Research Paper

Chlorine disinfection against *Legionella pneumophila* biofilms

Abdelwahid Assaidi, Mostafa Ellouali, Hassan Latrache, Hafida Zahir, Abdelaziz Karoumi and El Mostafa Mliji

ABSTRACT

Premise plumbing promotes the regrowth and survival of opportunistic pathogens, such as *Legionella pneumophila* (*L. pneumophila*), especially within biofilms. The purpose of this study was to investigate the effectiveness of chlorine disinfection against *L. pneumophila* serogroup 1 and serogroup 2–15 planktonic form and biofilms. Planktonic cells were able to survive during the study period in the presence of chlorine at recommended free chlorine levels (0.2–0.5 mg/L). Biofilms were developed on galvanized steel and polyvinyl chloride (PVC) for 18 and 30 days and exposed to 50, 100, 150, and 200 mg/L for 2 hours. No colony appeared immediately after chlorination; however, persistent cells were able to tolerate treatment and continue to grow on subsequent days. The biofilm formation was evaluated by atomic force microscopy. This study demonstrates that the biofilm formed on the surfaces of plumbing materials increases bacterial resistance against high levels of chlorination. A new approach towards monitoring and eradicating *L. pneumophila* from water systems is required.

Key words | biofilm, chlorine disinfection, *Legionella pneumophila*, water distribution networks

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HIGHLIGHTS

- Effect of chlorine against *Legionella pneumophila* biofilm was investigated, results confirmed using atomic force microscopy.
- Resistance of planktonic cells at recommended chlorine levels (0.2–0.5 mg L^{-1}).
- Biofilm formed on the surfaces of plumbing materials increase bacterial resistance against high levels of chlorination.

INTRODUCTION

Legionella bacteria have been known to cause Legionnaires' diseases (severe form of pneumonia) and Pontiac fever (a severe influenza-like illness) (Kim *et al.* 2002). Infection is commonly acquired by inhalation of aerosol originating from contaminated water systems such as cooling towers and hot-water networks of hospitals, hotels, cruise ships, industrial facilities, and family residences (Carratala & Garcia-Vidal 2010; Cooper & Hanlon 2010). *Legionella* are found normally attached to water systems surfaces, associated with other bacteria in an extracellular anionic matrix known as biofilm, and survive as an intracellular parasite of free-living amoebae. Several authors have reported that

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adopting this lifestyle, *Legionella*, like other bacteria, become more resistant to environmental stress and less susceptible to any treatment or disinfection (Assaidi *et al.* 2018a; Shaheen *et al.* 2019). Contamination of water systems occurs when opportunistic pathogens are released from a biofilm as a consequence of physical disturbance or active detachment of infectious cells, which then pose a potential threat to human health (Flemming *et al.* 2002). It has been shown that about 95% of all microbial cells present in water distribution networks exist as biofilms on plumbing material surfaces and only 5% occur in the water phase (Szewzyk *et al.* 2000; Flemming *et al.* 2002). Similarly, in a domestic hot water network, most of the culturable bacteria (72%) were found to be surface-associated (Bagh *et al.* 2004).

The characteristics of the plumbing materials may greatly influence the cell density and biofilm proliferation in water distribution systems (Assaidi *et al.* 2018a). It is known that plastic surfaces leach biodegradable organic compounds (plasticizers, antioxidants, lubricants, heat stabilizers) supplying additional nutrients to the biofilm organisms (van der Kooij *et al.* 2005). Moreover, steel materials (galvanized steel as an example) present a high roughness which provides the possibility of *Legionella* adhesion and biofilm formation (Assaidi *et al.* 2018b). The absence of biofilm formation was identified on copper surfaces; this is due to the bactericidal effect of copper ions (Assaidi *et al.* 2018a, 2018b). Copper temporarily limits the growth of *Legionella* biofilms, then the concentrations strongly increase after 200 days (Lehtola *et al.* 2004; van der Kooij *et al.* 2005).

There are a variety of disinfection methods involving physical (e.g. membrane filtration), thermal (e.g. heat pasteurization) and chemical (e.g. chlorination) means. Physical methods, such as membrane filtration, have not been widely used (Kim *et al.* 2002). For the disinfection of drinking water, chemical methods using disinfectants (with chlorine being the most popular) have been the most widely used. Two of the most common disinfection techniques used worldwide against *Legionella* are chlorination and thermal treatments. Residual chlorine is frequently used at a low concentration (0.2–0.5 mg/L) as a secondary disinfectant for the maintenance of water quality in distribution networks, or at higher concentrations as a systems disinfection treatment called hyperchlorination (Deborde & Von Gunten 2008; Cooper & Hanlon 2010). This process is usually effective just for short periods of time (Szewzyk *et al.* 2000; Garcia *et al.* 2007). In the case of thermal treatments, as recommended by the World Health Organization (WHO), water circulation temperature is kept at a minimum of 60 °C when leaving the heating unit and at least 50 °C when it reaches the tap (Bartram 2007). However, these temperatures have been shown to be insufficient to control *Legionella* proliferation in the hot water systems of several buildings (Mouchtouri *et al.* 2007; Serrano-Suarez *et al.* 2013). Many authors have tested the response of the *Legionella* in the presence of free chlorine with a concentration of 2 mg/L; but have not observed a significant reduction in the number of cells (Cooper & Hanlon 2010; Bodet *et al.* 2012). Several other studies have been conducted on the success of different methods for removing *Legionella* bacteria from internal plumbing systems (Stout & Yu 2003; Chen *et al.* 2005; Declerck *et al.* 2007; Zhang & Kuspa 2009; Lin *et al.* 2011). Opinions on the effectiveness of chlorine shock and other methods for the removal of these bacteria differ (Goldstone *et al.* 2012). Since most of the removal procedures are often unsuccessful, it is important to determine the reasons for the frequent appearance of these bacterial species in certain environments. The aim of the present study was to evaluate the effectiveness of chlorine against *L. pneumophila* serogroup (sg) 1 and *L. pneumophila* serogroup sg 2–15 planktonic form and biofilms formed on the surface of galvanized steel and PVC; two plumbing materials commonly used in water distribution systems in Morocco (Assaidi *et al.* 2018b).

MATERIAL AND METHODS

Bacterial strains, growth conditions and preparation of bacterial suspension

The strains used in this study were *L. pneumophila serogroup 1* and *L. pneumophila serogroup 2–15*, obtained as described previously (Assaidi *et al.* 2018b). *L. pneumophila* strains were cultured in Glycine-Vancomycin-Polymyxin-Cycloheximide (GVPC) at 37 °C ± CO_2 (2.5%) for 72 h. After culture, the cells were harvested by centrifugation for

15 min at 8,400 g and were washed twice and resuspended in KNO_3 solution with ionic strength 0.1 M.

Free chlorine effect on planktonic cells

Five different chlorine solutions were prepared to determine the response of *L. pneumophila* planktonic cells to residual chlorine, ranging from 0 to 0.5 mg/L. Yeast extract broth was prepared in 10 mL volume. All test liquids were inoculated with 100 µL of the test suspension (10^6 cfu/mL), sealed, and incubated at 37 °C for 72 h. For each test, 1 mL aliquots were removed at days 3, 6, 12, 18, 24, and 30. Subject to five-fold serial dilution, 100 µL was inoculated onto GVPC agar, which was incubated at 37 °C for 72 h. The analysis was performed in triplicate.

Biofilm growth experiments

The selected supports for this study were galvanized steel and PVC; plumbing materials commonly used in water distribution systems in Morocco (Assaidi *et al.* 2018a). Sections of each material were cut into 1 cm^2 and were cleaned as previously outlined (Assaidi *et al.* 2018b). For each test substratum, galvanized steel and PVC coupons were added to each well of a 6-well plate, and 3 mL of bacterial suspension containing 10^6 cfu/mL was added to each well in order to immerse the coupons. The plates were left at room temperature for 3 h in order to promote adhesion of the bacterial cells on the coupons. The coupons were then aseptically transferred into new sterile 6-well plates and were rinsed three times with sterilized distilled water to remove the non-adhering cells. Each coupon was then transferred to another sterile well and covered with 3 mL BYE plus L-cysteine. The plates were sealed with parafilm and incubated at 37 °C with BYE being replaced at 2- or 3-day intervals.

Biofilm development was followed over a period of 45 days, based on the determination of total cell counts. CFUs were counted by using the serial dilution technique of the bacterial suspension obtained after sonication. Counts were determined on GVPC after incubation at 37 °C (2.5% CO_2) for 72 h.

Hyperchlorination efficacy

Chlorine disinfection was performed after 18 and 30 days for the biofilm previously developed on each coupon. One milliliter of the appropriate chlorine solution (50, 100, 150, and 200 mg/L) was added to each well and left for 2 h at room temperature. After treatment, each coupon was placed into 3 mL of a 2 mg/L sodium thiosulphate solution in order to neutralize any residual chlorine and left for 3 min at room temperature. Each coupon was then transferred to another sterile 6-well plate, immersed in 3 mL of KON_3 (0.1 M), and agitated gently at 20 rpm for 3 min to remove any non-adherent bacteria as well as residual chlorine or sodium thiosulphate. Finally, the coupons were transferred into new sterile 6-well plates containing 3 mL BYE in each well; the plates were sealed with parafilm and incubated at 37 °C. The number of colony forming units was determined at days 3, 6, 12, 18, 24, 30, and 45.

Atomic force microscopy

Atomic force microscopy was used to perform quantitative measurements of surface topography. Before fixation, the coupons of plumbing materials were removed from the non-adherent cells by gentle rinsing in PBS. The biofilms were fixed with ethanol/acetic acid (3:1) for 10 min (Chao & Zhang 2011; Assaidi *et al.* 2018a). AFM images were acquired by an easy scan2 controller from Nanosurf. For scanning, the tapping mode in air was used under ambient conditions.

RESULTS

Efficacy of chlorine on planktonic cells

The ability of planktonic cultures of *L. pneumophila* to survive chlorine treatment at the levels similar to those used in potable water systems ranging from 0.2 to 0.5 mg/L was investigated.

Figures 1 and 2 show that *L. pneumophila* sg 1 and *L. pneumophila* sg 2–15 were able to survive and persist in BYE with an increasing number of 10^5–10^6 cfu/mL. Colony-forming units were detectable throughout the

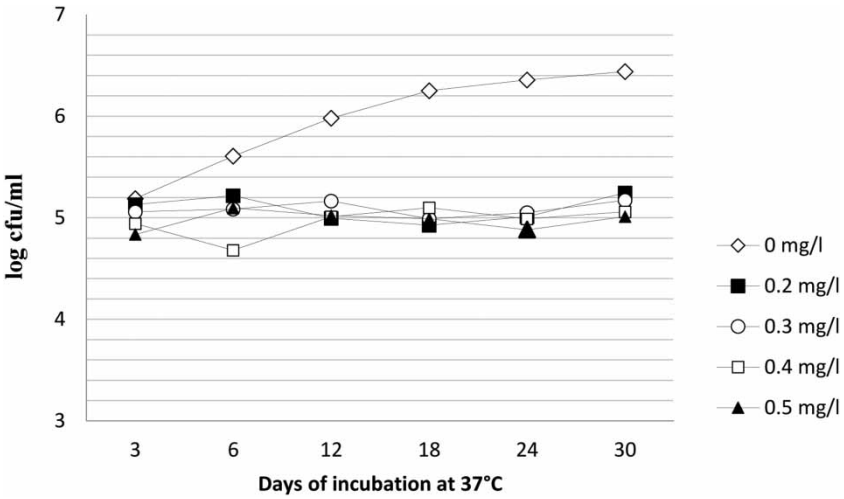

Figure 1 | The survival of *L. pneumophila* sg 2–15 in yeast extract broth (BYE) in the presence of chlorine.

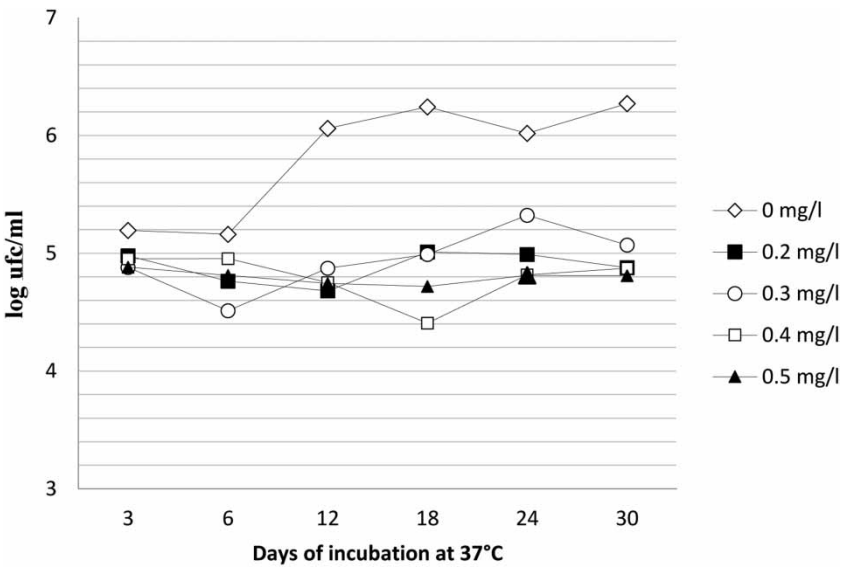

Figure 2 | The survival of *L. pneumophila* sg 1 in yeast extract broth (BYE) in the presence of chlorine.

experiment period in BYE supplemented with chlorine (0.2–0.5 mg/L) at 37 °C. These results suggest that the efficiency of these concentrations was not sufficient to reduce the numbers of *Legionella* that may occasionally survive in waters that have been judged to be microbiologically acceptable.

Efficacy of chlorine on biofilms

Biofilm was grown on coupons of galvanized steel and PVC; these two materials were rapidly biofouled and colonized by *L. pneumophila* as described previously (Assaidi *et al.* 2018a), and the chlorination was performed at day 18 and 30 to examine the effect of chlorine on 18- and 30-day *L. pneumophila* biofilms. Figures 3 and 4 show the growth of *L. pneumophila* sg 1 and *L. pneumophila* sg 2–15 biofilms on galvanized steel and PVC challenged with a range of concentrations at days 18 and 30. The number of cells forming biofilm increased rapidly on the surface of untreated coupons. Galvanized steel was the most colonized and supported biofilms which contained 9.81×10^6 CFU per coupon for *L. pneumophila* sg 1 and 1.25×10^7 CFU per

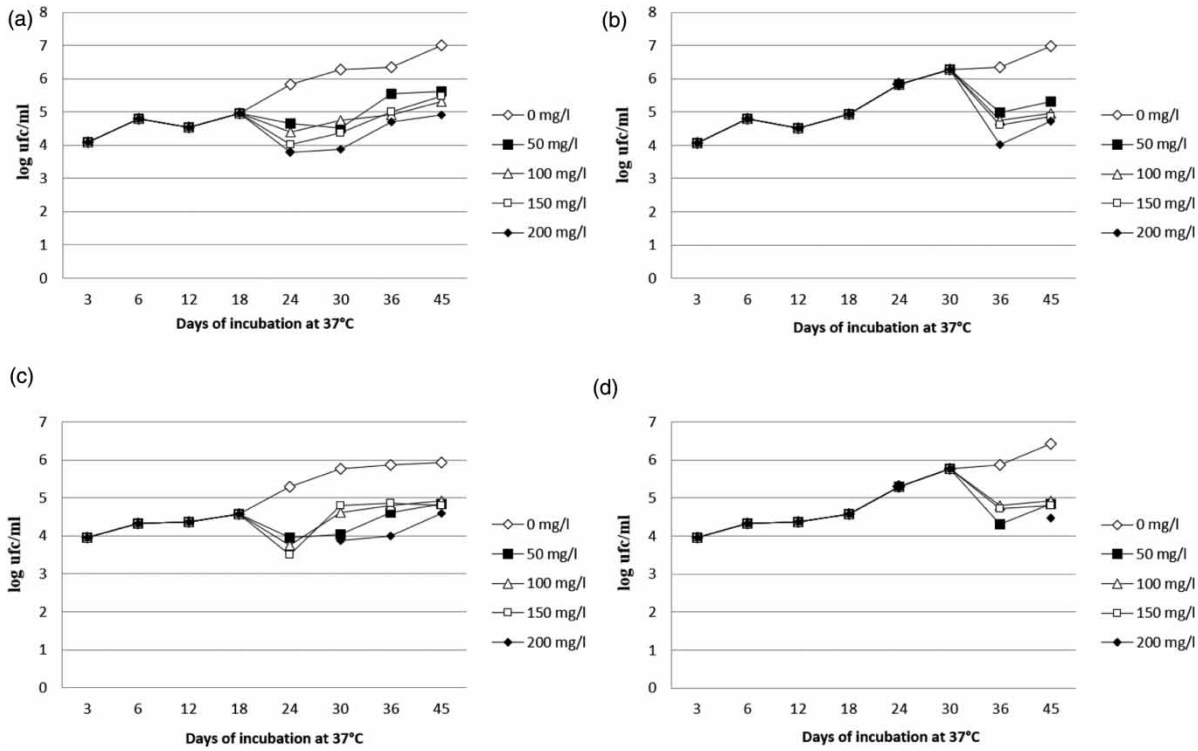

Figure 3 | The growth of *L. pneumophila* sg 1 on galvanized steel (a) and (b) and PVC (c) and (d) when challenged with a range of concentrations of chlorine at day 18 (a) and (c) and day 30 (b) and (d).

coupon for *L. pneumophila* sg 2–15. The results revealed that over the duration of the experiment (45 days), the biofilms of the two strains were developed at approximately the same rate on galvanized steel and PVC. Untreated biofilms exceed 7 $\log_{10}$ (CFU/cm^2) and 6 $\log_{10}$ (CFU/cm^2) on galvanized steel and PVC. The biofilms of *L. pneumophila* sg 1 and *L. pneumophila* sg 2–15 decreased in the number of colony forming units after exposure to chlorine at different concentrations. The more the concentration increases, the more the number of cells forming the biofilm decreases (Figure 4(a) and 4(b), respectively). However, for the two *L. pneumophila* strains, no colony was detected immediately after treatment.

For biofilms of 18-day *L. pneumophila* sg 1 (Figure 3(a)–3(c)), the greatest reduction rate was observed at 100 mg/L (1.5 $\log_{10}$), while for *L. pneumophila* sg 2–15 (Figure 4(a)–4(c)) this rate is 2.8 $\log_{10}$ after exposure to 150 mg/L.

Figures 3(b)–3(d) and 4(b)–4(d) show the response of *L. pneumophila* sg 1 and *L. pneumophila* sg 2–15 to chlorination at 50, 100, 150 and 200 mg/L on 30-days of incubation, respectively. After an initial drop in the number of viable cells, the biofilms became 100 times smaller than the negative controls. The number of cells forming biofilm exposed to chlorine reached 5.8 $\log_{10}$ (CFU/cm^2) for *L. pneumophila* sg 2–15 and 5.6 $\log_{10}$ (CFU/cm^2) for *L. pneumophila* sg 1 on galvanized steel, compared to 9.81×10^6 CFU/mL for *L. pneumophila* sg 1 and 1.25×10^7 CFU/mL for *L. pneumophila* sg 2–15 on untreated materials, indicating a measurable decrease in the number of detectable CFUs, resulting directly from the application of chlorine. For the 30-day biofilms of *L. pneumophila* sg 1, the greatest log reduction rate was observed after exposure to 100 mg/L (0.1). For *L. pneumophila* sg 2–15, the greatest reduction rate was also observed after exposure to 100 mg/L (0.6). As with the 18-day biofilms, the chlorine did not eradicate all the biofilm cells, rather they continued to grow, but at levels lower than the unchlorinated control.

Detectable colonies were observed 6 days after chlorination. The number of CFUs recovered during the rest of the experiment from biofilms exposed to the different

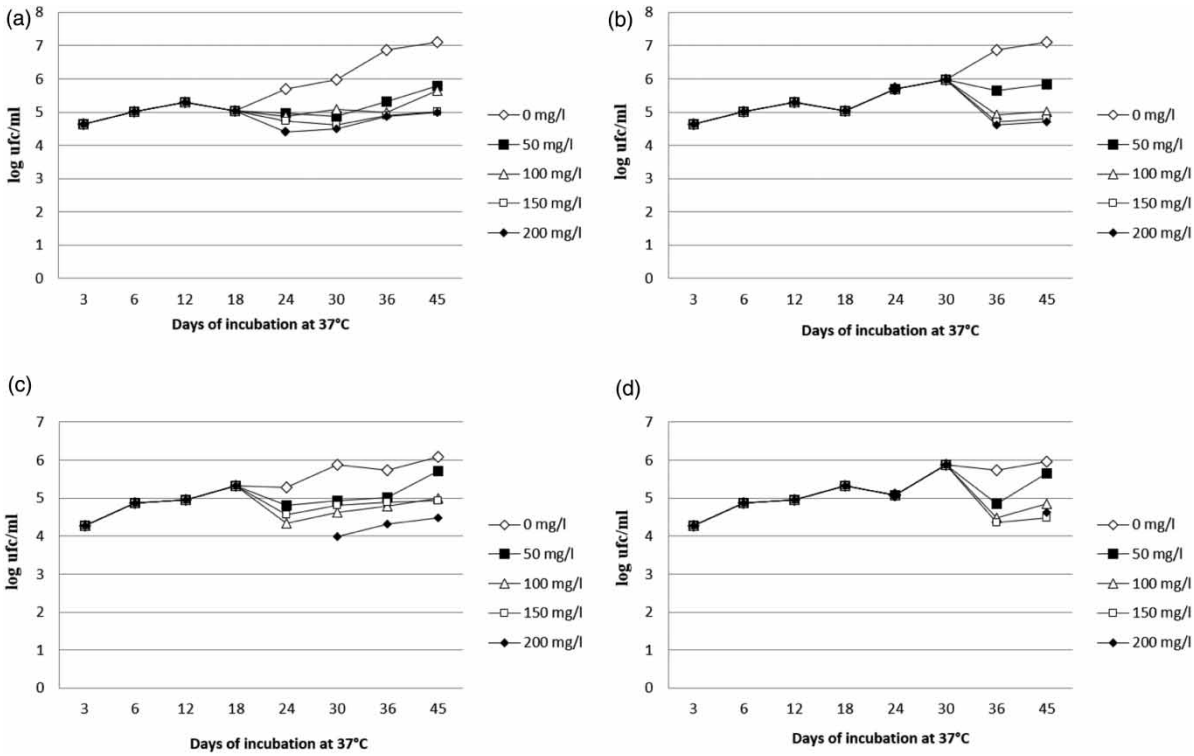

Figure 4 | The growth of *L. pneumophila* sg 2–15 on galvanized steel (a) and (b) and PVC (c) and (d) when challenged with a range of concentrations of chlorine at day 18 (a) and (c) and day 30 (b) and (d).

chlorine levels did not reach the magnitude of the non-chlorinated control biofilms, although the number of all chlorinated biofilms increased in subsequent days.

DISCUSSION

During the past several years, *Legionella* has been isolated from showerheads, taps, hot water systems of hotels, hospitals, and homes. In a number of cases, the occurrence of *Legionella* in plumbing systems was associated with Legionnaires' disease. Despite increased monitoring and advances in detection methods, there is still a lack of knowledge about the microbial ecology of *Legionella* and its response to treatment. Therefore, contamination of hot water systems with *Legionella* remains a persistent environmental challenge and a threat to public health.

In order to improve the efficiency of water treatment, the effect of chlorine disinfection against *L. pneumophila* in controlled conditions was examined. Steel and plastics are becoming the dominant materials frequently used as a conveyance device for potable water in new buildings. Based on the results obtained in this study, galvanized steel and PVC provide favorable conditions for *L. pneumophila* adhesion and biofilm growth. Chlorine disinfection against *L. pneumophila* planktonic form and biofilms revealed an important persistence of up to four times the recommended hyperchlorination levels. In addition, our results indicate that 18 and 30 days are sufficient for *L. pneumophila* to persist to chlorination treatment (50 mg L^{-1}). These findings would explain the survival and persistence of *L. pneumophila* in drinking water systems despite repeated cycles of chlorination.

Various disinfectants (chlorine, monochloramine, etc.) and physical treatments (heat, UV, etc.) are used in water systems to control *Legionella* colonization (Kim *et al.* 2002). Several disinfection studies have been performed on *Legionella* (Kim *et al.* 2002; Campos *et al.* 2003). Hyperchlorination of potable water is usually performed by the use of such chemical agents as sodium hypochlorite and is

recommended for the treatment of water distribution systems at residential facilities. This approach is appropriate for the treatment and removal of planktonic cells of *L. pneumophila*, but remains ineffective against biofilms (Figure 5). In the case of treatment failure, *L. pneumophila* may be able to recolonize water systems (Figures 3 and 4). It has been hypothesized that this recolonization is made possible because *Legionella* is protected in the biofilm or in amoebae (Barker *et al.* 1992; Murga *et al.* 2001; Donlan *et al.* 2005).

Hyperchlorination, frequently employed as a remedial treatment option, typically consists of a pulse injection of chlorine in water to achieve 20–50 mg L^{-1} of free chlorine in the system during a short period of time (Campos *et al.* 2003).

Preventive measures including chlorination have been used to prevent the colonization and proliferation of bacteria in water networks. Environmental strains may have been exposed to repeated cycles of chlorination, which may have led to the development of a mechanism of resistance to chlorine disinfection treatments. The results obtained from this study provide an overview of resistance to chlorination of *L. pneumophila* strains, where exposure to chlorine at regular intervals has facilitated greater tolerance to the disinfectant.

This study was conducted in laboratory conditions using a rich medium, the biofilm was formed and treated in batch systems not a continuous flow/circuit, however, studying the response of bacteria to disinfection in laboratory conditions is essential in order to perform several testing and controlling conditions, then, moving to a continuous system and representing real conditions of premise plumbing systems to applicate and disinfect on a high level. Moreover, caution should be taken when interpreting the results. It should be considered that the experience conducted in laboratory conditions sometimes poorly correlates with continuous systems, as the physicochemical of pipes' materials surfaces, temperature, presence of other microbes and nutrients can affect or protect the bacteria from an otherwise effective treatment.

The percentage of the inhibition and reduction (chlorine effect) of *Legionella* biomass on materials surfaces is the same for galvanized steel and PVC, which suggests that the choice of materials is essential and fundamental to control the biofilm formation. Once adhered, *Legionella* strains

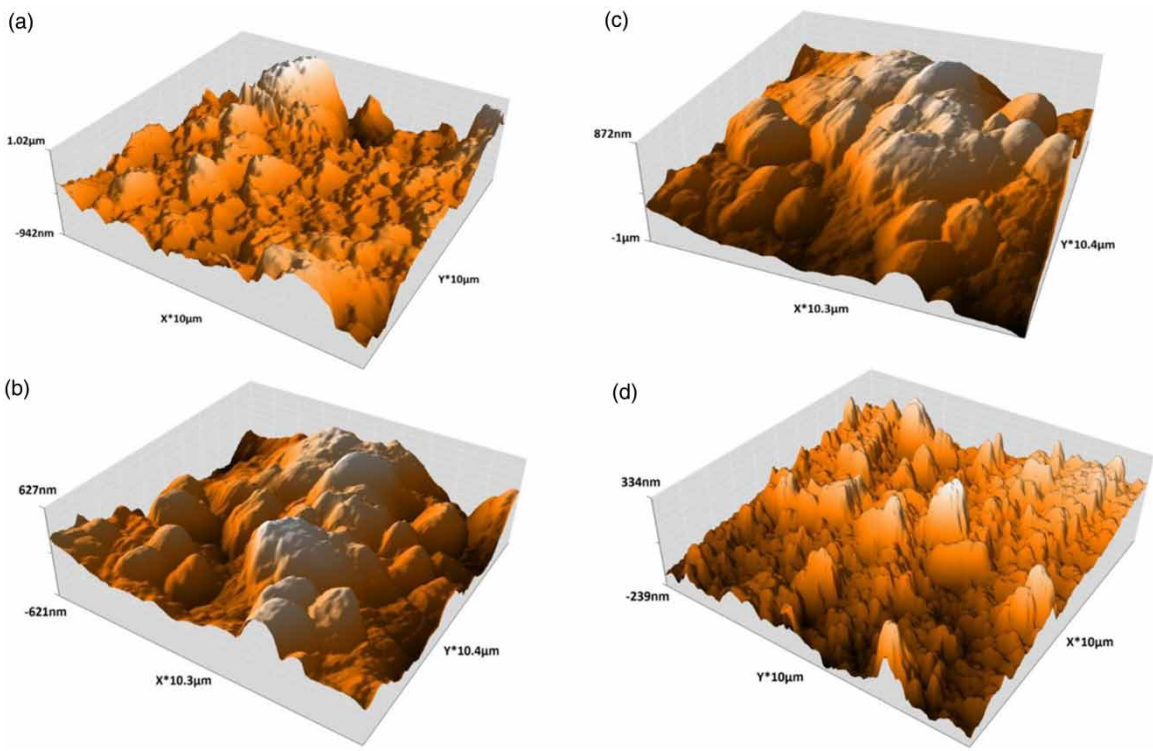

Figure 5 | Atomic force microscopy 3-dimensional images of 30-day biofilm of *L. pneumophila* sg 2–15 grown on galvanized steel (a) and (b) and PVC (c) and (d) after (a) and (c) and before (b) and (d) chlorination.

have the ability to form biofilm, which makes its eradication difficult despite a repetitive cycle of treatment (Figure 5). Furthermore, municipal water providers and consumers alike should take into consideration the risk of *Legionella* in water systems and select the most hygienic materials that limit bacterial adhesion and biofilm formation. These findings could have an important implication for the water in residential buildings and industry, which consider chlorine as an effective disinfectant. Therefore, the introduction of new approaches of disinfection and treatment is unavoidable to prevent biofilm formation and water contamination, as well as minimizing the risk of *Legionella* infections.

CONCLUSIONS

This study provides a better understanding of *L. pneumophila* survival within premise plumbing biofilms and demonstrates that the biofilm formed in the surfaces of plumbing materials increase bacterial persistence against high levels of chlorination. This persistence among *L. pneumophila* planktonic form and biofilms was higher, therefore demonstrating that the recommended levels of chlorine disinfection are not appropriate for removing these pathogens from water networks. Understanding these mechanisms is essential to find ways not only to prevent biofilm formation and water contamination, but also to minimize the risk of Legionnaires' disease.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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