

# MONOCHLORAMINE TREATMENT INDUCES A VIABLE-BUT- NONCULTURABLE STATE INTO BIOFILM AND PLANKTONIC *LEGIONELLA PNEUMOPHILA* POPULATIONS

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*Legionella pneumophila*, the causative agent of an atypical severe pneumonia in humans called Legionnaires' disease, is ubiquitous in aquatic environments. This gram-negative bacterium is normally found in water as an intracellular pathogen of amoebae. As a free-living organism, *L. pneumophila* can persist for long periods in biofilms commonly found in man-made water systems such as plumbing systems, air-conditioning equipment, or whirlpool spas (4). Grazing amoebae should consume these sessile *L. pneumophila* in biofilm communities and allow them to multiply (3). It is widely admitted that biofilms play a critical role in the persistence of these bacteria within water systems. Biofilm provides shelter and nutrients for the embedded community and prevents disinfectants from gaining access to the bacteria through the exopolysaccharide matrix (4).

The strategies of *L. pneumophila* to adapt and resist stressful environmental conditions include not only interaction with amoebae and biofilm localization but also the ability to enter in a viable-but-nonculturable (VBNC) state. Cells in a VBNC state fail to grow on the

routine bacteriological media on which they would normally grow. They typically exhibit very low levels of metabolic activity but can recover their culturability in favorable conditions. On resuscitation, these cells again became culturable (12). 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 (2, 11, 13). Addition of protozoa, e.g., the amoeba *Acanthamoeba castellanii*, to *L. pneumophila* in a VBNC state, resulted in the resuscitation of these bacteria to a culturable state (13). Such observations demonstrate that VBNC *L. pneumophila* regain pathogenic potential with a reactivation in amoebae and are therefore a public health concern. Many strategies have been used to eradicate *Legionella* in water and plumbing systems, such as chlorination, chloramination, overheating, and 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, 14). Eradication of *L. pneumophila* may require continuous treatment. Some authors suggested that continuous treatments with monochloramine have a better impact than free chlorine alone on *Legionella* eradication (8, 9) and on biofilm formation

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(10). Monochloramine's biocidal action is slower than that of free chlorine, but it is more stable, and a disinfecting residual activity can be maintained over long distances in a water distribution system (7, 9). Such an impact was also observed in biofilm-associated *L. pneumophila* in a potable water model and cooling towers systems (6, 15).

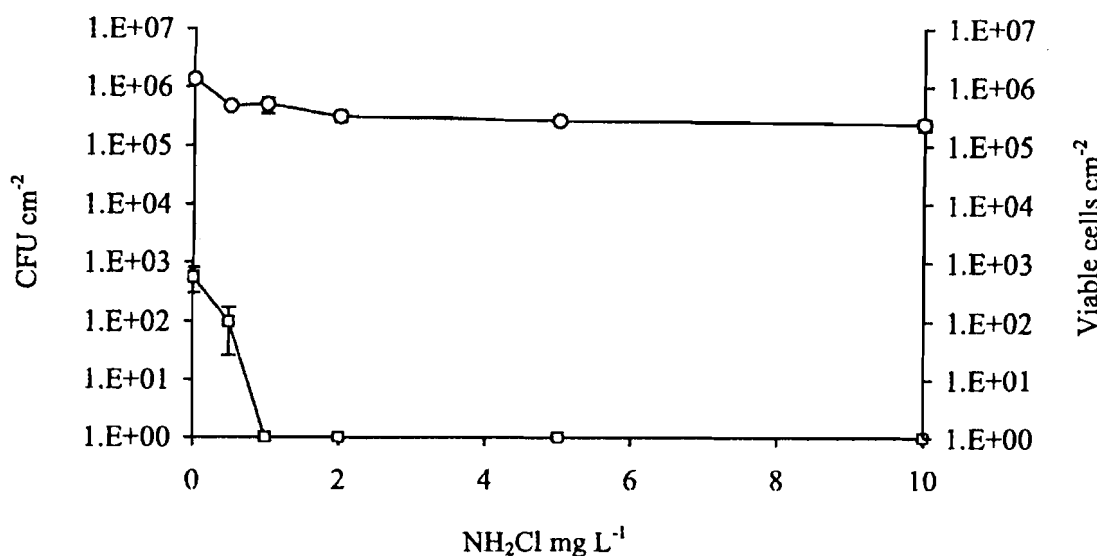
The aim of our work was to study the impact of monochloramine on sessile and planktonic *L. pneumophila* populations. For this purpose, we used the two recently sequenced Paris and Lens strains (5) obtained from the CNRL (Centre National de Référence des Légionelles, Lyon, France).

### SCREENING VBNC AFTER MONOCHLORAMINE TREATMENTS

We have produced *Legionella* biofilms on glass beads (0.5 mm diameter) in buffered-yeast extract (BYE) broth. Bacteria were grown for one week at 37°C, under static conditions. Planktonic cells were obtained in same growth conditions without glass beads. Planktonic and sessile cells were washed twice with sterile water and then treated with 0.25 to 10 mg liter<sup>-1</sup> monochloramine solutions. Solution concen-

trations were measured using the DPD method. After a 1-h treatment at room temperature, sterile sodium sulfite was added. Glass beads were transferred into a resting medium consisting of BYE broth diluted 10<sup>3</sup>-fold and incubated at 20°C for various durations. We used 10<sup>3</sup> diluted BYE broth as the resting medium in order to obtain total organic carbon concentration close to that of surface water (2 mg liter<sup>-1</sup> total organic carbon). *L. pneumophila* was unable to grow in this diluted medium but was able to survive for several weeks without detectable culturability decline (data not shown).

Sessile bacteria were collected from glass beads by sonication and enumerated on buffered charcoal yeast extract (BCYE) agar. We looked for VBNC cells in samples containing no culturable bacteria. Enumerations of viable and dead bacteria were determined by epifluorescence microscopy using the BacLight LIVE/DEAD Bacterial Viability Kits (Molecular Probe). Viable bacteria appear green and membrane damaged bacteria appear red. Enumeration results are presented Fig. 1 for samples kept 6 days in the resting medium after treatments of biofilms by monochloramine. We observed a loss of cultivability for monochloramine doses



**FIGURE 1** Culturability and viability of *L. pneumophila* Paris after monochloramine treatment. After monochloramine treatments, biofilms were transferred into resting medium (see text for details) and incubated at 20°C for 6 days. Squares indicate culturable cells enumerated on BCYE agar, and circles, viable cells enumerated by epifluorescence using the BacLight Kit, as cells cm<sup>-2</sup> (Molecular Probes).

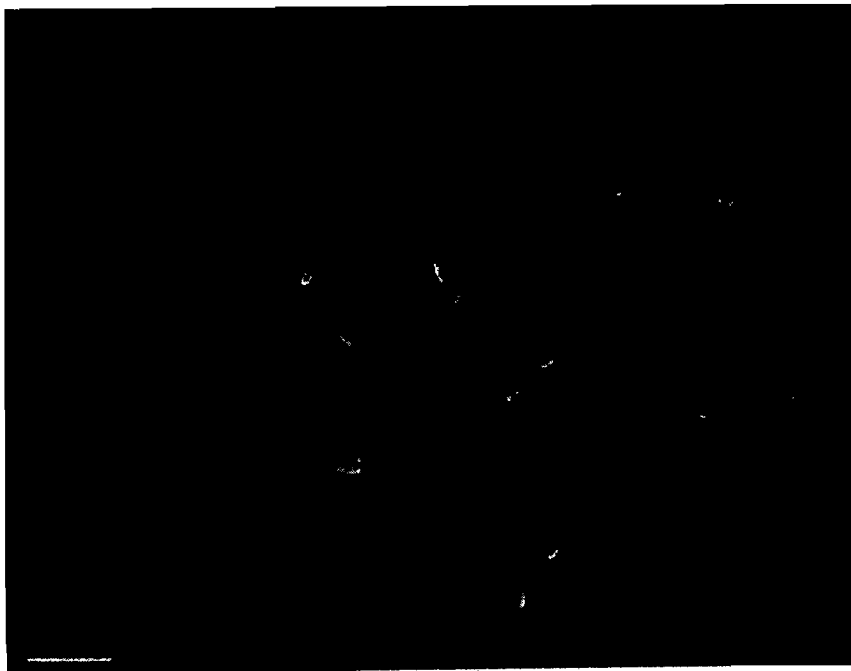
greater or equal to  $1 \text{ mg liter}^{-1}$  but a very low decrease in viable cells. Similar results were obtained for samples kept 12 or 20 days in the resting medium (data not shown). These results suggest that these samples contained VBNC cells. Nevertheless, green cells detected using BacLight staining indicate only that those cells possess integer membranes. In order to detect metabolic activity in VBNC cells, planktonic *L. pneumophila* Lens cells were treated with  $0.75 \text{ mg liter}^{-1}$  of monochloramine in order to obtain no culturable cells. After 8 days in the resting medium, we were able to detect esterase activity and membrane integrity by fluorescence microscopy using the ChemChrom V6 substratum (Chemunex) in samples without culturable cells (Fig. 2). This demonstrates that VBNC cells produced by monochloramine retained metabolic activity.

Steinert et al. (13) have observed that addition of amoebae to VBNC *L. pneumophila* led to resuscitation of these bacteria to a culturable state. Then, in order to check VBNC resuscitation, sessile bacteria collected after sonication were cocultivated with *A. castellanii* ATCC 50739. After 3 and 7 days of coincubation, the amoebae were lysed using centrifugation and vortexing. *L. pneumophila* culturability was thus

determined on BCYE agar. Infection attempts were realized with biofilms kept for 30 days in resting medium (Table 1). Amoeba addition to untreated biofilms led to an important increase of CFU, but not for biofilms treated with doses higher than  $1 \text{ mg liter}^{-1}$  of monochloramine, even after 7 days of coincubation. Recovery of culturability was observed for sessile bacteria treated with  $1 \text{ mg liter}^{-1}$  of monochloramine and coincubated with *A. castellanii*. However, when the same treated bacteria were not coincubated with the amoebae, no culturable cells were detected. We were not able to resuscitate sessile bacteria treated with higher monochloramine doses, despite detection of viable cells in these samples. It may be possible that these cells are progressively degenerating since they have more oxidative damages than those treated with only  $1 \text{ mg liter}^{-1}$  of monochloramine or that they required more than 30 days to resume these repairs to be able to infect amoeba.

#### CONCLUSION

*Legionella* samples treated with monochloramine contained viable and dead bacteria. We have observed that moderate monochloramine treatments of *L. pneumophila* biofilm could



**FIGURE 2** Esterase activity of *L. pneumophila* Lens 8 days after a  $0.75 \text{ mg liter}^{-1}$  monochloramine treatment. The white line represents  $10 \mu\text{m}$ .

**TABLE 1** Influence of *Acanthamoeba castellanii* on culturability of sessile *L. pneumophila* Paris treated with monochloramine

Monochloramine treatment (mg liter <sup>-1</sup> ) <sup>a</sup>	CFU obtained on BCYE- $\alpha$ agar <sup>b</sup>	
	Without amoeba	With amoeba <sup>c</sup>
Untreated	Yes	Yes
1	No	Yes
2	No	No
5	No	No
10	No	No

<sup>a</sup>Biofilms were processed with the indicated monochloramine doses and kept 30 days in the resting medium.

<sup>b</sup>Yes, colonies were observed on BCYE- $\alpha$  agar; no, no culturable cells were obtained on BCYE- $\alpha$  medium.

<sup>c</sup>Bacteria from the biofilms were cocultivated with *Acanthamoeba castellanii* for 3 days at 37°C.

induce VBNC cell formation. These nonculturable *L. pneumophila* are able to recover their culturability after amoeba infection. These organisms are commonly found in water systems, suggesting that our observations should be effective in the natural environment. We have treated *L. pneumophila* biofilms only for short periods. It will be necessary to investigate continuous treatments with low monochloramine doses as is recommended for municipal drinking water (9) to know if these conditions also produce VBNC *L. pneumophila*.

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