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Validation of SYTO 9/Propidium Iodide Uptake for Rapid Detection of Viable but Noncultivable *Legionella pneumophila*

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Abstract *Legionella pneumophila* is an ubiquitous environmental microorganism that can cause Legionnaires' disease or Pontiac fever. As a waterborne pathogen, it has been found to be resistant to chlorine disinfection and survive in drinking water systems, leading to potential outbreaks of waterborne disease. In this work, the effect of different concentrations of free chlorine was studied (0.2, 0.7, and 1.2 mg l^{-1}), the cultivability of cells assessed by standard culture techniques (buffered charcoal yeast extract agar plates) and viability using the SYTO 9/propidium iodide fluorochrome uptake assay (LIVE/DEAD® *Bac*Light™). Results demonstrate that *L. pneumophila* loses cultivability after exposure for 30 min to 0.7 mg l^{-1} of free chlorine and in 10 min when the concentration is increased to 1.2 mg l^{-1}. However, the viability of the cells was only slightly affected even after 30 min exposure to the highest concentration of chlorine; good correlation was obtained between the rapid SYTO 9/propidium iodide fluorochrome uptake assay and a longer cocultivation with *Acanthamoeba polyphaga* assay, confirming that these cells could still recover their cultivability. These results raise new concerns about the assessment of drinking water disinfection efficiency and indicate the necessity of further developing new validated rapid methods, such as the SYTO 9/propidium iodide uptake assay, to assess viable but noncultivable *L. pneumophila* cells in the environment.

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Introduction

Legionella pneumophila is a Gram-negative rod-shaped bacterium that can cause Legionnaires' disease (pneumonia with a high mortality rate) or Pontiac fever (a mild nonpneumonic form of illness) [19, 25, 37]. Contrary to most other pathogens that appear in drinking water due to fecal contamination [12, 32], *L. pneumophila* is known to be ubiquitous in natural aquatic systems such as groundwater, lakes, and rivers [6, 9, 25]. When disinfection procedures are not effective, viable *L. pneumophila* cells can remain in water and continue to be the cause of outbreaks. This pathogen has been isolated from shower heads, whirlpools spas, cooling towers, air conditioning systems, humidifiers, etc. [9, 25, 33–35], being transmitted to humans when contaminated aerosols are formed and inhaled.

Chlorine is the disinfectant most commonly used to ensure drinking water quality and has been used since the nineteenth century [28]. Comparing the disinfectants that can be used in drinking water systems, chlorine is one of the most effective as residual chlorine can remain in water to control the microbiological water quality between the application and the distribution points [14]. However, in the last few years, it has been found that emerging pathogens have increased resistance to chlorine, especially *L. pneumophila*. On the other hand, studies investigating the effect of chlorine concentration on this bacterium used a simple culture method to assess viability [15, 16] which is now known to have limitations [10]. It has been demon-

strated that, after exposure to stress conditions, a range of different bacterial species can enter a viable but non-cultivable (VBNC) state. In this state, cells are not able to grow and replicate in artificial media but are still viable and might maintain their pathogenic properties [8, 10, 20, 23, 36, 37]. In fact, it has been demonstrated that *L. pneumophila* can recover cultivability after being exposed to stress conditions when cocultured with amoeba species [7, 30]. This procedure demonstrates that *L. pneumophila* is able to remain infective but is tedious to perform, taking several days of coculture followed by four or more days for recovery of cultivable cells on agar media.

The aim of this work is to develop a rapid viability assay procedure and show that the assessment of *L. pneumophila* in water after exposure to chlorine stress by the use of standard methods can lead to false results: the pathogen can completely lose cultivability but still maintain membrane integrity, which we have validated to be indicative of cell viability by demonstrating infection of amoebae in coculture.

Methods

Strains *L. pneumophila* NCTC 12821 was grown on buffered charcoal yeast extract (BCYE) agar (Oxoid, UK) for 24 h at 30°C. Cells were suspended in 50 ml of dechlorinated and filtered tap water to give a final concentration of approximately 10^7 cells ml^{-1}.

Chlorine Preparation and Measurements Chlorine tablets (H-8801, Guest Medical, UK) were added to filtered distilled water to obtain a 5 g l^{-1} stock solution. The measurement of chlorine was done using the *N*,*N*-dimethyl-*p*-phenilenediamine (DPD) colorimetric method, as described in the standard methods for the examination of water and wastewater [2] with the exception of the absorbance wavelength reading which was adjusted to 492 nm [21].

Chlorine Disinfection Tests After considering the chlorine demand due to organic matter, an appropriate amount of the stock solution was added to the suspension in order to obtain a final concentration of free chlorine of 0.2, 0.7, and 1.2 mg l^{-1}. A control assay with no chlorine addition was also performed. Experiments were carried out in amber flasks (to avoid chlorine degradation by light) at room temperature (20°C) and stirred at 620 rpm. Samples were taken at 0, 10, 20, and 30 min and cells quantified as explained below. At times 0 and 30 min, the concentration of free chlorine was measured by the DPD method as described previously. The chlorine reaction was inactivated by the addition of sodium thiosulfate (Sigma, UK) applied at a final concentration of 5 mg l^{-1}. For each chlorine concentration, the experiment was repeated at least three times.

Assessment of Cultivable Cells A 40 µl aliquot of each sample was diluted (to give between 15 and 150 colony-forming units (CFU) per agar plate) and spread onto BCYE agar plates (in triplicate for each experiment) and aerobically incubated at 30°C for 4 days. After this time, the number of colonies was counted to determine the number of cultivable cells remaining in the chlorinated solution. When, after 4 days, no colonies were grown on BCYE agar plates, the plates were returned to the incubator for 14 days. Using this method, the limit of detection is 8.33 CFU ml^{-1}.

Assessment of Membrane Integrity To assess the membrane integrity, the LIVE/DEAD® *Bac*Light™ Bacterial Viability kit (Molecular Probes, Invitrogen, UK) was used. A 50-µl aliquot of each sample was diluted in 0.950 ml of dechlorinated, filtered tap water and stained with SYTO 9/ propidium iodide (PI). A 3-µl volume of an equal proportion of SYTO 9 and PI mixture was added to the sample and incubated in the dark, at room temperature (20°C) for 15 min followed by filtration through a black polycarbonate Nucleopore® membrane (0.2 µm pore size; Whatman, UK). Subsequently, the membranes were air dried and mounted onto glass slides with nonfluorescence immersion oil and a cover slip. The slides were examined using a Nikon Eclipse E800 episcopic differential interference contrast/epifluorescence (EDIC/EF) microscope under oil immersion (Best Scientific, UK) [13].

Coculture of L. pneumophila and Acanthamoeba polyphaga An axenic culture of *A. polyphaga CCAP1501/18* was maintained in Proteose Peptone Glucose Medium (PPG; CCAP, UK) at room temperature and subcultured every week. Five-milliliter samples of the bacterial suspension exposed to 1.2 mg l^{-1} of free chlorine for 0 (control) and 30 min were centrifuged at 3,000 rpm for 10 min (Heraeus, UK) and washed three times in PP medium (PPG but with glucose omitted) before resuspending in a final volume to achieve the concentration of approximately 5×10^5 cells ml^{-1}. Infection of *A. polyphaga* by *L. pneumophila* was performed as described by Garcia et al. [7]. Briefly, monolayers of *A. polyphaga* were formed in 96-well plates in the presence of PP medium at a concentration of 10^4 cells per well and infected with 200 µl of the *L. pneumophila* suspension prepared as described above. The plates were then centrifuged at 500×*g* for 5 min and incubated at 30°C for 1 h. After this time, plates were washed three times with PP medium and incubated at 30°C for 1 h in 50 µg ml^{-1} of gentamicin followed by three washes with PP media. The infected monolayers were then incubated at 30°C, and the cultivability of *L. pneumophila* was assessed after 24, 48, and 72 h of infection. For that, *A. polyphaga* cells were lysed with 0.05% Triton X-100 (Sigma, UK), supernatants before and after lysis were combined, and 40 µl aliquots were plated onto BCYE as described above.

Assessment of RNA Injury A 1.0-μl aliquot of SYBR® Green II RNA gel stain (SYBR II; Molecular Probes, Invitrogen, UK) was added to 50 μl of each sample diluted in 0.95 ml of dechlorinated filtered tap water and incubated in the dark, at room temperature (20°C) for 30 min. The stained suspension was then filtered through a black polycarbonate Nucleopore membrane (0.2 μm pore size), air dried, and mounted onto glass slides with nonfluorescence oil and cover slips and examined using EDIC/EF microscopy [13].

DNA Electrophoresis L. pneumophila NCTC 12821, grown under the same conditions as previously described, was suspended in 100 ml of dechlorinated and filtered tap water to give a final concentration of approximately 10^7 CFU ml^{-1}. This cell suspension was exposed to the same range of chlorine concentrations for 30 min. Following this, cells were concentrated by centrifugation at 4,000 rpm for 10 min and the DNA extracted and purified using a DNA extract kit (Sigma, Spain). The DNA obtained was run in a horizontal electrophoresis system for 2 h at 100 V using 1% (*w*/*v*) agarose gel (Bio-Rad, Portugal) containing ethidium bromide (50 μl l^{-1} of a 10 mg ml^{-1} stock; Bio-Rad). Finally, the gel was visualized under UV light.

Statistical Analysis Results obtained for cultivable cells, membrane integrity, RNA injury, and total cell counts were transformed on a logarithmic scale. The average for each was calculated from at least three experiments, and the homogeneity of variances across these parameters was checked by the Levene test for equality of variances using a statistical package (SPSS Inc., Chicago IL, USA). Differences between the parameters measured were subsequently compared by a one-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test. Differences were considered relevant if $P<0.05$.

Results

The chlorine concentrations measured in the cell suspension at time 0 and 30 min indicated that there was consumption of chlorine by reaction with *L. pneumophila* cells, as indicated in Table 1. Preliminary results showed that there was consumption of chlorine by the organic matter present in the tap water (results not shown), so the values presented (0.2, 0.7, and 1.2 mg l^{-1}) are the values following subtraction of chlorine consumption by organic matter. However, at time 0 min, the values obtained were much lower than theoretically expected (i.e., compared to values obtained in tap water without cells), meaning that there was immediate consumption by cells.

The effect of chlorine on *L. pneumophila* cells was evaluated by quantification of cells by two different methods: standard culture techniques and direct count of cells by observation under epifluorescence microscopy after staining with the SYTO 9/PI fluorochrome reagents (Fig. 1).

Table 1 Chlorine concentration demand immediately after and 30 min after the chlorine addition to the sterile-filtered tap water and to the *L. pneumophila* suspension

Cl_2 concentration (mg l^{-1})	Cl_2 measurement in the cell suspension (mg l^{-1})	
	0 min	30 min
0.2	0.058	0.030
0.7	0.483	0.083
1.2	0.858	0.140

The assay where no chlorine was added to the suspension served as a control and showed that cells maintain their physiological state in dechlorinated filtered tap water for at least 30 min. In fact, ANOVA results show that time does not influence any of the parameters studied, including cultivability ($P>0.95$). Cultivable cells represented 55% of the total number of cells, and as expected, differences between cultivability and either the total number of cells or viable cells (assessed by SYTO 9/PI membrane integrity staining) were statistically significant ($P<0.05$). When cells were exposed to 0.2 mg l^{-1} chlorine, there were no alterations in the viability status, but the cells appeared to start losing their cultivability in the first 10 min. This result was confirmed by the statistical analysis on the effect of time on this parameter ($P<0.05$). Increasing the chlorine concentration up to 0.7 mg l^{-1} caused a complete loss of growth capacity on agar plates (total loss of cultivability). Although no statistically significant differences were detected between the number of viable and total cells ($P>0.05$), it was observed that some of the cells did not fluoresce true green but had become yellow/orange; however, only cells fluorescing red were considered as dead cells due to a compromised cell membrane. Cells exposed to the maximum concentration of chlorine (1.2 mg l^{-1}) showed a loss of cultivability during the first 10 min of chlorination, and it was observed that there was an increase in the number of red fluorescent cells. Therefore, at this concentration, the difference in numbers between viable and total cells was statistically significant ($P<0.05$).

To validate the results obtained with the LIVE/DEAD viability kit, a sample of *L. pneumophila* exposed to 1.2 mg l^{-1} of free chlorine for 30 min was treated and cocultured with *A. polyphaga*. Figure 2 shows that cells were able to recover cultivability between 24 and 48 h of infection. Simultaneously, a control experiment was performed in which cells not stressed with chlorine were also cocultured with *A. polyphaga*. After 72 h of coculture, the numbers of cultivable cells were lower than before exposure to *A. polyphaga*.

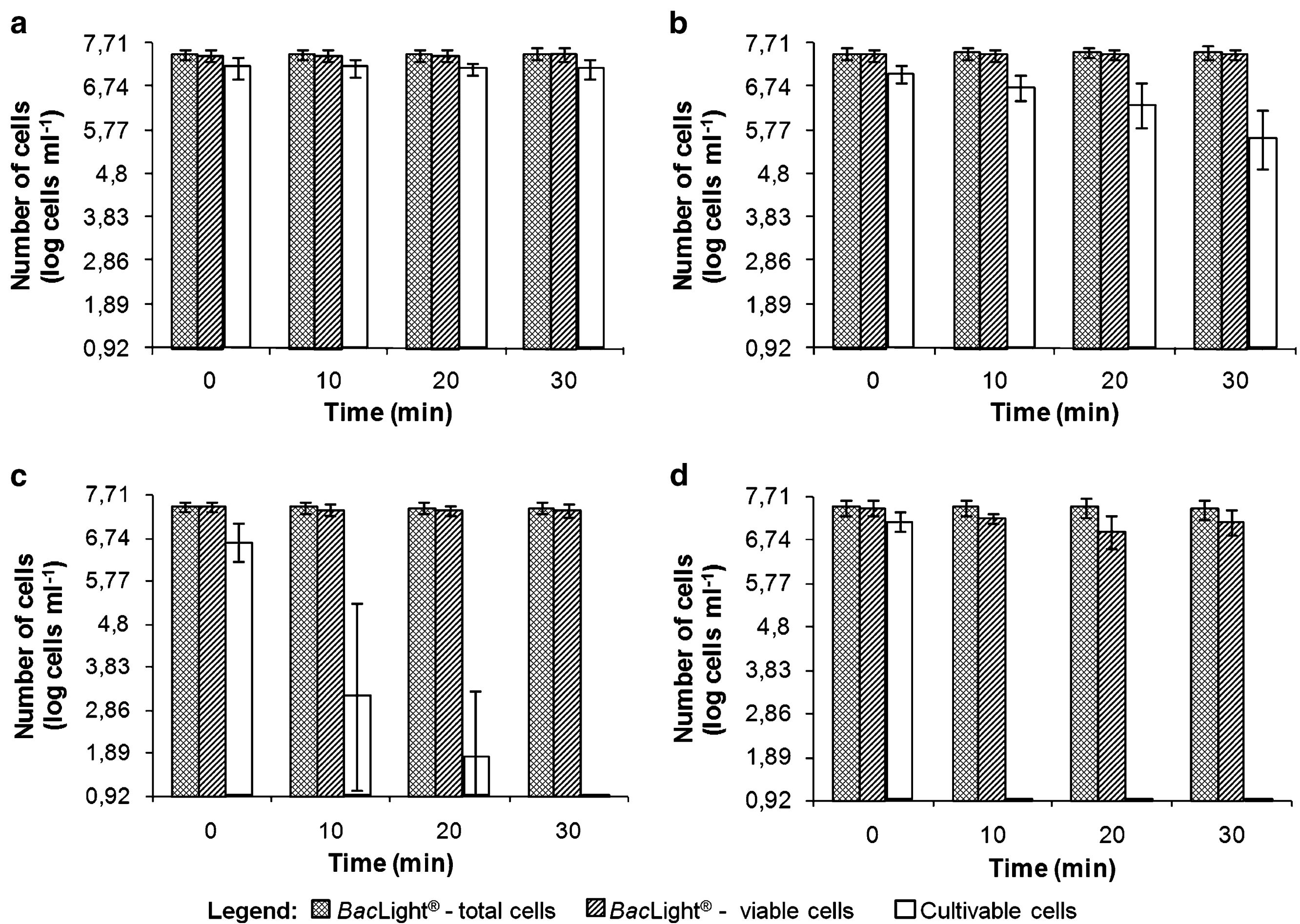

Figure 1 Variation in the total cell number, viability of SYTO 9^{-}/PI^{+} stained cells, and cultivability on BCYE agar after exposure to free chlorine concentrations of 0.0 mg l^{-1} (**a**), 0.2 mg l^{-1} (**b**), 0.7 mg l^{-1} (**c**), and 1.2 mg l^{-1} (**d**). *Error bars* represent standard deviation of at least three experiments. The 0.92 log cells ml^{-1} value represents the detection limit of the cultivability method

To investigate if *L. pneumophila* cells lost cultivability so quickly due to nucleic acid damage, chlorine-treated cells were stained with the SYBR II fluorochrome. This stains nucleic acids, although fluorescence intensity is much stronger when it binds to RNA compared to double-stranded DNA [17]. It was observed that neither fluorescence intensity nor cell numbers changed with increasing chlorine concentrations, suggesting that there are no variations in the RNA content in the cells under these conditions (results not shown). The DNA bands obtained by electrophoresis also show that there are no changes in the DNA content (results not shown).

Discussion

All the experiments described here were carried out by suspending the cells in dechlorinated, filtered tap water to represent realistic conditions. As expected when added to tap water (with no previous inoculation), there was a decrease in the chlorine concentration. This happens because tap water contains organic matter that can react with chlorine, the so-called chlorine demand, meaning that the real concentration available to interact with *L. pneumophila* cells is lower than the concentration added to the suspension. Prior studies showed that the chlorine consumed by organic matter is approximately 0.3 mg l^{-1} and occurs rapidly in the first 10 min (results not shown).

When chlorine was added to the *L. pneumophila* suspension, a sample was immediately taken and analyzed (corresponding to time zero). As seen in Table 1, a high proportion of the chlorine reacts instantly, with values significantly lower than the concentration added. The results obtained in tap water with no cells show a lower reduction confirming that chlorine reacts with the cells and that the reaction starts immediately after the addition. This can also explain the rapid loss of cultivability in the first 10 min after the addition of 1.2 mg l^{-1} of free chlorine, as seen in Fig. 1.

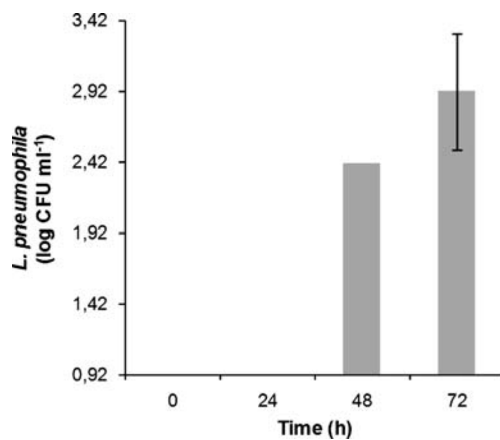

Figure 2 Number of cultivable *L. pneumophila* after 24, 48, and 72 h of coculture with *A. polyphaga*. *0 h* represents the number of cultivable *L. pneumophila* after 30 min of exposure to 1.2 mg l^{-1} of free chlorine. The 0.92 log cells ml^{-1} value represents the detection limit of the cultivability method

The *L. pneumophila* cell suspensions were prepared using a 24-h culture, so each batch of cells were in the same physiological conditions. To control this parameter and because the chlorine reaction with cells seemed to be immediate and fast, a sample was taken before chlorine addition, and cells were quantified by spreading on BCYE agar plates and by SYTO 9/PI double staining. SYTO 9/PI can be successfully used to stain *L. pneumophila* cells, as previously demonstrated by Ohno et al. [22]. Although the cultivability was found in the present study to be slightly variable, these values do not seem to be significant; the viability results showed that the cells were in a very similar state (percentage values of viable cells were always around 95% of total cells).

In the absence of chlorine, there was no loss of cultivability and viability of the cells in tap water. This was expected as *L. pneumophila* can survive for long periods in tap water [11, 29, 36] and can even grow and replicate under particular conditions [35, 38].

When 0.2 mg l^{-1} of free chlorine was added to the suspension, it was found that *L. pneumophila* cells lost some cultivability, but there were cells that could still be recovered by standard culture techniques. Microscopic observation of cells stained with SYTO 9/PI revealed that the number of viable cells was constant with time and also that the cells maintained a bright green color. The chlorine measurement after 30 min demonstrated that all chlorine was consumed by the cells. In fact, it can be considered that, at this concentration, chlorine is completely consumed immediately after its addition, as can be seen in Table 1 where the value at 0 min is very close to 0, and after 30 min no chlorine remains in the solution. The fact that some cells can still be grown on agar plates demonstrates that this level of chlorine represents a sublethal concentration and as such does not cause any damage to the membrane integrity since no cells take up PI. The loss of cultivability when cells are exposed to stress conditions (such as extreme temperatures, nutrient starvation, or chlorine oxidative stress exposure) is an effect which can be explained by injury to the cytoplasmic membrane and transport processes which reduce the membrane potential and respiratory activity and, consequently, cultivability [1, 3, 18]. However, Yamamoto et al. [37] have already demonstrated that, even after losing cultivability due to nutrient starvation, cells retain intact DNA and RNA and, in favorable conditions, can recover, elongate, and multiply.

When the free chlorine concentration was increased up to 0.7 mg l^{-1}, the cells lost cultivability completely between 20 and 30 min of exposure time; however, the number of viable cells assessed using SYTO 9/PI was not altered. Although the green cells lost their brightness and some of them became yellow and orange, there was no increase in the number of cells that fluoresced red. Some authors have suggested that, when cells change their fluorescence color from green to orange or yellow but are not exactly red, this means that there is some injury to the cellular membrane that allows some of the PI to penetrate the cell. However, when the injury is minor, the concentration of PI that can penetrate the cell and bind to DNA is not high enough to exclude all the SYTO 9 in the cell bound to DNA, so they appear yellow and orange and are considered as viable cells [5]. Indeed, no residual free chlorine remained at the end of the incubation period which might account for the extended viability of the cells.

The maximum chlorine concentration used (corresponding to 1.2 mg of free chlorine per liter) was sufficient to cause complete loss of cell cultivability in 10 min, but once again, there was an insignificant decrease in viability. In contrast, it was observed that most of the cells were not green or red but orange, which suggests that low level injury to the cytoplasmic membrane had occurred due to the chlorine concentration. At the end of these experiments, some free residual chlorine was still detectable, indicating that this concentration of chlorine was in excess of that needed to completely react with the cells within 30 min.

The use of LIVE/DEAD to assess the viability of cells is still controversial. Some authors are skeptical in accepting that green cells that have lost their cultivability are effectively viable [4, 31]. To validate the results obtained in this work, a sample of *L. pneumophila* previously exposed to 1.2 mg l^{-1} of free chlorine for 30 min was used to infect *A. polyphaga*. Results demonstrated that *L. pneumophila* has, in fact, entered into a viable but noncultivable state as cells recovered their capability of growth on artificial media (BCYE) between 24 and 48 h of coculture with amoebae. Alternatively, in the control, where cells were not exposed to

chlorine, the number of cultivable cells after 72 h was lower than before coculture with amoebae, which indicates that cells were not multiplying but were instead resuscitating inside of the amoebae. These results clearly demonstrate that *L. pneumophila* cells that appear green when stained with *Bac*Light™ kit were effectively viable, although not cultivable. The resuscitation to a cultivable state of VBNC *L. pneumophila* using amoeba species has been demonstrated before [7, 30] and therefore used in this work to demonstrate that LIVE/DEAD is a technique that can be successfully used to access the effectiveness of disinfection. The advantage of this assay is the short time it takes to obtain the results: using this method, the results can be obtained in a few hours, while when using the amoeba cocultivation assay, the results are not available in less than 1 week.

The resuscitation of VBNC cells that have been exposed to stress conditions such as chlorination is sometimes difficult to achieve. Oliver et al. failed to resuscitate chlorine-treated *Escherichia coli* cells when using several methods previously demonstrated as successful [24]. However, and unlike the present work, they have not used live eukaryotic hosts for resuscitation. It has been clearly demonstrated here that *L. pneumophila* very quickly loses cultivability without losing viability, which is not surprising as it had been demonstrated previously that noncultivable cells can still be viable [8, 22]. Moreover, Hussong et al. [10] demonstrated that noncultivable *L. pneumophila* cells are not just viable but can cause infection of embryonated eggs. On the other hand, Steinert and colleagues [30] had suggested that Pontiac fever, a mild form of disease caused by *L. pneumophila*, could be due to VBNC cells. However, it is important to note that this study is only valid for chlorine or any other biocide that disrupts the bacterial membrane as the PI is membrane-integrity-dependent. Biocides that might not compromise the membrane integrity should be validated in an independent study.

At all concentrations of chlorine investigated (0.2, 0.7, and 1.2 mg l^{-1}) the total number of cells remained constant during the 30 min of experimental time. SYTO 9 and PI are both fluorochrome stains that bind with nucleic acids, and when there is some damage to the nucleic acids, they are not able to bind, and the cells cannot be visualized. The fact that the number of total cells observed by epifluorescence microscopy was always the same suggests that there was little or no injury to the nucleic acid structure, as expected at this low concentration of free chlorine. These results were corroborated by DNA electrophoresis which indicated that genomic DNA remained intact following chlorine treatment (results not shown). Although Phe et al. [26, 27] have previously shown that chlorine is able to damage the nucleic acids in *E. coli* cells, the disinfectant dosage used was much higher and, in addition, it is already known that *L. pneumophila* is more resistant to chlorine than *E. coli*. [16].

This study has clearly demonstrated that the standard culture methods used to assess the presence of *L. pneumophila* are not ideal to study the presence of this pathogen and especially its viability because, even after completely losing the capability of growth on BCYE agar plates, cells remained viable and able to infect amoebae. This raises a new concern for water quality assessment and requires the development, as reported here, of new validated, rapid methods to detect viable *L. pneumophila* cells in drinking water after disinfection.

In addition, it also suggests that viable *L. pneumophila* are probably more widespread in drinking water distribution systems than previously thought, despite the presence of residual chlorine at concentrations indicated by the international health protection agencies to control microbiological quality. The fact that a relatively low number of outbreaks with this bacterium are observed is perhaps more related to the fact that, for disease to occur in humans, unlike many waterborne pathogens causing gastrointestinal infection, *L. pneumophila* has mainly to be inhaled in the form of aerosols to gain access to the lung.

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