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Viability and infectivity of viable but nonculturable *Legionella pneumophila* strains induced at high temperatures

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Abstract

Thermal disinfection is commonly used to prevent the proliferation of culturable *Legionella* in engineered water systems (EWS). In response to such stress, culturable *Legionella* populations can switch into a viable but nonculturable (VBNC) state. The importance of such VBNC *Legionella* cells is currently hotly debated. Here, we investigated the stress response patterns and transitions of the bacteria to the VBNC state at 55°C, 60°C and 70°C on two *L. pneumophila* strains for >80 days using a combination of cell-based viability indicators. Complete loss of culturability at 55°C, 60°C and 70°C occurred after 3-8 hours, 60 min and <2 min, respectively. In contrast, *L. pneumophila* strains required 9 days at 55°C, 8 hours at 60°C and 20 min at 70°C to achieve a 2 log reduction in cells with intact membranes and high esterase activity; a 4 log reduction was achieved only after 150, 8-15 and 1-4 days, respectively. In parallel, the presence of diagnostic outer-membrane epitopes (OMEs) and changes in the infectivity patterns of the two strains towards amoebae and THP-1 cells were assessed. OMEs were more persistent than viability indicators, showing their potential as targets for VBNC *Legionella* detection. *L. pneumophila* strains infected amoebae and THP-1 cells for at least 85 days at 55°C and 60°C and for up to 8 days at 70°C. However, they did so with reduced efficiency, requiring prolonged co-incubation times with the hosts and higher *Legionella* cell numbers in comparison to culturable cells. Consequently, infection of amoebae by thermally induced VBNC *L. pneumophila* with lowered virulence can be expected in EWS. Although the gold standard method cannot detect VBNC *Legionella*, it provides important information about the most virulent bacterial subpopulations. Our results indicate that a prolonged thermal regime 60°C at the central parts of warm water

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systems is not only effective against culturable *L. pneumophila* but in the long run even against VBNC cells.

Keywords

Legionella; VBNC; *Acanthamoeba*; macrophages; thermal disinfection; viability

1 Introduction

Temperature is one of the key factors that influences the proliferation of *Legionella* in natural and man-made water systems. This has been observed for temperatures ranging between 12°C and 50°C (ESGLI, 2017; Lesnik et al., 2016); however, optimal replication temperatures are between 32°C and 40°C (ESGLI, 2017; Lesnik et al., 2016). These temperatures are normally encountered in cooling towers and spas which are common sources of large outbreaks of Legionnaires' disease (LD). LD cases also occur sporadically from cold and specifically from hot water systems colonized with this bacterium (Bartram et al., 2007; ECDC, 2017).

To prevent and control the proliferation of *Legionella* in engineered water systems (EWS), several disinfection strategies are applied (ESGLI, 2017). Among them, a temperature control regime is recommended for the EWS of large buildings (e.g. hospitals). In European hot water systems, for example, water leaving the heaters should have a minimum temperature of 60°C and should return at a minimum of 55°C (ESGLI, 2017). In cold water systems, the well-characterised range of temperatures in which *Legionella* replicates (>20°C) should be avoided. Although such measures have proven efficient to reduce culturable *Legionella* concentrations from EWS, they are not successful in eradicating the bacterium (Allegra et al., 2011; Mouchtouri et al., 2007; Rhoads et al., 2015). Thermal shocks at higher temperatures such as 70°C and 80°C are therefore also suggested as disinfection measures (reviewed in Whiley et al., 2017). However, *Legionella* may not only survive (Farhat et al., 2010) but also recolonise the water system weeks after thermal treatment (Steinert et al., 1998; Vervaeren et al., 2006).

A number of factors may be responsible for hampering the effectiveness of thermal treatments: (i) within water pipes *Legionella* lives in biofilms in a free-living form or intracellularly within free-living amoebae (FLA), which not only protect the bacteria from external stressors but also provide nutritional sources to favour their replication (Declerck, 2010); (ii) different *Legionella* species and strains have different thermal susceptibilities (Cervero-Aragó et al., 2015; Sharaby et al., 2017); (iii) *Legionella* and/or other thermophilic bacteria can adapt and transfer genes (Lesnik et al., 2016; Sharaby et al., 2017); (iv) improperly managed or designed system hydraulics (Bédard et al., 2015) and (v) the presence of viable but nonculturable (VBNC) cells not detectable by culture-based methods, which could overestimate the performance of the thermal treatments applied (Allegra et al., 2011; Kirschner, 2016).

Legionella, as other gram-negative bacteria, may enter the VBNC state under conditions of environmental stress such as starvation (Schrammel et al., 2018a), high temperatures

(Allegra et al., 2008; Chang et al., 2007) and oxidising agents (Türetgen, 2008). In this state, the bacterium does not grow on standard artificial media, the gold-standard method for the enumeration of the bacteria and risk assessment, but is still active, maintaining low metabolic activity, membrane integrity, virulence-related protein expression (Alleron et al., 2013; Schrammel et al., 2018a) and a low level of virulence (Al-Bana et al., 2014; Dietersdorfer et al., 2018). The importance and potential health relevance of such VBNC *Legionella* is intensively discussed in the current literature. Moreover, the presence of nonculturable *Legionella* and the higher numbers of *Legionella* detected by cultivation-independent methods such as qPCR or direct detection with antibody based assays (Füchslin et al., 2010) in comparison to the standard culture-based method, may confuse the operators of water systems (Kirschner, 2016). For example, the diversity on the lipopolysaccharide (LPS) and specifically the outer-membrane epitopes (OMEs) of *L. pneumophila* have been used in the past years for serotyping *L. pneumophila* strains for diagnostic purposes (Helbig et al., 1997). However, little is known about the persistence of such OMEs under different environmental conditions and their relation to *Legionella* viability and infectivity (Schrammel et al., 2018b).

In the present study, we assessed the impact of three temperatures currently used to control culturable *Legionella* in EWS — 55°C, 60°C and 70°C — on the viability of two *L. pneumophila* strains in ultrapure water microcosms for >80 days. The microcosms were monitored by analysing the total cell count (TCC) and using viability indicators that comprise culturability, cellular integrity, DNA content, membrane integrity, esterase activity of the cells, and the presence of OMEs. Notably, we also semi-quantitatively assessed changes in the infectivity pattern of the two *L. pneumophila* strains using an *Acanthamoeba* host as an amoeba model and a macrophage-like cell model (differentiated from THP-1 cells).

By comprehensively studying the viability and infectivity of culturable and specifically VBNC *L. pneumophila* in a multiparametric way we intended to elucidate the impact that elevated temperatures have on the two states of these important opportunistic waterborne pathogens.

2 Material and Methods

2.1 Selected *L. pneumophila* strains

A *L. pneumophila* SG1 subtype mAb Benidorm strain isolated from a clinical source (LpClin) and a *L. pneumophila* SG1 mAb subtype Philadelphia strain (LpParis) were used for all experiments (Schrammel et al., 2018a, 2018b). The two strains belong to the most virulent mAb 3/1 subtype according to the Dresden panel (Helbig et al., 1997). Strains were stored at -80°C in cryotubes (Rotistore, Carl Roth, Karlsruhe, Germany).

2.2 Amoeba culture

The *Acanthamoeba castellanii* genotype T4 strain (SPA08) isolated from a keratitis patient (Dietersdorfer et al., 2018) was chosen as a host amoeba model system. The strain was cultivated and prepared as explained elsewhere (Dietersdorfer et al., 2018). First, 0.4 mL

axenic trophozoite suspensions of 1×10^5 amoeba cells in peptone-yeast-glucose (PYG) medium were transferred to 12-well cell culture plates. The plates were incubated at 30°C for 1 h to allow amoeba adherence to the bottom of the wells. After that, the PYG medium was removed by aspiration and replaced by 1 mL of 1:10 PYG medium diluted in Page's amoeba saline (PAS, ATCC1323).

2.3 Generation of THP-1 macrophages

THP-1 cells, acquired from V. Mündler (Medical University of Vienna, Austria), constitute a human monocytic cell line derived from an acute monocytic leukaemia patient. Cells were maintained and prepared as explained elsewhere (Dietersdorfer et al., 2018). First, monocytes were counted and adjusted to a final concentration of 1×10^5 cells/mL using a haemocytometer. One millilitre of monocyte suspension was transferred to a 12-well cell culture plate. Then, 50 ng/mL of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) was added for 72 h to differentiate the cells into macrophage-like cells. After 3 days, the cells were washed twice with pre-warmed phosphate-buffered saline (PBS). Finally, 1 mL of fresh RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) without antibiotics was added to each well.

2.4 Generation of microcosms, heat treatments and sample monitoring

All *Legionella* microcosms were prepared as detailed in Schrammel et al. (2018a) and in the SI. First, three independent cultures of the two *L. pneumophila* strains were grown in liquid buffered yeast extract (liBYE, modified from Edelstein, 1981) until they reached the stationary phase. In this phase *L. pneumophila* expresses similar traits to its transmissive state in the environment (Al-Bana et al., 2014). Cultures were then washed twice by centrifugation at $3,500 \times g$ at 20°C. Supernatants were discarded, and cells were resuspended in ultrapure water (Simplicity® Ultrapure water (Merck, Darmstadt, Germany)) by vortexing. After checking the OD₆₀₀ value, the volume required to achieve a cell concentration of 1×10^8 cells/mL in 350 mL of autoclaved ultrapure water pre-heated at 55°C, 60°C or 70°C was inoculated (T_0). Triplicate bottles of the *Legionella* microcosms were incubated without shaking at the selected temperatures and monitored over time (T_{min} , T_h , T_{days}). For each temperature, different sampling time points were chosen. Bottles were shaken 20 times and from each microcosm, samples of 15-20 mL were taken and cooled by placing the tube in cold water (approximately 18°C) for 2 min and vortexed for 20 s. Samples were then prepared for further analyses: culturability, analysis of cellular integrity and DNA content, viability indicators, presence of OMEs and infection assays (Dietersdorfer et al., 2018; Schrammel et al., 2018a, 2018b).

2.5 Analysis of *Legionella* culturability

Tenfold dilution series of *Legionella* microcosm samples were transferred to buffered charcoal-yeast extract (BCYE) agar plates without antibiotics (BioMerieux, Vienna, Austria). Complete loss of culturability was confirmed when no colonies were observed after plating 0.5 mL of direct microcosm samples after 10 days of incubation at 37°C in triplicate. When a positive *Legionella* infection in amoebae and THP-1 macrophages was confirmed by microscopy (see below), 1 mL of the co-culture suspension was harvested and directly

plated on BCYE agar. All plates were incubated at 37°C for up to 10 days and checked every 3-4 days for *L. pneumophila* colony growth.

2.6 Characterisation of *L. pneumophila* cells by flow cytometric (FCM) analyses

The details of all staining procedures, the FCM protocol analysis, the gating strategy and the FCM results calculation are described extensively in our previous publications (Schrammel et al., 2018a, 2018b) and can be found in the Supplemental Information (SI). *Legionella* microcosms were analysed using an Attune NxT Flow Cytometer (Life Technologies, Darmstadt, Germany) equipped with a 488 nm flat-top laser at 50 mW. The flow cytometric assays had a limit of quantification (LOQ) of 1.3×10^4 cells/mL (Schrammel et al., 2018a, 2018b).

2.6.1 Viability indicators—Total cell counts (TCC), cell integrity and DNA content of the bacterial cells were analysed using SYBR Green I (SGI) staining. The membrane integrity of the cells was assessed using a combination of SYBRGreen I/propidium iodide (SGI/PI) staining. The esterase activity of the cells was analysed by staining with 5(6)-carboxyfluorescein-diacetate (CFDA).

2.6.2 Quantification of the presence of specific OMEs—Four monoclonal antibodies (mAbs) from the Dresden panel used in typing assays for the lipopolysaccharide (LPS) of *L. pneumophila* SG1 and a mAb targeting major outer-membrane protein (MOMP) were used to assess the presence of the respective OMEs (Helbig et al., 1997). MAb 3/1, 8/5 and MOMP, specific for both test strains; mAb 20/1, specific for LpClin; and mAb 8/4, specific for LpParis were assessed by immunofluorescence staining (Schrammel et al., 2018b). The number of stained cells and the staining intensity were analysed by FCM.

2.7 Infection of amoebae and THP-1 macrophages

For each temperature and strain, three *L. pneumophila* microcosms were analysed in parallel. Each bacterial suspension was directly added to amoeba and macrophage cultures at different multiplicities of infection (MOI): 1, 100 or 400 legionellae per amoeba; and 50 or 100 legionellae per THP-1 macrophage. To enhance the interaction between legionellae and host cells, amoeba plates were centrifuged at $500 \times g$ for 5 min and THP-1 macrophages at $300 \times g$ for 5 min. Amoeba co-cultures were then incubated at 30°C, whereas co-cultures with human cells were incubated at 37°C with 5% CO₂ (Dietersdorfer et al., 2018).

2.8 Monitoring of *L. pneumophila* infections

Co-cultures were observed daily using an inverted microscope (Olympus CK 2, Vienna, Austria). When host cells showed any sign of infection (intracellular replication of *Legionella* cells) the respective well was harvested, fixed and stained using the Monofluo *L. pneumophila* IFA Test Kit (Bio-Rad Laboratories Diagnostics Group, Redmond, WA, USA) as previously explained (Dietersdorfer et al., 2016). All slides were observed using a Nikon Eclipse 8000 epifluorescence microscope and pictures were taken using the Nikon DS-QiMc camera and NIS Elements BR 2.3 software (Nikon, Surrey, UK). Infection was considered positive when vesicles of more than five bright *Legionella* cells were observed within one host cell. Cultures of hosts without bacteria were used as negative controls.

2.9 Statistics

The data set obtained was analysed using Spearman's rank correlation (SPSS vers. 24, IBM International), as most of the data did not follow a normal distribution. Correlations between viability indicators were accepted as significant for p-values <0.05 and as highly significant for p <0.01 (Tables SI 4-6).

3 Results

We monitored the impact that temperature had on *L. pneumophila* in a multiparametric way, using viability indicators that comprised culturability, an assessment of cell integrity and DNA content, membrane integrity and esterase activity of the cells as well as the presence of virulence-related epitopes. Most importantly, we also semi-quantitatively assessed changes in the infectivity pattern of the bacteria using amoeba and a macrophage-like host model.

3.1 Loss of *L. pneumophila* culturability

At 55°C, LpClin and LpParis completely lost culturability on plates (8 log reduction, <1 CFU/mL) at T_{3h} and T_{8h}, respectively. At 60°C, the total loss of culturability was observed already at T_{30min} for LpParis and at T_{1h} for LpClin. At 70°C, neither of the two strains were culturable at T_{2min}. Once nonculturable, none of the *L. pneumophila* strains formed colonies on standard media plated directly from the water microcosms (Figure 1).

3.2 Total cell counts, cell integrity and DNA content

Despite the rapid reduction in culturability observed at different temperatures, the concentration of intact cells (TCC) remained constant throughout the different experiments (~ 8 log cells/mL, Figure 2A). However, variations in the mean fluorescence intensity (MFI) of SG1-stained cells were observed (Figure 2B). After an initial constant phase of 15 days, the MFI of *L. pneumophila* strains incubated at 55°C and 60°C was reduced by 1 log at T_{152days} (55°C) and 1.5 log at T_{200days} (60°C). At 70°C, after an initial constant phase of 24 h, the MFI decreased by 1.5-2 log at T_{80days} indicating a degradation of the DNA content.

3.3 Membrane integrity

Although all *L. pneumophila* microcosms were generated from bacterial liquid cultures to promote homogeneous bacterial populations (SI), three different subpopulations were observed for all *Legionella* strains and temperatures analysed immediately after the inoculation into ultrapure water (SI Table 2). One subpopulation of the cells comprised cells with intact membranes, a second subpopulation comprised cells with harmed membranes and a third subpopulation comprised cells in the so-called intermediate-membrane state, located between the previous subpopulations in the flow cytometry plots (Figure 3).

At 55°C, the number of cells with intact membranes (approximately 70% of the TCC, SI Table 2) gradually decreased over time, following a different pattern depending on the strain analysed. LpClin, which was nonculturable at T_{3h}, showed a simultaneous 1 log reduction in cells with intact membranes (Figure 3). After that, it continued decreasing by up to 5 log at T_{152days}. Despite the loss of culturability, the concentration of LpParis cells with intact membranes remained stable during the first 72 h (approximately 80% of the TCC, SI Table

2). After that, it started decreasing by more than 6 log at T_{152days} (Figure 3). Cells of both test strains shifted from the intact-membrane subpopulation gate to the subpopulation of cells with intermediate-state membranes over time. Then cells shifted to the subpopulation of cells with harmed membranes. Thus, at T_{152days}, intermediate-state cells were 1 log (LpParis) and 3.6 log (LpClin) lower (Figure 3). Consistently, the concentration of cells with harmed membranes (dead cells) was proportionally higher.

At 60°C, the number of intact cells decreased faster for LpClin than for LpParis (Figure 3). Intact cells decreased below the LOQ (>4 log reduction) at T_{15days} for LpClin and at T_{88days} for LpParis. Although the concentration of cells with intermediate membranes increased between 0.5 log (LpClin) and 1.2 log (LpParis) during the first 24 h, after longer incubation times (T_{88days} and T_{217days}), the same subpopulations were reduced by ~3 and 4.5 log, respectively.

At 70°C, the subpopulations with intact membranes decreased between 0.5 (LpParis) and 1 log (LpClin) already at T_{10min} (Figure 3) and at T_{4days} concentrations were below the LOQ (>4 log reduction). After a slight (0.8-0.9 log) initial increase the cells with intermediate membranes state were reduced by >3 log at T_{8days} and by >5 log at T_{98days}.

3.4 Esterase activity

According to the mean fluorescence intensity of the CFDA staining, two different subpopulations per *L. pneumophila* strain were distinguished in the flow cytometry histograms: a subpopulation with high esterase activity (HE) and one with low esterase activity (LE). At 55°C, LE cells showed a similar pattern for LpClin and LpParis. LE cell numbers (approximately 30% of the TCC, SI Table 2) were stable over 16 days. However, at T_{152days} they were reduced by 7 log (LpClin) and 2.7 log (LpParis) (Figure 3). In the case of HE cells (approximately 70% of the TCC, SI Table 2), the LpClin subpopulation decreased gradually immediately after inoculation, reaching a 4 log reduction (below the LOQ) at T_{152days}. In contrast, the number of HE cells of LpParis was stable for the first 8 h and then followed the same trend as LpClin, decreasing >4 log at T_{152days} (Figure 3).

At 60°C, the LE subpopulations of both *L. pneumophila* strains remained stable for 24 h. At T_{88days} LE cells were reduced by >4 log. The HE subpopulations of the two strains remained stable for the first hour. A >4 log reduction was already observed at T_{8days} (Figure 3).

At 70°C, the LE subpopulations of both strains remained relatively stable only for the first hour. A ~4 log reduction was already observed at T_{8days}. The concentration of HE cells immediately decreased after inoculation and was reduced by 1 log at T_{2min} of thermal exposure. Already at T_{1day}, both HE subpopulations were below the LOQ (Figure 3).

3.5 Presence of specific OMEs

Four monoclonal antibodies (mAbs) from the Dresden panel were used to assess the presence of OMEs. mAb 3/1, 8/5 and MOMP, specific for both test strains; mAb 20/1, specific for LpClin; and mAb 8/4, specific for LpParis. Two different subpopulations of cells were distinguished for all mAbs analysed, according to their mean fluorescence intensity

(MFI): low fluorescent (LF) and high fluorescent (HF) cells. In line with our previous study, the MFI accounts to the average number of epitopes on the surface of the cells available for antibody binding (Schrammel et al., 2018b).

Our results showed no differences between strains or temperatures during the first 8 days with MOMP staining, during which stable numbers of LF and HF cells were observed (approximately 70% and 30% of the TCC, respectively). At 55°C, the LF subpopulation of LpClin and LpParis remained stable for 194 days (Figure 4). At 60°C, LF cells decreased between 4 log (LpParis) and 5 log (LpClin) after T_{200days}, and at 70°C, a 3 log reduction was observed for both strains at T_{60days}. The HF subpopulation had a dramatic 7 log reduction at incubation times >T_{15days}, for both strains and the three temperatures analysed, with the exception of the HF subpopulation of LpClin at 55°C, which was reduced by only 1 log at T_{194days}. Thus, at increasing temperatures the number of epitopes per cell strongly decreased (HF) and subsequently, at longer exposure times the number of cells expressing the MOMP epitope also decreased.

The mAb 3/1 binds to a specific LPS epitope of *L. pneumophila* SG1 strains that belong to the most virulent Pontiac group; the two strains used in the current study belonged to that group. At 55°C, the number of cells expressing the 3/1 epitope (LF) remained stable throughout the experiment (37% of the TCC). However, at higher temperatures, a reduction of 0.5 log (60°C) and 0.5-1 log (70°C) was observed for both, LpClin and LpParis, at T_{200days} and T_{59days}, respectively (Figure 4). Regarding, the HF subpopulation (approximately 63% of the TCC), at 55°C a reduction of <1 log occurred at T_{194days}. At higher temperatures, this subpopulation was reduced by >7 log at T_{8days} and T_{15days} at 70°C and 60°C, respectively (Figure 4).

The isotype-IgM antibody 8/5 binds to a specific LPS epitope of all *L. pneumophila* SG1 strains. The number of cells expressing the 8/5 epitope (LF subpopulations) remained stable (60% of the TCC) for both strains and all temperatures analysed. A similar trend was observed for the cells highly expressing the 8/5 epitope (HF subpopulations) except for LpParis at 70°C which was 5 log reduced at T_{59days} (Figure 4).

The mAb 20/1 binds specifically to certain mAb subtypes of *L. pneumophila* SG1, such as OLDA and Benidorm (LpClin) and the mAb 8/4 binds specifically to certain mAb subtypes of *L. pneumophila* SG1, such as OLDA, Oxford and Philadelphia (LpParis). The results obtained show that temperature did not impact the number of cells expressing these epitopes (LF) neither the number of epitopes expressed by cell (HF) (data not shown).

3.6 Infectivity of *L. pneumophila* strains after incubation at 55°C

In parallel to the viability parameters, we analysed the infectivity patterns of the two *L. pneumophila* strains. The methodology used enabled us to semi-quantitatively assess changes in the efficiency of *L. pneumophila* to cause infection in amoebae and THP-1 macrophages. The infectivity efficiency was represented by the different MOIs tested, the days in co-culture required to observe an infection and the number of replicates in which such infections were observed (Figure 5). In general, at all temperatures tested and for both host models, the two strains required longer co-culture times to observe an infection in

comparison to T_0 (with culturable cells). Neither LpClin nor LpParis recovered culturability after passage through amoebae or THP-1 macrophages.

At 55°C, the two *L. pneumophila* strains were able to replicate in amoebae and THP-1 macrophages for as long as they were culturable for all MOIs tested. Once nonculturable, the two strains infected amoebae for up to 85 days with high variability between replicates, especially for the lower MOIs. Moreover, the time required to observe infection in amoeba changed from 1 day at T_0 for up to 16 days in some cases (e.g., LpClin at $T_{3\text{days}}$ or $T_{16\text{days}}$) (Figure 5). No infections of amoebae were observed at $T_{130\text{days}}$. A similar pattern was observed for THP-1 macrophages. Once nonculturable, LpClin and LpParis continued to infect THP-1 macrophages at the highest MOIs (50 and 100), with high variability between replicates. Although both strains did not infect THP-1 macrophages at single time points (LpClin at $T_{85\text{days}}$, and LpParis at $T_{16\text{days}}$), the two *L. pneumophila* strains were infective at $T_{130\text{days}}$, the latest time point tested using MOI 100 (Figure 5, SI Figure 2).

3.7 Infectivity of *L. pneumophila* strains after incubation at 60°C

At 60°C, the infection patterns were comparable to those at 55°C. With the reduction in the number of culturable cells, the MOIs and incubation times needed to observe infection were extended. Once nonculturable, LpClin and LpParis infected amoebae at MOIs 100 and 400 for up to 15 days with a high variability between replicates specifically for the smallest MOI (Figure 6). At $T_{15\text{days}}$, *L. pneumophila* microcosms needed prolonged incubation times (11-17 days) for a detectable infection. LpParis infected amoebae until $T_{43\text{days}}$ only at the highest MOI, 400 (Figure 6). LpClin also infected amoebae also at later time points until $T_{86\text{days}}$ at MOI 400 and occasionally at MOI 100, always requiring longer co-culture times in comparison to T_0 .

Regarding co-cultures with THP-1 macrophages, on-going infections were observed for the two *L. pneumophila* strains for up to 15 days with a slight variability between replicates and requiring longer co-culture times in comparison to T_0 . In the long exposure period, the two strains continued to infect THP-1 macrophages until the latest time point tested ($T_{83\text{days}}$ for LpParis and $T_{114\text{days}}$ for LpClin) for at least one of the two MOIs tested (Figure 6).

3.8 Infectivity of *L. pneumophila* strains after incubation at 70°C

LpClin and LpParis infected amoebae for up to 8 days for at least one of the MOIs tested (mostly at MOI 400), and a high variability between replicates was observed (Figure 7). At least two of the triplicate microcosms of the two strains infected the THP-1 macrophages at all times analysed for up to 8 days at 70°C (Figure 7, SI). Thereafter, no more infections were observed in amoebae or in THP-1 macrophages (Figure 7).

3.9 Correlation between the different viability indicators

In a comprehensive statistical approach, we analysed how the different parameters are related to each other. Overall, the correlation pattern between parameters was strongly influenced by the temperature applied. At 55°C, we observed a significantly positive correlation between the concentrations of culturable cells and the number of cells with intact

membranes and high esterase activity ($p < 0.01$). At this temperature both subpopulations were >4 log reduced after 150 days of incubation.

In comparison to 55°C, at 60°C a steeper decreasing trend of the concentration of viable cells was observed. It took 15 days to observe a >4 log reduction in cells with intact membranes and low esterase activity. However, HE cells showed the same reduction already at $T_{8\text{days}}$ of heat exposure. At 70°C, again the most sensitive viability indicators, such as the cells with intact membranes and HE activity, were reduced by >4 log at $T_{4\text{days}}$ and $T_{24\text{h}}$, respectively; it took between 4-8 days to observe the same reduction in the number of LE cells. At higher temperatures, the aforementioned parameters also significantly correlated with the concentration of cells with intermediate-state membranes ($p < 0.01$), low esterase activity ($p < 0.01$) and degradation of DNA ($p < 0.05$), represented by the MFI of cells stained with SGI. Thus, higher temperatures synchronised the *L. pneumophila* subpopulation dynamics, showing a common decreasing trend over time.

Conversely, cells with membranes in an intermediate state and low esterase activity withstood 55°C for a longer time than the more active subpopulations (Tables SI 4-6).

Among the OMEs, several correlations were observed. In most cases, the concentration of cells with a high number of epitopes (HF subpopulations) of mAb 3/1 and mAb 8/5 were positively inter-correlated ($p < 0.01$). The concentration of HF and LF subpopulations also correlated with some viability indicators, specifically with cells with intact and intermediate-state membranes, high esterase activity and the MFI of cells stained with SGI ($p < 0.05$ and $p < 0.01$ depending on HF/LF mAb subpopulation and strain) (Tables SI 4-6).

4 Discussion

LD is an atypical pulmonary infection that causes substantial morbidity and mortality worldwide (Phin et al., 2014). Approximately 90% of LD infections are caused by *Legionella pneumophila*, the transmission of which is generally associated with exposure to aerosols produced from colonised water systems (Buse et al., 2012; Phin et al., 2014). Within such systems, *Legionella* survives in existing biofilms, replicating inside FLA (Declerck, 2010). Once inhaled, *L. pneumophila* encounters alveolar macrophages in the human body and uses mechanisms similar to those utilised in the environment, eventually causing human pulmonary infection (Buse et al., 2012). To control *Legionella* proliferation in EWS, preventive measures and disinfection strategies based on specific thermal regimes are currently used. However, the bacteria are often not successfully eliminated from the system (Farhat et al., 2010; Steinert et al., 1998; Whiley et al., 2017). The ability of *Legionella* to switch into a VBNC state as a survival strategy against stressors and the implications that this may have on the disinfection strategies is currently hotly debated.

In the present study, we monitored the impact of three temperatures currently used to control culturable *Legionella* in EWS (55°C, 60°C and 70°C) on the viability of two *L. pneumophila* microcosms in ultrapure water for >80 days, one of the longest and most comprehensive studies performed for culturable and specifically for VBNC *L. pneumophila* thermal exposure.

For each temperature tested, we observed distinct phases of VBNC cell formation and, depending on the parameters analysed, how temperature impacted the differential development of *L. pneumophila* subpopulations. The results obtained showed that culturability, among the viability indicators used, was the most sensitive to temperature as previously reported by Hoefel et al. (2003). At 55°C, a complete loss of culturability (8 log reduction) was observed at T_{3-8h} for both *L. pneumophila* strains. Although differences between strains were observed, both strains were more resistant to temperature than the strains used by Cervero-Aragó et al. (2015) which were reduced by 8 log in approximately 18 min at 55°C. At 60°C, the two *L. pneumophila* strains stopped growing on plates at T_{0.5h} and T_{1h}. The results are in agreement with a study by Stout et al. (1986), who observed the same reduction after approximately 28 min. Differences between strains could be explained by a distinct composition of the bacterial membranes (Shevchuk et al., 2011) and, as suggested in Sharaby et al., (2017), by a distinct isolation source (clinical vs environmental) that might have exerted a selective pressure on the strains. At 70°C, the two strains were nonculturable at T_{2min} of incubation, as also observed by Cervero-Aragó et al. (2015) despite the different strains used to perform the heat treatments. Thus, at 70°C differences between strains do not longer play a role.

Following culturability, the subpopulations of cells with HE activity and cells with intact membranes were consecutively the most sensitive. At 55°C, it took 150 days to observe a >4 log reduction in the concentration of cells with intact membranes and HE activity. In comparison to 55°C, at 60°C a steeper decreasing trend of the concentration of viable cells was observed. It took 8 days to observe a >4 log reduction in cells with HE activity and 15 days for cells with intact membranes and LE activity. At 70°C cells with intact membranes and HE activity, were reduced by >4 log at T_{4days} and T_{24h}, respectively; it took between 4-8 days to observe the same reduction in the number of LE cells. These data suggest that at temperatures above 55°C, *L. pneumophila* reduces its metabolic activity before the thermal stress harms the bacterial membranes. Once membranes are harmed, degradation of the DNA takes place as observed by the reduction in the MFI of SGI positively stained cells (McDougald et al., 1998). Strong correlations between viability indicators were observed; particularly at the highest temperatures in which cells could not withstand the stressor and viable cells were uniformly reduced following similar subpopulation dynamics.

The two *L. pneumophila* strains remained infective for *Acanthamoeba* and THP-1 cells depending on the temperature used. At 55°C the two *L. pneumophila* strains infected amoebae and THP-1 cells after heat treatments for 85 and 130 days and after 114 days at 60°C. At 70°C, the two *L. pneumophila* strains infected amoebae and THP-1 cells for a maximum of 8 days. However, after the total loss of culturability, they infected the host cells with reduced efficiency, requiring higher bacterial concentrations (higher MOIs) and longer co-incubation times as only part of the cells remained infective. Moreover, a high variability between replicates was observed. Similar results were also observed for the same strains under starvation conditions (Dietersdorfer et al., 2018).

The LPS of *L. pneumophila* is one of the virulence factors associated to the pathogenesis of the bacteria as, as part of the bacterial envelope, it mediates the contact between pathogens and host cells (Shevchuk et al., 2011). Moreover, the high diversity in the LPS composition

has been used as serotyping tool for *L. pneumophila* strains and serogroups, e.g. the Dresden panel. Specifically, the LPS provides several epitopes/binding sites for monoclonal antibodies (mAbs). Among them, the epitope recognized by the mAbs 3/1 from the Dresden panel has been detected in 78% of community acquired and travel-associated LD cases (Reichardt et al., 2010). In their study Reichardt et al., (2010) showed a phase-variable expression of *L. pneumophila* mAb 3/1 that regulates the hydrophobicity of the bacterial membranes and suggested that such modulation ability maybe linked to the high virulence observed for the mAb 3/1 strains. The major outer-membrane protein (MOMP) is one of the most abundant proteins of *L. pneumophila*'s envelope and it has been described as an enhancer of bacterial phagocytosis by human monocytic cells (Shevchuk et al., 2011).

In the present study we studied for the first time the impact of thermal stress on the expression of several OMEs likely to correlate with the infectivity of thermally produced VBNC *L. pneumophila* cells. Our results showed that although in some cases the number of epitopes per cell was reduced at increasing temperatures (e.g. MOMP, 3/1); the total number of cells expressing such epitopes remained stable over time even when neither infection nor viable cells were observed. This supports the idea that their presence *per se* is not necessarily associated with viability or infectivity (Schrammel et al., 2018b). Hence, we considered VBNC *L. pneumophila* subpopulations viable when they showed high metabolic activity, an intact membrane with a cytoplasmic space containing DNA (SGI-positive) and, ultimately, were infective. Unfortunately, comparisons between viability indicators and the number of thermally induced VBNC *L. pneumophila* cells that remained infective at the latest sampling points were not possible because some of the viable subpopulations analysed had a very low cell number (close or below the LOQ). In our previous study on the infectivity of starved VBNC *Legionella*, we estimated that between 0.7-6.3% of 1×10^5 cells remained infective at T_{221days} under starvation conditions (Dietersdorfer et al., 2018). Moreover, Al-Bana et al. 2014 calculated that 1-3 out of 100,000 starved VBNC *L. pneumophila* cells resuscitated in amoebae.

Epalle et al. (2015) monitored the membrane integrity of three *L. pneumophila* SG1 strains exposed to 50°C, 56°C, 60°C, 65°C and 70°C over 1 h and checked whether thermally induced VBNC *L. pneumophila* cells at 60°C and 70°C were able to infect macrophage-like cell lines U937, HL-60 and the epithelial cell line A549, as well as the amoeba *A. polyphaga*. Major differences in the methodology used, such as different preparation of initial cell suspensions, concentrations of cells analysed and a different gating strategy complicate the comparison between studies regarding membrane integrity (e.g. when comparing Allegra et al., 2011, 2008), emphasising the need for standardised protocols in cell-based analyses by flow cytometry (Schrammel et al., 2018a). Overall, it seems that the two *L. pneumophila* strains in the present study maintained the integrity of the membranes longer than the strains used by Epalle et al. (2015), specifically at temperatures <70°C. That could explain why, in addition to the lower MOIs tested, they did not observe direct infection of human cells after 30 min at 60°C or 70°C. After 30 min at 70°C, 2 out of 3 *L. pneumophila* strains used in their study infected *A. polyphaga* using lower MOIs and recovered culturability on BCYE agar plates. After infecting amoebae, the two resuscitated strains were able to infect the U937 and HL-60 cell lines. Although in our study heat-induced infective VBNC *L. pneumophila* cells were observed in co-culture with the two

hosts, they never recovered culturability. In a previous study from our group, the same *L. pneumophila* strains in a VBNC state induced by starvation conditions recovered culturability only after infecting primary monocyte-derived macrophages (Dietersdorfer et al., 2018) proving again that resuscitation on agar plates is a complex mechanism that requires further research (Pinto et al., 2015).

4.1 Implications of the results obtained on the actual prevention strategies against *Legionella*

By maintaining the water heater set point temperature at 55°C or 60°C, parts of the *L. pneumophila* populations — most likely nonculturable — may still infect amoebae for up to 85 days, whereas at 70°C, they may remain infective for up to 8 days. Assuming that VBNC *L. pneumophila* cells are characterized by an intact membrane and high esterase activity (Schrammel et al., 2018a) at least 1% of the cells were viable after 9 days at 55°C, 8 h at 60°C and 20 min at 70°C. These values represent minimum values as it cannot be ruled out at the moment that cells with intermediate membranes and low esterase activity are viable as well. Although 70°C proved to be effective in reducing the viability of *L. pneumophila* by >4 log during continuous long-term treatment, the maintenance of an EWS at 70°C for prolonged periods of time through the entire system is neither realistic nor affordable. At the same time, our results showed that a short thermal treatment at 70°C (<1 h) is less effective in reducing viability of culturable and nonculturable *L. pneumophila*; therefore, a recolonisation of the system cannot be excluded (reviewed in Whitley et al., 2017).

Relatively recent studies have shown not only that short thermal shocks at high temperatures can potentially favour the development of thermophilic *Legionella* strains (Allegra et al., 2011) due to the intrinsic potential of the genus to adapt to a wide range of temperatures (Lesnik et al., 2016; Sharaby et al., 2017) but also that they could enhance a faster recolonisation of the EWS by eliminating competitive bacteria (Ji et al., 2018; Vervaeren et al., 2006). Moreover, among all *Legionella* species, *L. pneumophila* is the most commonly detected species in hot water systems, suggesting a better adaptation of the bacteria to high temperatures (Lesnik et al., 2016; Mouchtouri et al., 2007). Such adaptation is likely to be related to a species- or even strain-specific membrane composition (Shevchuk et al., 2011).

Chang et al. (2007) observed that starved VBNC *Legionella* cells showed a higher resistance towards temperature in terms of membrane integrity than the culturable cells. Thus, the activation of the stringent response due to nutrient scarcity probably activates physiological or structural changes that protect the cells against temperature as well (Chang et al., 2007). Here, we used ultrapure water to produce the bacterial microcosms because it was a more standardisable water matrix. Therefore, an impact of starvation on the results obtained in the present study cannot be ruled out.

In EWS, *Legionella* lives associated with FLA. Cervero-Aragó et al. (2013) reported that trophozoites of *Acanthamoeba* and *Vermamoeba*, two of the most prevalent amoebae in EWS (Thomas and Ashbolt, 2011), were sensitive to temperatures >50°C, whereas their cystic forms withstood higher temperatures. Thus, maintaining a temperature of 60°C would inactivate amoebic trophozoites and promote their encystment, blocking their availability as natural replicators of *Legionella*. Moreover, as noted by Bédard et al. (2015), the thermal

regime must be combined with good system hydraulics that ensure a delivery of high temperature to the terminal ends (e.g. taps, showers) the common areas likely to be colonised by *Legionella* due to the lower temperatures and chlorine concentrations. Additionally, more control on the water use frequency might help managing the microbial community in these terminals (Ji et al., 2018). Finally, the effectiveness of the thermal regime implemented should be monitored over time. According to legislation, in many countries, such surveillance is made using standard culture-based methods. Although these methods are not useful for detecting bacteria in the VBNC state, here we observed that they provide useful information about the concentration of the most virulent bacterial subpopulations.

Conclusions

- Currently, there is a big debate about the significance and health relevance of the VBNC state of *Legionella*. In this study, we could show that thermally-induced VBNC *L. pneumophila* may still be infective on amoebae and THP-1. However, they do so with reduced efficiency, most likely due to a decreasing number of infective cells and to a lower infectivity of VBNC compared to culturable cells. Whether they would be infective for humans needs to be further studied. In any case, infection of amoebae could lead to a recolonisation of the system.
- An EWS that maintains a constant thermal regime at 60°C in the water heater tank and recirculating lines and has good hydraulics that ensure that a high temperature reaches all parts of the system may not only avoid the proliferation of culturable but also of VBNC *L. pneumophila* according to our results. This temperature would also reduce the availability of amoebae as natural replicators of the bacteria. Our results also showed that a short (<1 h) exposure to 70°C is less effective in reducing viability of culturable and nonculturable *L. pneumophila*. Further research is needed to see how different thermal treatments in simulating plumbing conditions impact the viability of *L. pneumophila* within mixed microbial communities.
- To provide effective system surveillance, culture-based methods provide information about the concentration of the most virulent *Legionella*. Cell-based methods and the use of OMEs might be potential complementary tools to study and detect *Legionella* VBNC cells. However, before their application in risk assessment, further development is needed to use them in mixed environmental samples with low *Legionella* concentrations, with similar efficiency as culture based methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Culturability of the two *L. pneumophila* strains at 55°C, 60°C and 70°C on BCYE agar plates. Results are shown as triplicate average values $\pm$ Standard deviation.

Figure 2.

Total cell counts, cell integrity (A) and DNA content represented by the MFI of cells positively stained with SGI (B) of the two *L. pneumophila* strains at 55°C, 60°C and 70°C. Results are shown as triplicate average values $\pm$ Standard deviation. RFU (relative fluorescence intensity).

Figure 3.

Membrane integrity (left column) and esterase activity (right column) of the two *L. pneumophila* strains at 55°C, 60°C and 55 60 70°C. For membrane integrity, three different subpopulations are displayed: cells with intact membranes (green), cells with membranes in an intermediate state (orange) and cells with harmed membranes (red). For esterase activity two subpopulations per strain are displayed: cells with high activity (HE, regular lines), and cells with low activity (LE, dashed lines). The results are shown as triplicate average values

± standard deviation. Dashed black lines show the LOQ, 1.3×10^4 cells/mL (Schrammel et al., 2018a).

Figure 4.

Concentration of mAbs-stained cells of the two *L. pneumophila* strains at 55°C, 60°C and 70°C. For each mAb tested two different subpopulations are displayed: highly fluorescent cells (HF, dashed lines), low fluorescent cells (LF, solid lines). Results are shown as triplicate average values $\pm$ Standard error. Dashed black lines show the LOQ, 1.3×10^4 cells/mL.

55°C	T0	T8h	T22h	T3d	T9d	T16d	T85d	T130d
LpClin_amoebae								
Moi 1	1d	16d		15d				
Moi 50	1d				5d	7d		
Moi 100	1d	7d	11-14d	15d	2d	16d		
Moi 400			4d	15d	2d	2-6d	7d	
culture	+	-	-	-	-	-	-	-
LpClin_THP-1								
Moi 1	1d							
Moi 50	1d	7d	3d	8d		3d		
Moi 100		2d	4d	8d	3d			3d
culture	+	-	-	-	-	-	-	-
LpParis_amoebae								
Moi 1	1d							
Moi 50	1d	3d	7d	5d				
Moi 100	1d	1d		5d		8d	7d	
Moi 400		1d	2-7d	3d	4d	4d		
culture	+	-	-	-	-	-	-	-
LpParis_THP-1								
Moi 1	1d							
Moi 50	1d	2-7d	5d	3d	3d		3d	
Moi 100		7d	6d	3d	3d		3d	3d
culture	+	-	-	-	-	-	-	-

Figure 5.

Culturability and infectivity patterns of the two *L. pneumophila* strains incubated at 55°C. Legionella microcosms were analysed at different times. Nonculturable cells (8 log reduction) are plotted within the pink square box. The dashed line accounts for differences between replicates. Infectivity was analysed by co-culture with Acanthamoeba and THP-1 cells at different MOIs. Culturability after passage through hosts is plotted below the MOIs; (+) culturable and (-) nonculturable. The numbers within the wells represent the days required to observe an infection in co-culture with the hosts. Green wells: all replicates

infective; yellow wells: two replicates infective; orange wells: one replicate infective; red wells: no infection observed. White wells without number: not analysed; h=hours; d=days.

60°C	T0	T10m	T20m	T60m	T24h	T8d	T15d	32d	39d	43d	46d	60d	72d	74d	79d	83d	86d	100d	114d
LpClin_amoebae																			
Moi 1	1d																		
Moi 100	1d	1-4d		1d		3d	14-17d							11d			6d		
Moi 400	1d	1-8d	1-3d	1-11d	3-7d	3d	2-3d				4-11d	4d		4d			4d		
culture	+	+	-	-	-	-	-				-	-		-			-	-	-
LpClin_THP-1																			
Moi 50	1d	3-4d	4-7d	3-4d	2-3d	4-7d	3d				4d	4d		4d			6d	6d	6d
Moi 100	1-3d	1-4d	1-6d	3-4d	2-7d	4-7d	3d					4d		4d				6d	3d
culture	+	+	-	-	-	-	-				-	-		-			-	-	-
LpParis_amoebae																			
Moi 1	1-3d																		
Moi 100	1d	12d	12d	8d	10d	3d	3-11d	4d											
Moi 400	1d	3d	3-6d	4d	3-7d	3-7d	3d	4d	11d	3-11d									
culture	+	+	-	-	-	-	-	-	-	-			-		-	-			
LpParis_THP-1																			
Moi 50	1d	3-4d	3-4d	3-4d	3-7d	4-7d	4-7d	4d	4d	4d			6d		6d	6d			
Moi 100	1d	3-4d	3-4d	3-6d	3d	3-4d	3-4d	4d	4d	4d						3d			
culture	+	+	-	-	-	-	-	-	-	-			-		-	-			

Figure 6.

Culturability and infectivity patterns of the two *L. pneumophila* strains incubated at 60°C. *Legionella* microcosms were analysed at different times. Nonculturable cells (8 log reduction) are plotted within the pink square box. The dashed line accounts for differences between replicates. Infectivity was analysed by co-culture with Acanthamoeba and THP-1 cells at different MOIs. Culturability after passage through hosts is plotted below the MOIs; (+) culturable and (-) nonculturable. The numbers within the wells represent the days required to observe an infection in co-culture with the hosts. Green wells: all replicates infective; yellow wells: two replicates infective; orange wells: one replicate infective; red wells: no infection observed. White wells without number: not analysed; h=hours; d=days.

70°C	T0	T2m	T10m	T60m	T24h	T4d	T8d	T56-98d
LpClin_amoebae								
Moi 1	7d				11d			
Moi 100		7d	7d	7-14d		7-14d	3-10d	
Moi 400	7-12d	4d		7d	3d	3-14d	3d	
culture	+	-	-	-	-	-	-	-
LpClin_THP-1								
Moi 50	1-7d	1-4d	6d	4-6d	3d	7d	3-6d	
Moi 100	1-4d	1-7d	4-7d	4d	3-6d	3-7d	3-7d	
culture	+	-	-	-	-	-	-	-
LpParis_amoebae								
Moi 1		4d						
Moi 100	4d	4d	4d	4d	3d	3-4d	3d	
Moi 400	1-4d	4d	4d	4-8d	3d	3-4d	3d	
culture	+	-	-	-	-	-	-	-
LpParis_THP-1								
Moi 50	4-7d	3-4d	4-7d	4-7d	3-6d	3-7d	3d	
Moi 100	1d	3-7d	4d	4d	3-7d	3-4d	3-4d	
culture	+	-	-	-	-	-	-	-

Figure 7.

Culturability and infectivity patterns of the two *L. pneumophila* strains incubated at 70°C. *Legionella* microcosms were analysed at different times. Nonculturable cells (8 log reduction) are plotted within the pink square box. The dashed line accounts for differences between replicates. Infectivity was analysed by co-culture with *Acanthamoeba* and THP-1 cells at different MOIs. Culturability after passage through hosts is plotted below the MOIs; (+) culturable and (-) nonculturable. The numbers within the wells represent the days required to observe an infection in co-culture with the hosts. Green wells: all replicates infective; yellow wells: two replicates infective; orange wells: one replicate infective; red

wells: no infection observed. White wells without number: not analysed; m=minutes; h=hours; d=days.