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Inactivation of *Pseudomonas aeruginosa* Mature Biofilm by Monochloramine

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Abstract: Monochloramine has been recognized for its capacity to penetrate biofilms better than other oxidizing agents. In this study, the action of monochloramine on mature biofilms of *Pseudomonas aeruginosa* developed *in vitro* on different plumbing materials (polypropylene, polyethylene, electron-ray cross-linked polyethylene reticulate, PVC, copper, stainless steel, and Teflon) was investigated. Its biofilm viability effectiveness was assessed using fluorescence microscopy and confocal microscopy with LIVE/DEAD BacLight. Concentrations of 1 ppm and 2 ppm eradicated the biofilm after 3 h, whereas a lower contact time or a lower concentration (0.5 ppm) had little effect. The results showed that monochloramine was very effective against mature biofilms of *P. aeruginosa*. Moreover, concentration and contact time greatly affected its activity.

Keywords: *Pseudomonas aeruginosa*; monochloramine; biofilm; plumbing materials; confocal microscopy; LIVE/DEAD BacLight

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1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen widely recovered from the environment and recognized for its adhesiveness and capacity to form biofilms [1, 2]. Bacterial biofilm confers cell protection against hostile environments, and it is a strategy for microorganisms to colonize, adapt and survive in different and changing habitats [3]. Moreover, *P. aeruginosa* biofilm is able to antagonize the presence of other bacterial species, such as *Legionella pneumophila*, through the production of bacteriocins or the release of homoserine lactone quorum sensing molecules [4, 5]. The composition is the basis of the peculiarities of the biofilms produced by these bacteria. Indeed, surface colonization is linked to extracellular polysaccharide production, composed of polysaccharides, nucleic acids, and proteins, which allow cell-to-surface adhesion [6, 7].

Moreover, microelements such as calcium, magnesium, and iron have been reported to influence the attachment of *Pseudomonas* to solid surfaces [6]. Biofilms can tolerate various antibiotics and disinfectants more than their planktonic counterparts [8-11]. For these reasons, *P. aeruginosa* is responsible for a wide spectrum of human infections, especially in patients with compromised host defense mechanisms. Moreover, infections occur mainly in healthcare settings, especially in critical care units [12, 13], and they can also be acquired from different water reservoirs (i.e., faucets, sinks, and wastewater drainage systems) [14, 15]. Indeed,

hospital water is one of the reservoirs of healthcare-associated pathogens most dangerous for outbreaks [16, 17], especially in immunocompromised patients [18]. In this context, bacterial eradications are particularly challenging [19].

From this perspective, biofilms spread from the sink trap to the skin of patients or can transiently contaminate the hands of healthcare workers, who can then contaminate the patients [20-23]. Furthermore, the difficulty of eradicating these strains from water reservoirs lies precisely in the characteristics of the biofilm matrix: with the result that translates into greater resistance due to the chemical and mechanical protections provided by this matrix and also due to the physiological modification of bacteria in these conditions [24-26]. For these reasons, new strategies have been developed in recent years to enhance the efficiency and effectiveness of common water disinfection methods and reduce the consumption of chemicals [27, 28].

At present, chlorine is the most commonly used disinfectant for drinking water, but monochloramine (MC), produced by the reaction of free chlorine and ammonia, can be used as an alternative to free chlorine, mainly because it penetrates biofilm better [29-32] has a higher stability. It does not react readily with natural organic matter, thus producing fewer disinfection by-products [33].

As far as we know, there is little research on the effectiveness of MC against biofilm produced by *P. aeruginosa*. For this reason, the present study aimed to verify the effectiveness of MC in inactivating *P. aeruginosa* biofilm developed under laboratory conditions onto the most commonly used plumbing materials.

2. Materials and Methods

2.1. Plumbing materials.

Seven different materials in the form of coupons (26 mm×76 mm) were employed for the biofilm production: copper, polyethylene (PE), polypropylene (PP), electron-ray cross-linked polyethylene reticulate (PE-Xc), PVC, stainless steel, and Teflon. Before use, each coupon was treated with 70% (v/v) ethanol for 10 min, washed in deionized water, and air-dried for 24 h.

2.2. Preparation of the bacterial strain.

P. aeruginosa ATCC 15422 strain was grown at 30°C in Tryptic Soy Broth (TSB, Sigma), harvested by centrifugation (20 min, 1000×g), washed twice in 20 mL of filter-sterilized tap water, and then suspended in 200 mL of filter-sterilized tap water. The final bacterial concentration was equal to 3×10^5 cells/mL. Suspensions were incubated statically at 30°C for 24 h.

2.3. Cultivation and inoculation of drinking water biofilms.

Drinking water biofilms on different coupons were grown into a stainless-steel tank connected to a cold-water laboratory tap and perfused with drinking water at a flow rate of approximately 15 L/h. Preliminary investigations on the drinking water used for the experiments did not detect the presence of *P. aeruginosa* (data not shown). Therefore, after 14 days of perfusion, each coupon was transferred to a flow-through reactor. As described above, the reactor was filled with the suspension of P. aeruginosa prepared by injecting 100 mL of the bacterial suspension. After static incubation at room temperature for 24 h, the reactor was

connected to the cold-water laboratory tap and continuously perfused with drinking water for 4 weeks.

2.4. MC preparation and measurement.

MC stock solution was prepared in 5 mM pH 8.0-8.5 buffer by adding a calculated amount of sodium hypochlorite (ENOXIN P15 Plus, Sanipur S.p.A.) solution and ammonium sulfate solution (ZEBION P35DW, Sanipur S.p.A.) (100 mg N/L).

MC concentration was measured by colorimetric test kits (total chlorine, Hach-8167; monochloramine, Hach-10200) and a DR/900 spectrophotometer (Hach Co.). The MC stock solution was stored at 6°C in the dark before the experiments (maximum for 24 hours).

2.5. Observation of MC effect on P. aeruginosa biofilm.

For each assay, three different concentrations of MC (0.5, 1, and 2 ppm) were tested for 4 exposure times (30 min, 1, 2, and 3 h). Briefly, after 4 weeks, coupons were taken out of the reactor and put into 1000 mL of fresh MC at different concentrations (0.5 ppm, 1 ppm, and 2 ppm). The coupons at each MC concentration were tested in triplicate. The viable bacterial cells remaining on the coupon surfaces were assessed by fluorescent microscopy and confocal microscopy observation after biofilm staining with the LIVE/DEAD BacLight Bacterial Viability Kit (L13152, Molecular Probes, Inc., Eugene, OR).

The LIVE/DEAD BacLight Bacterial Viability Kit was useful for monitoring the viability of bacterial populations as a function of the membrane integrity of the cells. It utilizes mixtures of SYTO 9 green-fluorescent nucleic acid stain, which penetrates all bacterial cells, and fluorescent green, and propidium iodide, which penetrates only bacteria with damaged membranes resulting in red fluorescence. Thus, cells with an intact membrane stain green, whereas nonviable cells stain red. In brief, a 2X stock stain solution of the LIVE/DEAD BacLight staining reagent mixture was prepared by dissolving the contents of SYTO 9 dye and propidium iodide in 5 mL of filter-sterilized dH_2O. A volume of 300 µL of the stain solution was added to each biofilm sample. The stained biofilm samples were incubated in the dark for 15 min at room temperature.

Observations were performed before (T0) and after 30 min, 1h, 2h, and 3h of MC contact time. Fluorescent microcolonies in each biofilm were observed by fluorescence microscopy and confocal microscopy.

3. Results and Discussion

Except for copper (Figures 1a and 1b), biofilms grown on all the other coupons revealed very high concentrations of viable bacteria after 4 weeks without any significant difference among the different materials.

For this reason, copper coupons were not considered for the experiments. Viable *P. aeruginosa* biofilms were treated with MC doses of different concentrations and at different exposure times in three experiments for each coupon. Good reproducibility was obtained. No significant difference was observed in the effect of MC on biofilm grown on different materials. Here we showed the results of the experiments performed on Teflon coupons. Figure 2 showed biofilm grown on Teflon coupons after 4 weeks prior to the exposition to MC.

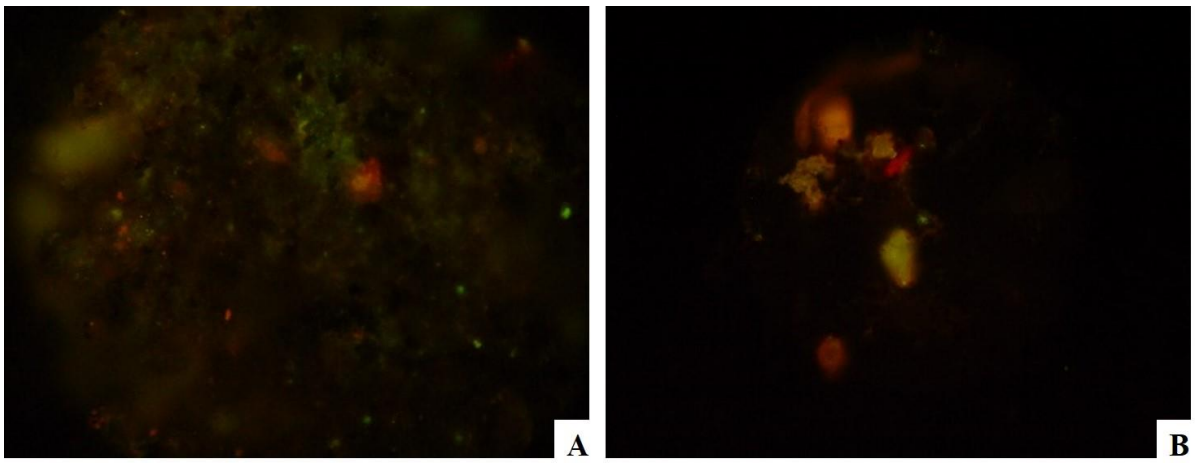

Figure 1. Biofilms developed on two different copper coupons (**a** and **b**). 2D observation by fluorescent microscopy.

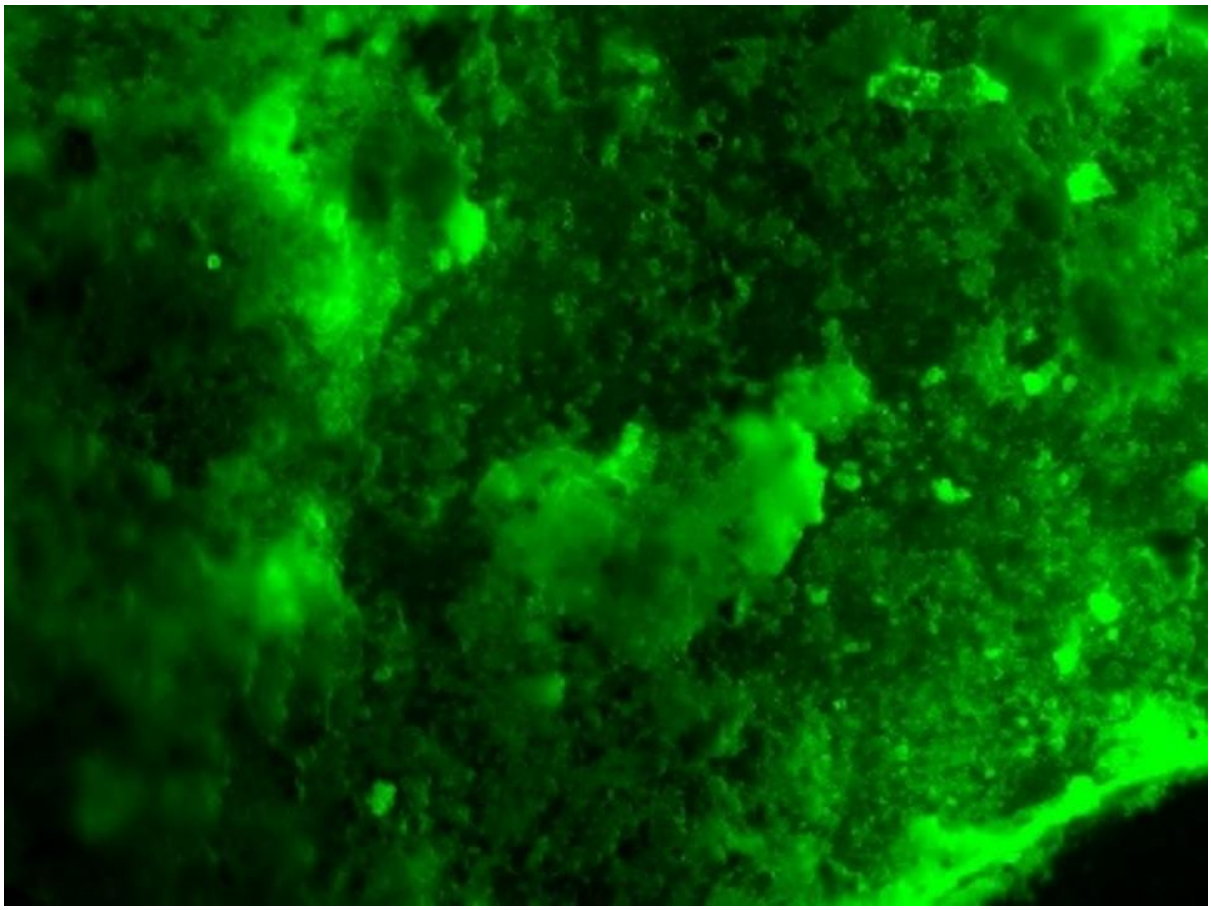

Figure 2. Pseudomonas aeruginosa biofilm developed on teflon coupons. 3D observation by confocal microscopy.

The effect of 0.5 ppm MC on biofilm developed on Teflon observed by fluorescent microscopy is shown in Figure 3. A modest inactivation effect was observed after 1 h (Figure 3a). The efficiency of inactivation increased with the increase of the contact time (Figure 3b) and was relatively higher after 3 h (Figure 3c).

An MC concentration of 1 ppm was significantly more effective than 0.5 ppm. Figure 4 illustrates the biofilm behavior after a 1 ppm MC dose observed by confocal microscopy. With this dosing protocol, a very high decrease in viable cells was observed 2 h after 1 ppm MC addition (Fig. 4a).

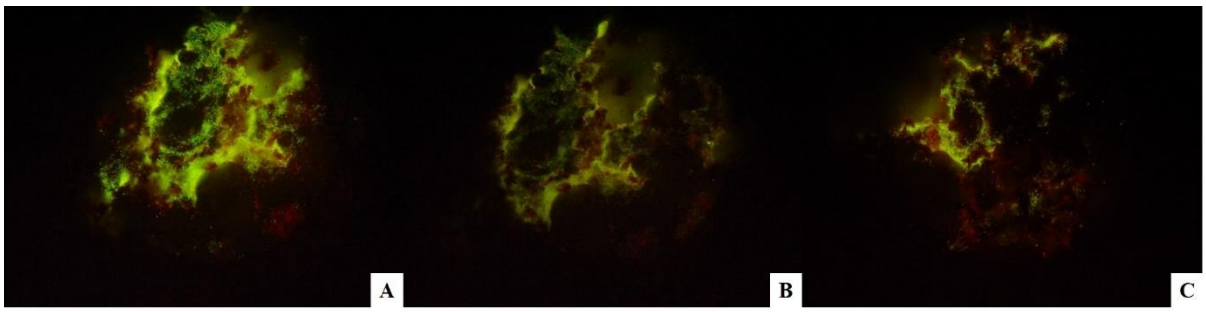

Figure 3. Effect on biofilm produced on Teflon coupons of 0.5 ppm MC after 1 h (**a**), 2 h (**b**), and 3 h (**c**). 2D observation by fluorescent microscopy.

Close examination of the treated biofilm showed that 2h after the MC release, *P. aeruginosa* cells were killed in the deeper layers of the biofilm (Fig. 4b). Nonetheless, the reduction in total viable cells was maximum after 3h (Fig. 4c and 4d). Thus, at 1 ppm MC as contact time increased, the viability partially decreased until the biofilm appeared completely nonviable at 3 h. Similar results were obtained with 2 ppm MC.

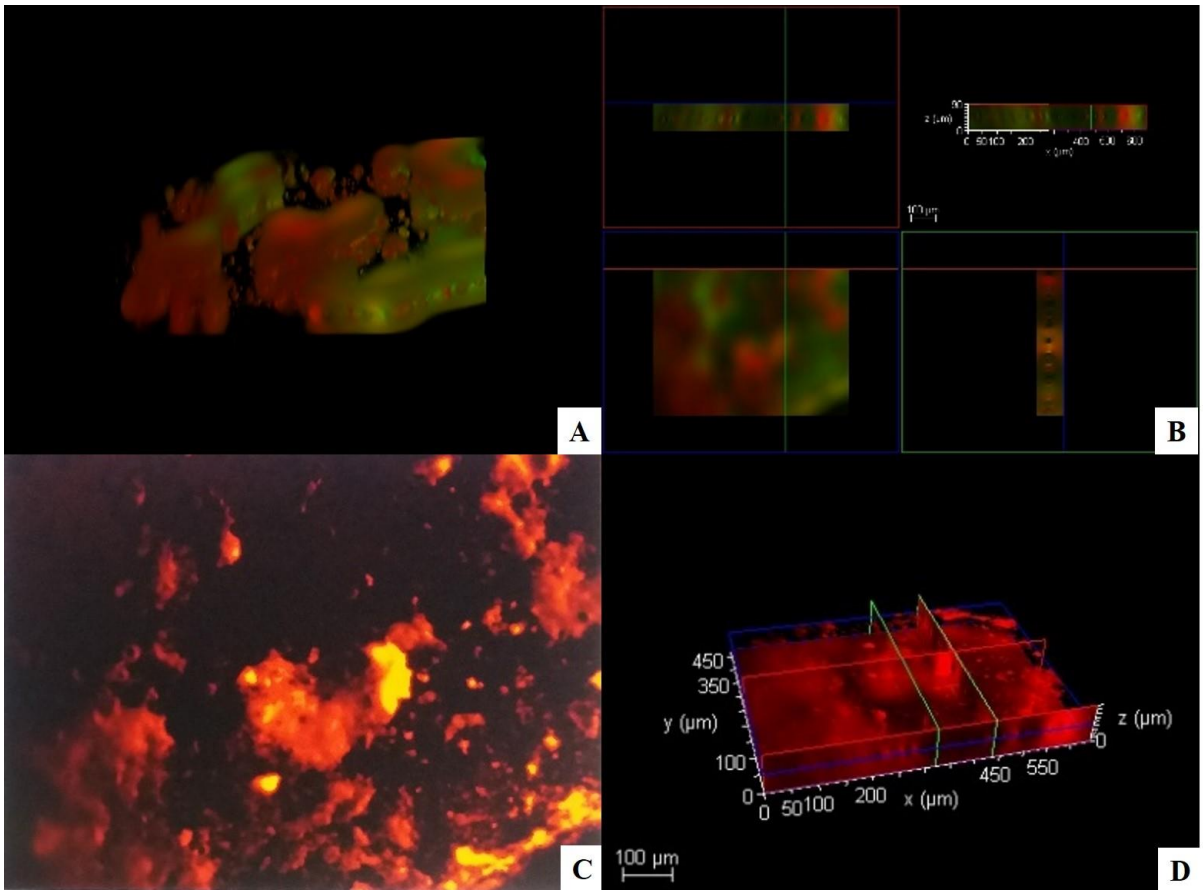

Figure 4. Effect against biofilm developed on Teflon coupons 2 h (**a** and **b**) and 3 h (**c**) after exposure to 1 ppm MC. 3D observation by confocal microscopy (**d**).

Finally, the investigation of cell viability in the planktonic phase showed a detachment of viable cells into the liquid medium after 3 h in the presence of 0,5 ppm MC (Fig. 5). The observation of the same coupon exposed to an MC concentration of 1 ppm and 2 ppm for 3 h showed no detachment of viable cells.

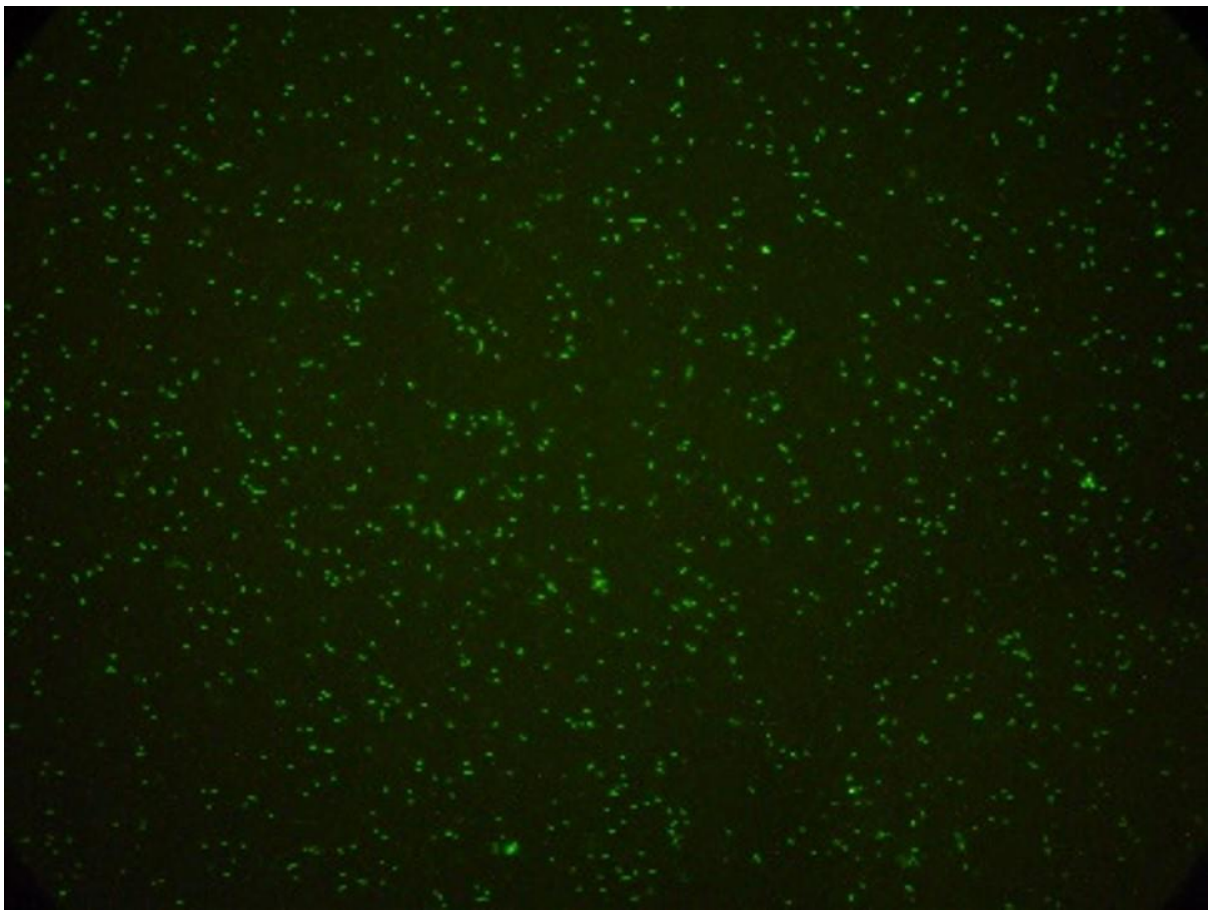

Figure 5. Effect on biofilm detachment of viable cells from Teflon coupons after 0.5 ppm MC exposure. 2D observation by fluorescent microscopy.

The aim of the present study was to investigate the effect of MC against *P. aeruginosa* drinking water biofilms grown on different plumbing materials under laboratory conditions. For this purpose, *P. aeruginosa* ATCC 15422 biofilms were allowed to form onto coupons of the most common materials used in the domestic pipelines in a tank under continuous flow-through of drinking water for 4 weeks.

The viability of *P. aeruginosa* biofilms on the different plumbing materials was determined before and after the treatment with different MC concentrations at different exposure times. Since chloramines in high doses have been found to cause blood damage and liver damage in laboratory animals [34, 35], the EPA has set the upper limit of the allowable usage of chloramines at 4 ppm. Anyway, for drinking water, disinfection is lower concentrations are frequently used (i.e., 1-2 ppm) [32]. For this reason, we decided to test a maximum MC concentration of 2 ppm in our experiments.

Biofilm formation based on cell viability with LIVE/DEAD BacLight before the MC release was very low on copper coupons. This was not surprising because it is well-known that the type and characteristics of pipe materials can influence the formation of microbial biofilms in drinking water systems [36]. In particular, biofilm development was dramatically affected by the presence of toxic metals like copper [37]. Therefore, copper coupons were not considered for the experiments.

Our data showed that as contact time with the disinfectant increased, the biofilm viability partially decreased until it appeared completely nonviable after 3 h at 1 ppm and 2 ppm.

Moreover, a very strong reduction of viable cells was observed after 2 h in the deeper layers of the biofilm. This could be because MC can penetrate biofilms quickly and deeply [30]. It has been seen that the ability of MC to penetrate biofilms better follows a dose-dependent effect [38, 39].

In our experiments, no difference was observed between 1 ppm and 2 ppm MC concentration, while a 0.5 ppm MC concentration had little effect. This could be due to the fact that only at concentrations as low as 1 ppm is the disinfectant able to penetrate biofilm matrixes [40]. Another possible explanation is that MC killed bacteria by breaking down cell membranes, but in mature biofilms, the cell lysis process was generally delayed [41]. Thus, a disinfectant concentration lower than 1 ppm probably was not sufficient to disrupt cell membranes.

Finally, measurements of cell viability of cells in the planktonic phase showed that exposure to 0.5 ppm MC for 3 h caused a detachment of *P. aeruginosa* viable cells into the liquid medium. This was not observed at higher MC concentrations, where biofilm was completely inactivated. As a possible explanation of this evidence, it could be argued that, as already written, MC penetrates complex biofilm matrixes starting from a concentration of 1 ppm [40]. Thus, it is possible that a 0.5 ppm MC concentration partially caused the biofilm detachment, but it could not kill the cells.

4. Conclusions

Although good reproducibility was obtained in our experiments, a major limitation of the present study was that biofilm growth techniques tend to differ between each research group, producing biofilms often dissimilar structurally and physiologically [42, 43]. This could prevent comparison among experiments assessing the effect of MC on *P. aeruginosa* biofilm using alternative growth techniques. As a consequence, a second limitation of the study was that biofilm should always be observed *in situ*. In our experiments, a confocal laser scanning microscope could have allowed the biofilm to be observed under flow conditions. Real-time observations would probably provide a greater understanding of the mechanisms of MC.

As far as we know, the effect of MC against biofilm produced by *P. aeruginosa* on the most common materials used for piping has not been published yet. Our results showed that MC is very effective against mature biofilms of *P. aeruginosa*, causing the reduction in viable and total cell areal densities without difference among different plumbing materials.

In our experiments, MC concentration and contact-time variables showed to be very important. Data demonstrated that 1 ppm and 2 ppm MC eradicated the biofilm after 3 h, whereas a lower contact time or a lower concentration (0.5 ppm) had little effect.

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Conflicts of Interest

The authors declare no conflict of interest.

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