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Assessment of viable but non-cultivable cells of *Legionella pneumophila* after chlorination

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Legionella pneumophila is a waterborne bacterial pathogen that can cause death worldwide. In the present study it is demonstrated that after disinfection, with chlorine at different concentrations, bacteria that are no longer detectable using standard culture techniques may still be viable.

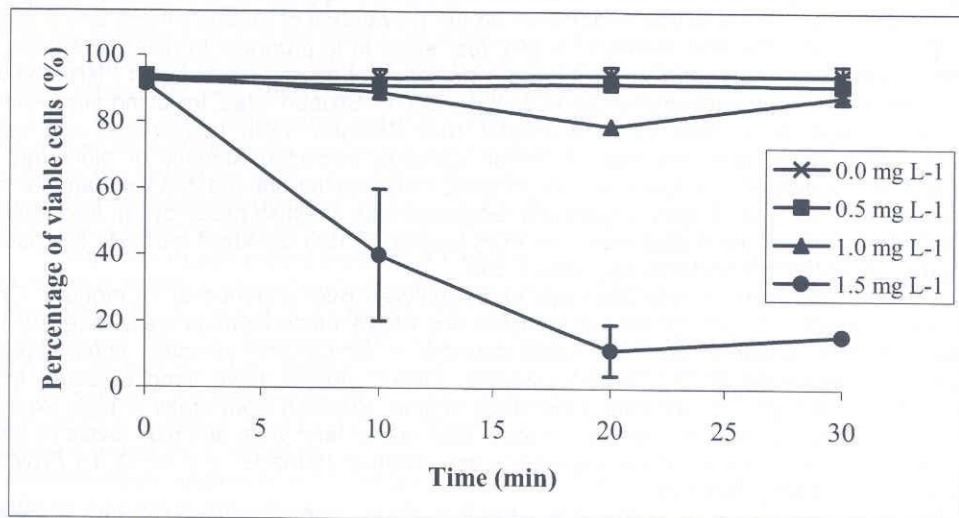
A cell suspension of *Legionella pneumophila* NCTC 12821 was prepared in dechlorinated and filter-sterilised drinking water. Chlorine was added (at final concentrations of 0.5, 1.0 and 1.5mg/L) and samples were taken after 0, 10, 20 and 30min for cell quantification by culture on Buffered Charcoal Yeast Extract (BCYE) agar plates and viability assessment using the BacLight[®] kit.

In the control assay, where no chlorine was added, there was no observed loss of cultivability and the number of live and dead cells, as indicated by the SYTO-9/Propidium Iodide (PI) double staining method, remained constant with time. Similar results were obtained in the assay where 0.5mg/L chlorine was added (the

percentage of viable cells remained around 90%). However, when the chlorine concentration was increased up to 1.0mg/L and 1.5mg/L there was a rapid loss of cultivability although it was possible to detect live cells by the SYTO-9/PI method. For example, after 30min contact time with a chlorine concentration of 1.5mg/L, 15% of the cells stained green but not red indicating that their cytoplasmic membrane remained intact (figure).

While cultivability using standard plating techniques has been used for a long time as the gold-standard to assess the survival of a microorganism in a given environment, the development of new biochemical methods to evaluate the same parameter has questioned the ability of a single cell to divide in artificial nutrient media as a suitable indication of the cell's life and death. The notion of viable but non-cultivable (VBNC) has subsequently arisen to describe organisms that are not cultivable at a given time or condition, but may revert to a state of cultivability later or under different circumstances. For *Legionella pneumophila*, SYTO-9/PI results demonstrated that, even after completely losing the cultivability on BCYE agar medium, there was no total loss of membrane integrity since there are still green cells remaining in the suspension excluding PI.

Earlier studies had already shown that concentrations of chlorine of 1.0mg/L (or even lower) could cause a rapid loss of *L pneumophila* cultivability. Results obtained in this work were in agreement with these studies; however, they also demonstrated that, even after completely losing the cultivability, it is possible to find live cells in drinking water in the VBNC state. When bacteria are pathogenic, such as *Legionella pneumophila*, the VBNC state can result in serious public health problems. Therefore, with this work it has been shown that cultivability is not the best method to analyse the water after disinfection. This brings new concerns about the necessity of developing new methods to detect pathogens in water after disinfection to ensure that there are no viable cells, guaranteeing a safe distribution of drinking water.



Variation in the percentage of *Legionella pneumophila* NCTC 12821 viability with time after exposure to different concentrations of chlorine