

Long-Term Survival of *Legionella pneumophila* in the Viable But Nonculturable State After Monochloramine Treatment

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Abstract *Legionella pneumophila*, a facultative intracellular human pathogen, can persist for long periods in natural and artificial aquatic environments. Eradication of this bacterium from plumbing systems is often difficult. We tested *L. pneumophila* survival after monochloramine treatment. Survival was monitored using the BacLight Bacterial Viability Kit (Molecular Probes), ChemChrome V6 Kit (Chemunex), quantitative polymerase chain reaction and culturability on buffered charcoal–yeast extract agar. In nonculturable samples, regain of culturability was obtained after addition of the amoeba *Acanthamoeba castellanii*, and esterase activity and membrane integrity were observed after >4 months after treatment. These results demonstrate for the first time that *L. pneumophila* could persist for long periods in biofilms into the viable but nonculturable (VBNC) state. Monitoring *L. pneumophila* in water networks is generally done by enumeration on standard solid medium. This method does not take into account VBNC bacteria. VBNC *L. pneumophila* could persist for long periods and should be resuscitated by amoeba. These cells constitute potential sources of contamination and should be taken into account in monitoring water networks.

Introduction

Legionella pneumophila, the causative agent of a severe pneumonia in humans called Legionnaire's disease, is

ubiquitous in aquatic environments. This Gram-negative bacterium is normally found in water as an intracellular pathogen of amoeba. Replication inside these eukaryotic hosts is most probably the only way for this pathogen to multiply in the environment [1]. Contaminated aerosol droplets transmit this facultative intracellular pathogen. Once in the lungs, this bacterium penetrates alveolar macrophages where it replicates, leading to severe and potentially fatal Legionnaires' disease [2–5].

The intracellular life cycle of *L. pneumophila* in macrophages and in amoeba is similar [6]. When ingested, the microbe can resist digestion and replicates before killing its host. In water, amoeba should consume *L. pneumophila* and allow them to multiply. Intracellular replication occurs in a ribosome-studded phagosome that does not fuse to lysosomes [1]. The *L. pneumophila* life cycle consists of at least two phases. The first, called multiplicative or replicative phase, takes place in the host. The second phase of growth, the transmissive or active infective phase, occurs after bacteria have been liberated from the host to the aquatic reservoir. Bacteria that have recently lysed of host cells have been shown to be more resistant to chemical disinfectants and to treatment with biocides [7].

As a free-living organism, *L. pneumophila* can persist for long periods in water and biofilms commonly found in man-made water systems, such as plumbing systems, air-conditioning equipment, or whirlpool spas [8]. It is widely accepted that biofilms play a critical role in the persistence of these bacteria within water systems. Biofilms provide a shelter and nutrients for embedded community and prevents disinfectants from gaining access to the bacteria through the exopolysaccharide matrix [8].

The strategies of *L. pneumophila* to adapt and resist to stressful environmental conditions not only include interaction with amoeba and biofilm localization but also the

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ability to enter in a viable but nonculturable (VBNC) state. Cells in the VBNC state fail to grow on the routine bacteriologic media on which they would normally grow. They typically exhibit a low level of metabolic activity, but they can recover their culturability under favorable conditions. On resuscitation, these cells became again culturable [9]. Different stressful conditions such as poor nutrient conditions, heat, and concentrated salts of hot spring waters or chlorine treatment can lead to a VBNC state in *L. pneumophila* populations. Addition of protozoa, e.g., the amoeba *Acanthamoeba castellanii* or *A. polyphaga*, to *L. pneumophila* in VBNC state resulted in the resuscitation of these bacteria to a culturable state [10–14]. Such observations demonstrate that VBNC *L. pneumophila* regain pathogenic potential with a reactivation in amoebae. Moreover, these bacteria infect amoeba and macrophages in a similar way, so we can suppose that VBNC can recover their culturability and their pathogenicity in macrophage. Therefore, these VBNC could be a public health concern.

Many strategies have been used to eradicate *Legionella* in water and plumbing systems, such as chlorination, chloramination, overheating, and ultraviolet (UV) irradiation of the water. Treatments of contaminated systems by these strategies have been successful, but only for short periods, after which the bacteria have again been found in these sources [1, 15]. In fact, eradication of *L. pneumophila* may require continuous treatment. Some investigators suggested that continuous treatments with monochloramine have a better impact than free chlorine alone on *Legionella* eradication [16, 17] and on biofilm formation [18]. Monochloramine's biocidal action is slower than that of free chlorine, but it is more stable, and a disinfecting residual activity can be maintained during long distances in a water-distribution system [17, 19]. Such an impact was also observed for biofilm-associated *L. pneumophila* in a potable water model and in cooling tower systems [20, 21]. The aim of this work was to study the effect of monochloramine on *L. pneumophila*. We demonstrated herein that monochloramine induce a VBNC state, and such dormant cells should survive for long periods in water.

Materials and Methods

Microbial Strains and Growth Conditions

L. pneumophila serogroup 1 CIP 105349 (Paris) strain obtained from the Centre National de Référence des Légionelles, Lyon, France) was grown on buffered charcoal–yeast extract (BCYE) plates or in buffered YE (BYE) broth at 37°C, 5% CO₂. CIP 105349 biofilms were developed on 5-mm diameter glass beads (Bioblock,

Fisher Scientific, France) in BYE broth (4.6 cm² of glass beads/ml broth). Glass beads were carefully washed with water and pyrolyzed (3 hours, 500°C) before biofilm development. Bacteria were grown for 1 week at 37°C under static conditions. Nonadherent cells were removed by washing twice the glass beads with sterile water into Petri dishes before application of treatments or collecting cells by sonication (see later text). *A. castellanii* ATCC 50739, a gift from Michael Steinert (Würzburg, Germany), was grown at 25°C in peptone–yeast extract–glucose (PYG) medium [22] for 3 days in polystyrene flasks.

Oxidant Solutions Preparation

Hypochlorite solutions were prepared from NaOCl commercial solutions. Monochloramine (NH₂Cl) solutions were prepared by adding 0.3 g Cl₂/L NaOCl (commercial solution of sodium hypochlorite [47%/50%]; Bioblock, Fisher Scientific) to 4 mM NaHCO₃, 5.6 mM NH₄Cl solutions. The pH was adjusted to 9.1 with NaOH or H₂SO₄. Solution concentrations were measured using the *N,N*-diethyl-*p*-phenylenediamine method (Association Française de Normalisation [AFNOR], specification NFT90-038) and expressed in mg chlorine equivalent per liter. All other chemicals were purchased from Sigma-Aldrich (France).

Monochloramine Treatments

Monochloramine solutions were applied on biofilms at concentrations ranging from 0.25 to 10 mg/L to determine the threshold dose corresponding to samples without culturable bacteria. After 1 hour at room temperature, sterile sodium sulfite was added (1.5 moles Na₂SO₃/mole of chlorine) to inactivate monochloramine. Glass beads were transferred into “RM” consisting of 10³-fold BYE-diluted broth and incubated at 25°C for various durations in the same surface-to-volume ratio used for the generation of the biofilms installation.

Enumeration of *L. pneumophila*

Enumerations of bacterial suspensions were done by the plate-count method on BCYE agar medium. To obtain bacterial suspensions from sessile bacteria, glass beads were transferred into sterile water (2 cm² glass beads/per ml sterile water) and gently sonicated at 51 Watts with a Bioblock 86480 apparatus (Fisher Scientific) for 3 minutes. These conditions were established in the framework of a 3 years study on biofilm communities (N. Merlet, data not shown) to optimize for maximal numbers of

colony-forming units (CFU). Numbers of *L. pneumophila* cells capable to form colonies on solid medium (i.e., culturable *L. pneumophila*) were obtained from enumeration on BCYE agar medium. Numbers of viable and dead bacteria were determined by epifluorescence microscopy (Olympus Bx 41 microscope, excitation filters wavelength 430 to 490 nm) using the BacLight LIVE/DEAD Bacterial Viability Kits (Molecular Probes, Eugene, Oregon) as recommended by the supplier. Observation of metabolically active cells was realized using the ChemChrome V6 Kit (Chemunex, Ivry-sur-Seine, France) as recommended by the supplier.

Enumeration of total bacteria by quantitative polymerase chain reaction (qPCR) was done as follows. Cells were washed twice with sterile water and lysed with 0.09 M Tris HCl pH 7, 0.9 mg/mL proteinase K, and 0.9 % (wt/vol) sodium dodecyl sulfate for 1 hour at 60°C. Crude DNA solutions were purified using standard methods [23]. Real-time PCR was performed on an ABI Prism 7700 Sequence Detection System apparatus (Applied Biosystems, France) using Taqman procedure and the forward primer LPMIP.1 (5'-CAA TTC AGC GCC ACT CAT AGC-3'), the reverse primer LPMIP.2 (5'-GCA TAG ATG TTA ATC CGG AAG CA-3'), and the Taqman probe LPMIP MGB-VIC (5'-VIC-TGC ATG CCT TTA GCC_MGB-3'). Reaction mixtures were composed of 7.5 µl TaqMan Universal Buffer PCR Master Mix (Applied Biosystems), 900 nM each primer, 250 nM Taqman probe, and 4.5 µl template DNA in a total volume of 15 µl. After activation of the AmpErase UNG enzyme present in the TaqMan Universal Buffer (2 minutes; 50°C), conventional PCR cycling were done (10 seconds at 95°C, 15 seconds at 95°C, and 1 minute at 50°C). Bacteria enumerations were obtained from standard curves established from standard DNA solutions prepared from enumerated fresh bacterial suspensions.

Resuscitation Essays Using Amoeba Infection

Three day-old amoeba cultures were adjusted to 5.10^5 cells/mL with amoeba dilution buffer (PYG without proteose peptone, yeast extract, and glucose). Bacteria suspensions were prepared from biofilms by transferring glass beads into sterile water (2 cm² glass beads/ml sterile water) and subsequent gentle sonication. Two mL amoeba suspension and 2.5 mL *Legionella* suspension (corresponding to 5 cm² biofilm) were introduced into a 25 cm² bottom surface polystyrene flasks and incubated at 37°C under a 5% CO₂ atmosphere. After 3 and 7 days of coin-cubation, the amoeba were lysed using centrifugation (12,000 g at 5 minutes) followed by 1 minute of vortexing on an MS1 Minishaker (IKA-Works). *L. pneumophila* culturability was determined on BCYE agar.

Results

Impact of Monochloramine on Cell Culturability

After treatments, we used 10³-fold diluted BYE broth as resting medium (RM) to obtain total organic carbon (TOC) concentration close to that of surface water (2 mg/L TOC). *L. pneumophila* is unable to grow in RM [10].

L. pneumophila biofilms were developed on glass beads. Under our conditions, biofilms density usually reached 10⁵ CFU/cm². Figure 1 shows the impact of 1-hour monochloramine treatments on sessile *Legionella* culturability. Monochloramine doses <1 mg/L led to a decrease of culturability. For concentrations ≥1 mg/L monochloramine, a total loss of culturability was observed, and no colony on BCYE plates was observed in samples corresponding to zero CFU/0.5 cm² of biofilm until at least 145 days in RM.

Quantitative PCR Enumeration from Biofilm

We monitored bacteria in biofilms after monochloramine treatments by a qPCR procedure for 20 days (Fig. 2). By this method, low differences in total bacteria enumerations were observed between treated and untreated populations (e.g., 9.7×10^4 genome unit (GU)/cm² for untreated populations vs. 4.3×10^4 GU/cm² for biofilms treated with 10 mg/L monochloramine 20 days after the onset of the experiment; Fig. 2), although culturability showed strong differences (e.g., 1.6×10^3 CFU/cm² for untreated populations versus zero UFC/0.5 cm² for biofilms treated with

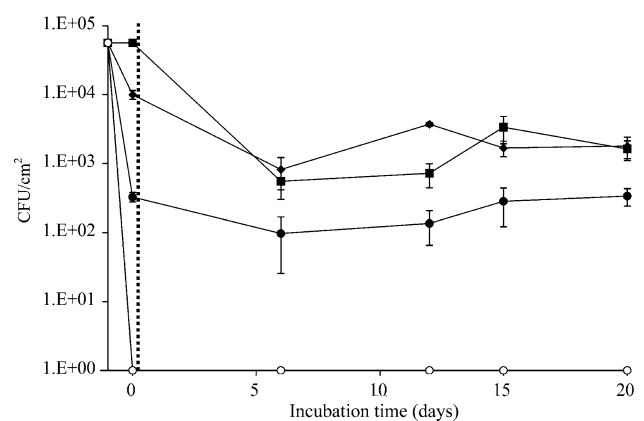


Fig. 1 Culturability of sessile *L. pneumophila* after treatment with monochloramine. Biofilms were treated with 0 (closed squares), 0.25 (closed diamonds), 0.5 (closed circles), 1 (x), 2 (+), 5 (open diamonds), and 10 mg/l (open circles) monochloramine. The broken line indicates sulfite addition and glass bead transfer to RM. Curves for 1, 2, and 5 mg/l, similar to the curve for 10 mg/l, are hidden on the graph

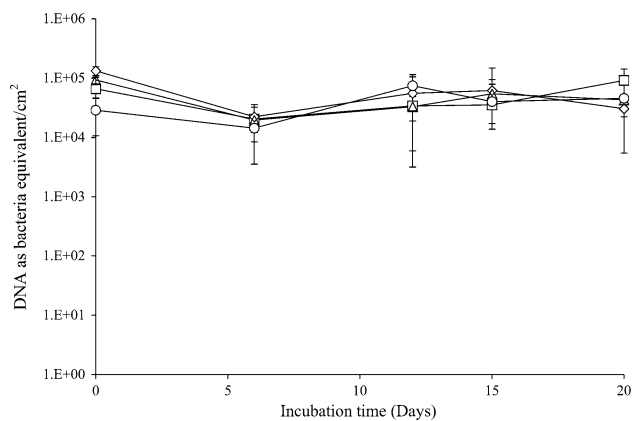


Fig. 2 Enumeration of *L. pneumophila* by quantitative PCR in biofilm after monochloramine treatment. Biofilms were treated with 0 (open squares), 1 (open diamonds), 5 (open circles), or 10 (open triangle) mg/l monochloramine (average of three independent biofilms for each monochloramine dose)

10 mg/L monochloramine 20 days after the onset of the experiment; Fig. 1).

Enumeration of Live and Dead Cells and Esterase Activity

To detect viable and dead bacteria, we analyzed samples with the Live/Dead BacLight Bacterial Viability (Table 1). Viable bacteria appear green, and dead bacteria still containing DNA appear red. Cells with damaged outer membranes show mixed colors [24]. This method has already been used for *L. pneumophila* population analysis [15, 25]. All monochloramine-treated biofilm samples from 0 to 10 mg/l contained viable bacteria (Table 1). After 15 days in RM, we observed approximately 29% of viable cells for all treated samples and approximately 45% of viable cells for untreated biofilm (Table 1).

Table 1 Influence of monochloramine on the culturability and viability of *L. pneumophila* in biofilm 15 days after treatment

| Monochloramine dose (mg/l) | CFU/cm ^{a,b} | Viable cells (%) ^c |
|----------------------------|---------------------------------------|-------------------------------|
| 0 | $3.4 \times 10^3 \pm 1.4 \times 10^3$ | 44.5 ± 9.9 |
| 0.5 | $2.8 \times 10^2 \pm 1.6 \times 10^2$ | 20.9 ± 4.2 |
| 1 | 0 ^d | 28.8 ± 4.5 |
| 2 | 0 | 29.0 ± 7.0 |
| 5 | 0 | 29.0 ± 3.3 |
| 10 | 0 | 29.4 ± 5.5 |

^a CFU obtained on BCYE agar

^b 0 indicates no culturable cells on BCYE plates in 0.5 cm² biofilm

^c Viable cells counts performed after BacLight staining

Average of at least 3 independent experiments (^d >10 independent experiments)

To confirm VBNC presence in analysed samples, esterase activity was assessed using the ChemChrome V6 Kit. This kit contains a nonfluorescent substrate that is enzymatically cleaved to liberate free fluorochrome within the cytoplasm of metabolically active cells. Only viable cells with intact membranes have the ability to perform this cleavage and retain the fluorescent label. This means that living cells can be easily discriminated from dead cells, which do not fluoresce. Biofilm treated with 1 mg/l monochloramine and incubated 145 days in RM contained approximately 10% of the bacteria with esterase activity (Fig. 3). Among the active cells shown in Fig. 3, some cells seem to have strong esterase activity according to the strong green color exhibited.

Legionella pneumophila Resuscitation with *A. castellanii*

Steinert et al. [14] observed that addition of amoebae to VBNC *L. pneumophila* led to resuscitation of these bacteria to a culturable state. To check VBNC resuscitation, samples were cultivated with *A. castellanii*. Infection attempts were realized with biofilms stayed 30 days in RM (data not shown). Amoeba addition to untreated samples led to a significant increase of CFU, but not for biofilms treated with doses >1 mg/mL monochloramine, even after 7 days of coinubation. Nevertheless, culturable bacteria were observed when amoebas were added to biofilm treated with 1 mg/L monochloramine, whereas without amoeba, culturable bacteria were not detected from the same biofilm batch. These results demonstrate that *L. pneumophila*

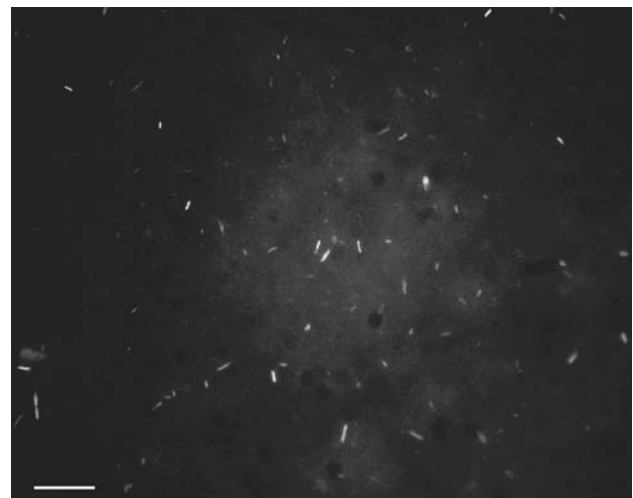


Fig. 3 Esterase activity and membrane integrity of nonculturable *L. pneumophila* cells in biofilm after 145 days in RM. Biofilm was treated with 1 mg/l monochloramine to obtain nonculturable cells; then it was kept in RM before assessment by ChemChrome V6 assay. Photograph surface correspond to 1.19×10^{-4} cm² biofilm. Bar represents 10 μ m

VBNC cells obtained after treatment with 1 mg/L monochloramine were resuscitated with *A. castellanii*.

Discussion

We applied moderate monochloramine treatments on biofilm containing *L. pneumophila* with doses just sufficient for culturability loss. We observed that these nonculturable *L. pneumophila* exhibited membrane integrity and esterase activity, suggesting strongly that these cells were in the VBNC state. Some of these VBNC cells were able to be resuscitated with amoeba. Nevertheless, we were unable to resuscitate the nonculturable bacteria detected in samples treated with monochloramine doses >1 mg/L. Reason for this failure is not clear. It may be possible that these damaged cells are unable to resume repair and thus progressively degenerate. Some investigators have demonstrated that *A. polyphaga* is also able to resuscitate VBNC *Legionella* [12]. It could be instructive to compare *Legionella* VBNC resuscitation by *A. polyphaga* with resuscitation by *A. castellanii*. These protozoan are commonly found in water systems, suggesting that VBNC resuscitation should be effective in natural environment.

qPCR Enumerates DNA from Culturable Cells as Well as from Nonculturable Cells

Some investigators have developed qPCR procedure to enumerate *L. pneumophila* in plumbing water and cooling towers [26–28]. These promising methods provide rapid bacteria quantification compared with the normalized method based on detection of culturable *Legionella* on Petri dishes (e.g., the AFNOR NFT90-431 [ISO 11731] method). qPCR-based technical procedures are currently evaluated by private and public laboratories to produce normalized qPCR methods for *Legionella* enumerations (AFNOR XP T 90-471 method; a group from the ISO technical committee 147 is currently working on this method). Nevertheless, it is known that enumeration by qPCR is unable to distinguish nucleic acid extracted from viable and culturable, VBNC, or dead cells [29]. Figure 2 shows that biofilm enumeration by qPCR gave similar values for all analyzed samples, i.e., between 10^4 and 10^5 bacteria equivalent/cm², whereas Table 1 indicates that some of these samples contained no more culturable cells and >50% of dead cells. Thus, despite the fact that we are unable to determine if our qPCR protocol is able to distinguish dead from live cells, we succeeded in enumerating VBNC bacteria, demonstrating the usefulness of qPCR to monitor *Legionella* persistence into biofilms.

Nonculturable monochloramine were kept in RM for approximately 4 months. In these samples, we were able to

detect VBNC cells harboring strong esterase activity at the end of the experiment (Fig. 3), indicating that these cells remained alive. This is the first report of long-term persistence of *L. pneumophila* in the VBNC state after monochloramine treatment. In water networks, these VBNC cells should be a source of recontamination after treatments applied for *Legionella* eradication, even several months after the end of treatment.

In this study, we treated samples only for short periods of time. It will be necessary to investigate continuous treatments with low monochloramine doses, as is recommended for municipal drinking water [17], perhaps in the presence of the amoeba *A. polyphaga*, to determine if these conditions also produce VBNC *L. pneumophila*.

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