flow; ■, laminar flow; ●, stagnant flow. Reprinted from Liu and colleagues (2006) *J Appl Microbiol* **101:** 437–442, doi:10.1111/j.1365-2672.2006.02970.x, with permission of the publisher (Wiley-Blackwell).

requirement for functional pilin expression in order to colonize pre-established biofilms. Using mutants of *L. pneumophila*, Lucas and colleagues showed that in the absence of suitable protozoan hosts the bacteria were able to utilize pili and or other unidentified pilin-independent adhesins to colonize biofilms. In the presence of protozoans, its unique lifestyle as a facultative intracellular parasite diminishes its need for primary attachment and retention mechanisms.

### *Growth and retention of biofilm-associated* Legionella*: more than one possible strategy to pick*

To become a productive member of society, attached bacteria must differentiate into biofilm-associated cells, which implies an up- and/or downregulation of a number of genes, e.g., repression of flagellum synthesis (Watnick and Kolter, 2000). In the case of *P. aeruginosa*, 22% of the genes are upregulated in the biofilm state, while 16% are downregulated. Hindré and colleagues (2008) performed the first transcriptome analysis of *L. pneumophila* biofilm cells. They revealed that a substantial proportion of *L. pneumophila* genes are differentially expressed, as 2.3% of the 2932 predicted genes exhibited at least a twofold change in gene expression.

In the next phase of the integration process, secondary colonizers start to grow and form microcolonies, which are the basic structural unit of biofilms (Donlan, 2002). Using immunogold and fluorscein immunolabelling, Rogers and Keevil (1992) were the first to detect microcolonies of *Legionella* in naturally occurring biofilms. Following further growth and an increase in volume the microcolonies become embedded in an EPS matrix, which reinforces the biofilm structure. Different bacterial species produce differing amounts of EPS (Prakash *et al.*, 2003). To date there is only one study, performed by Piao and colleagues (2005), suggesting that *L. pneumophila* might be able to produce EPS. Mycelial-like biofilms contained a thin layer of ruthenium red-stainable substance coating the cells that might be EPS.

In multispecies biofilms, where bacterial competition for food is high, it is advantageous to have more than one way to obtain the required growth nutrients. In the case of *L. pneumophila*, there exist two distinct ways. First, nutrients can be delivered by the biofilm environment itself. Numerous publications show that *L. pneumophila* is capable of obtaining nutrients either directly from other living microorganisms, like algae and some heterotrophic bacteria, producing them in excess, and/or indirectly from decaying organic matter. Second, *L. pneumophila* is able to infect and replicate inside protozoans. During its stay in the intracellular environment, peptides and proteins of the infected host are degraded and used as a nutrient source. Both ways of obtaining growth nutrients will be considered in detail.

*Extracellular replication in the biofilm matrix.* Biofilm-associated bacteria distribute themselves geographically based on the neighbours and environment that best suits their needs and requirements (Watnick and Kolter, 2000). The survival and growth of pathogenic microorganisms, generally characterized by a fastidious nature, within biofilms might be enhanced by the association and metabolic interactions with indigenous organisms (Donlan, 2002). Tison and colleagues (1980) were the first to demonstrate that *L. pneumophila* has the capability to grow on extracellular products provided by other bacteria. *L. pneumophila* was isolated from an algal-bacterial mat community, growing at 45°C in an anthropogenic thermal effluent. Under lab conditions, the *L. pneumophila* isolate grew in association with *Fisherella* sp. (a cyanobacterium) over a pH range of 6.9-7.6 in a mineral salts medium at 45°C. *Legionella* growth depended upon active photosynthesis of the cyanobacterium, implying the use of released algal products. Other algae that might support growth of *L. pneumophila* in basal salts medium are *Scenedesmus* spp., *Chlorella* spp. and *Gleocystis* spp. (Hume and Hann, 1984). In addition to algae, some heterotrophic bacteria appear able to support *L. pneumophila* growth. In complex media lacking *L*-cysteine or iron salts, and therefore unable to support *Legionella* growth, *L. pneumophila* forms satellite colonies around strains of some common aquatic bacteria, including *Flavobacterium breve*,

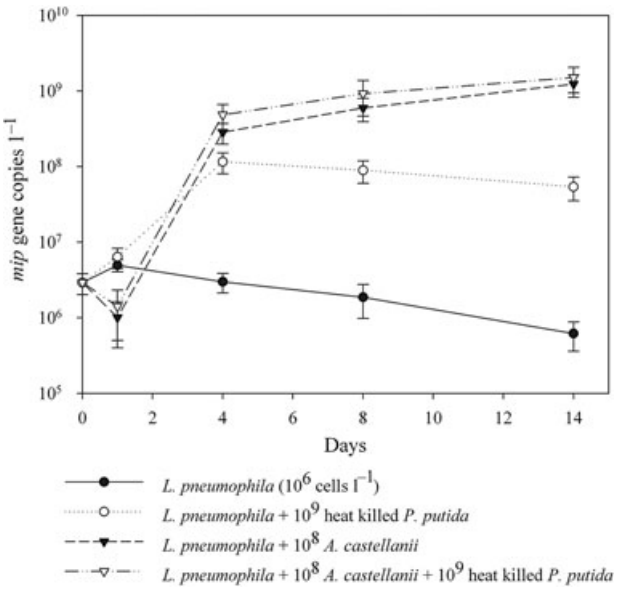


**Fig. 3.** Necrotrophic growth of *L. pneumophila* at 28°C in filter-sterilized tap water on heat-killed (30 min 60°C) *P. putida* cells and live *A. castellanii* cells. Quantification of *L. pneumophila* cells was performed during 2 weeks using real-time PCR targeting the *mip* gene. Reprinted from Temmerman and colleagues (2006) *Appl Environ Microbiol* **72:** 4323–4328, doi:10.1128/AEM.00070-06, with permission from the American Society for Microbiology.

*Pseudomonas* spp., *Alcaligenes* spp. and *Acinetobacter* spp. (Wadowsky and Yee, 1983; Stout *et al*., 1985).

In addition to nutritional commensalism, the ability to obtain carbon and energy sources from dead or decaying organic matter would significantly benefit the survival and growth of biofilm-associated *L. pneumophila*. Dead organic material can result from water disinfection measures (Van der Kooij *et al*., 2005) or from the biofilm itself, which as a constantly renewing entity can contain nearly 50% dead microbial cells (Tresse *et al*., 2003). A recent publication by Temmerman and colleagues (2006) demonstrated that *L. pneumophila* shows necrotrophic growth. Using several experimental setups, they clearly proved that *L. pneumophila* was able to consume and grow on dead Gram-negative bacteria like *Pseudomonas putida* and *E. coli* and amoebae like *Acanthamoeba castellanii*. However, when comparing necrotrophy with intracellular growth in *A. castellanii* as shown in Fig. 3, it appeared that the major route of *L. pneumophila* replication under environmental conditions occurs by means of protozoans.

Competition for substrate is considered to be one of the major evolutionary driving forces in the bacterial world, and numerous experimental data show how different microorganisms may outcompete others, as a result of a better accessibility or utilization of a given energy source. This means that, although a certain microenvironment in the biofilm may harbour sufficient nutrients for *L. pneumophila*, they might serve for the greater part to amplify faster-growing bacteria like *E. coli* and *P. aeruginosa*. By forming multinucleate, non-septate filaments, *L. pneumophila* may have hit upon another strategy to compete with fast-growing indigenous biofilm-associated bacteria (Piao *et al*., 2005). Under nutrient-rich conditions, the multinucleate *L. pneumophila* filaments can form septa. A septum is an inward growth of both the cytoplasmic membrane and cell wall, which separates compartments containing a nucleus. Each compartment represents one future daughter cell. In that way, one multinucleate, filamentous *L. pneumophila* bacterium can rapidly give rise to numerous daughter cells.

*Intracellular replication in protozoans.* The high concentration of microorganisms within biofilms provides excellent opportunities for predation by free-living protozoans, more specifically amoebae. This makes them important regulators of biofilm-associated bacterial populations, which contributes to the prokaryotic-eukaryotic co-evolution (Hilbi *et al*., 2007). It should therefore not be surprising that several biofilm-associated bacterial pathogens such as *Mycobacterium* spp., *Pseudomonas* spp., *Vibrio* spp. and *Legionella* spp. have evolved and acquired the capability to survive and multiply within free-living amoebae (Greub and Raoult, 2004; Molmeret *et al*., 2005; Weissenberger *et al*., 2007). In 1980, Rowbotham described for the first time the ability of *L. pneumophila* to replicate within amoebae (Rowbotham, 1980). Since then, *L. pneumophila* has been described to multiply in 14 species of free-living amoebae, two species of ciliated protozoans (*Cyclidium* spp. and *Tetrahymena pyriformis*) and one species of slime mold (*Dictyostelium discoideum*) (Fields *et al*., 2002).

Studies using flow-through or batch systems, containing a self-assembled or naturally occurring biofilm consortium, demonstrated that amoebae like *Hartmanella vermiformis* and *A. castellanii* are used for replication of biofilm-associated *L. pneumophila* in oligotrophic aquatic environments [Murga *et al*., 2001; Kuiper *et al*., 2004; Declerck *et al*., 2007b (Fig. 4)]. Moreover, in the absence of amoebae, biofilm-associated *L. pneumophila* numbers did not increase at all. Bacteria were only able to persist in the biofilm community and in some cases entered the viable but non-culturable (VBNC) state in order to promote their survival. Additionally, it has been suggested that biofilm-associated *L. pneumophila* may spend only a minor portion of its lifespan inside amoebae, using them solely as a host for replication (Declerck *et al*., 2007b). Forty-eight hours after inoculating *A. castellanii*, the whole amoeba population was heavily infected. Soon after, amoebic hosts lysed, releasing high numbers of *L. pneumophila* into the bulk phase of the reactor.

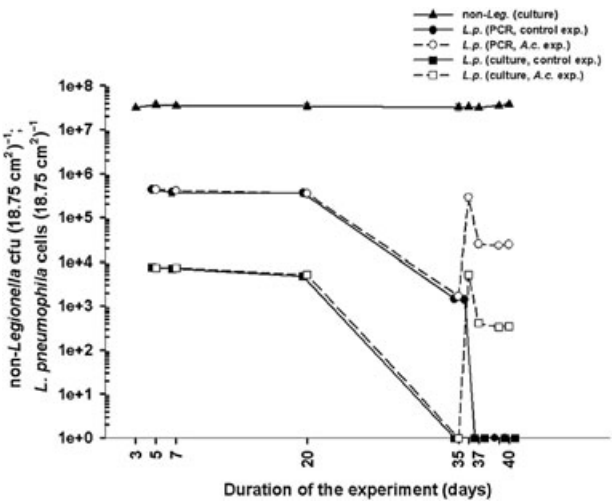


**Fig. 4.** Evolution of biofilm-associated non-*Legionella* and *L. pneumophila*. *Legionella pneumophila* and *A. castellanii* were inoculated in the rotating annular reactor (RAR) on days 5 and 35 respectively. The RAR was operating in flow-through mode and was fed with sterile dechlorinated tap water. Time-point samplings where *L. pneumophila* concentrations were below the quantification limit of both the culture and real-time PCR method are indicated by zero values. Reprinted from Declerck and colleagues (2007b).

To date, a lively discussion is ongoing concerning the survival and replication of *L. pneumophila* in biofilms. Do biofilm-associated *L. pneumophila* bacteria really require protozoan hosts to grow and increase their cell numbers or are they able to actively replicate in the community and thus obtain their nutrients from the biofilm environment? This discussion continues due to the lack of trustful data from pilot-scale or on-site experiments, using naturally occurring consortia. The problem of working with natural biofilm communities is that biofilms are microbial hotspots and the pathway of trophic interactions is immensely complex. In my opinion, both extracellular and intracellular replication can occur, depending on the environmental circumstances. Apparently, the intracellular pathway remains the most important one as demonstrated by Temmerman and colleagues (2006). Also, numerous benefits are related to intracellular replication in protozoans (reviewed by Molmeret *et al*., 2005), which have not yet been demonstrated for extracellular replication.

As a member of multispecies biofilms, the survival of *L. pneumophila* depends not only on its partners but also on its opponents. Toze and colleagues (1990) found that up to 32% of heterotrophic bacteria, e.g., *Pseudomonas* spp. and *Aeromonas* spp., isolated from chlorinated drinking water, inhibit growth of *Legionella* spp. Possible mechanisms of inhibition have not yet been unravelled. However, this effect could be partially attributed to bactericidal proteins, collectively known as bacteriocins that are continuously produced by both Gram-positive and Gram-negative bacteria. Guerrieri and colleagues (2008) performed a study to investigate the effect of bacterial interference on biofilm counts of *L. pneumophila*. They showed that certain bacteria are able to influence *L. pneumophila* in its survival and persistence in biofilms through production of bacteriocins. *Pseudomonas fluorescens* SSD, the best bacteriocin producer, inhibited both the formation and the stability of *L. pneumophila* biofilms more effectively than the other tested bacterial strains. *Pseudomonas aeruginosa*, inactive against *L. pneumophila*, showed a synergistic effect when tested with *P. fluorescens*. These observations confirm that microbial interference displays a variety of mechanisms, which are only partially known and may combine one with the other. Also, bacteria can influence *L. pneumophila* replication in the intracellular host environment. In a recent study, we observed that *P. aeruginosa* and *E. coli*, when present together with *L. pneumophila* in the same replication vacuole, significantly decreased *Legionella* growth (Declerck *et al*., 2005). This may be because both non-*Legionella* bacteria can consume important resources provided by the amoeba host, which are normally used by replicating *L. pneumophila*. Another explanation could also be that, although we did not detect any influence of *P. aeruginosa* or *E. coli* on the growth of *L. pneumophila* when they were incubated together in saline, it is possible that the 'intracellular amoeba environment' may induce the production of certain *Legionella* growth-inhibiting components by the other bacterial species.

### *Detachment*

Detachment of bacteria from biofilms followed by a subsequent dispersal in the environment is an integral part of the dynamic nature of life in surface-associated microbial communities (Stoodley *et al*., 2001; Wilson *et al*., 2004). Biofilm cells may detach and disperse either by shedding of daughter cells from actively growing microbes, as a result of nutrient levels or quorum sensing, or by shearing of biofilm aggregates because of flow effects (Donlan, 2002). Detachment caused by flow effects can be divided into two processes, namely: erosion and sloughing. Erosion is the continual detachment of single cells and small portions of the biofilm, whereas sloughing is induced by unexpected events and is characterized by the rapid, massive loss of biofilm (Characklis, 1990). Storey and colleagues (2004) demonstrated a continual erosion of *L. pneumophila* from biofilms, present in a pilot-scale water distribution system, under laminar flow conditions. This process turned into sloughing under turbulent conditions, with more than 90% of the *L. pneumophila* detached from the substratum and mobilized into the adjacent bulk phase. Fluorescent *in situ* hybridization was used to visualize *Legionella* bacteria within the detached

biofilm and cell numbers ranged from a few in small cell clusters to as many as 100 in larger aggregates. Problems may arise when high numbers of *L. pneumophila* become detached from the biofilm and mobilize into the bulk water phase. First, such an event can cause a significant decrease in the species richness of the resident planktonic *Legionella* spp. community (Wéry *et al*., 2008). Second, detached pathogenic bacteria like *L. pneumophila* have the potential to reach humans as an infective dose (Storey *et al*., 2004). Thus, a thorough investigation of bacterial detachment is of fundamental importance to the dissemination of infection and contamination in both clinical and public health settings.

Thanks to its intracellular lifestyle in protozoans, *L. pneumophila* has some supplementary strategies to become detached from the biofilm community. First, infected amoebae such as *Naegleria* sp. which possess an additional flagellate stage will swim away from the biofilm when environmental conditions become unfavourable, e.g., nutrient deficiencies. Second, infected amoebae have been shown to release vesicles of respirable size (< 5 μm), each containing 20–200 *L. pneumophila* (Berk *et al*., 1998). Such vesicles easily get incorporated into aerosols, which may lead to the transportation of numerous *L. pneumophila* over considerable distances.

## Substrate-associated versus floating biofilms

As the criteria for the biofilm mode of growth are very broad, the environments suitable for microorganisms to colonize and establish biofilms are practically limitless. Biofilms may occur attached to a surface, suspended in fluid as flocs or exist as pellicles at air–liquid interfaces, also referred to as floating biofilms. In general, floating biofilms are 30–300 μm thick and common in both anthropogenic and natural aquatic environments (Jennings *et al*., 2003; Knulst *et al*., 2003). Such films contain numerous microorganisms, some of which are harmful to humans like *E. coli*, *P. fluorescens*, *V. cholerae* and *Salmonella* spp. (Yildiz and Schoolnik, 1999; Zorga *et al*., 2001; Spiers *et al*., 2002; Scher *et al*., 2005). Disturbance of floating biofilms by mechanical or natural means may lead to the production of aerosols that allow transmission of biofilm-associated pathogens over considerable distances, until they are inhaled by susceptible persons (Jennings *et al*., 2003). In addition to the presence of bacteria, floating biofilms offer a suitable substratum to all protozoans (free-living amoebae and ciliates) capable of substratum-associated motility (Wotton and Preston, 2005). Preston and colleagues (2001) and Preston (2003) already demonstrated the locomotion and feeding of *Acanthamoeba* spp., *Naegleria* spp. and *Vannella* spp. at the water–air interface. In a recent study, we observed the presence of *Legionella* spp. in floating biofilms in both natural and anthropogenic aquatic environments (Declerck *et al*., 2007a). Moreover, it has been shown that *L. pneumophila* is able to actively colonize these niches, where it requires amoebic hosts in order to replicate (Declerck *et al*., 2007c).

## Concluding remarks

Recently, research attention towards the ecology of *L. pneumophila* has significantly intensified. This is not only because it will improve our understanding concerning its survival strategies in anthropogenic systems, but also because it will help us to develop better and more efficient control strategies to prevent the occurrence of legionellosis. Although microbiologists are rapidly gaining a greater understanding of fundamental aspects involved in the biofilm life cycle of *L. pneumophila*, many questions and issues still remain to be answered. One of the main challenges for the future is to provide additional data concerning the growth of *Legionella* in multispecies biofilms under environmental conditions. It has to be elucidated if, in addition to the known intracellular replication in protozoans, *Legionella* is able to grow and thrive as a commensal bacterium in biofilm communities. Until recently, it remained impractical to work with naturally occurring biofilm consortia, due to the limitations in identifying all community members. However, thanks to the development and introduction of high-throughput sequencing, we are entering a new era of microbial ecology. Thanks to sequencing data and metagenomic analyses, it has become feasible to determine the metabolic and functional potential of entire microbial communities. As biofilms contain both growth supporters and opponents, it will be very interesting to reveal which bacteria antagonize *L. pneumophila* growth or to the contrary which stimulate *L. pneumophila* growth.

The next step requires further investigation of how microbial interaction and growth of *L. pneumophila* in biofilms result in an infectious disease process. Does extracellular replication also induce the highly virulent *L. pneumophila* phenotype as it does in the case of protozoans? It might be that the heterogeneous microenvironments that occur within biofilms could promote and select a differentiated population of highly virulent *L. pneumophila* that facilitate infection and survival in human hosts.

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