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Influence of surface copper content on *Stenotrophomonas maltophilia* biofilm control using chlorine and mechanical stress

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ABSTRACT

This work aimed to evaluate the action of materials with different copper content (0, 57, 96 and 100%) on biofilm formation and control by chlorination and mechanical stress. *Stenotrophomonas maltophilia* isolated from drinking water was used as a model microorganism and biofilms were developed in a rotating cylinder reactor using realism-based shear stress conditions. Biofilms were characterized phenotypically and exposed to three control strategies: 10 mg l^{-1} of free chlorine for 10 min, an increased shear stress (a fluid velocity of 1.5 m s^{-1} for 30s), and a combination of both treatments. These shock treatments were not effective in biofilm control. The benefits from the use of copper surfaces was found essentially in reducing the numbers of non-damaged cells. Copper materials demonstrated better performance in biofilm prevention than chlorine. In general, copper alloys may have a positive public health impact by reducing the number of non-damaged cells in the water delivered after chlorine exposure.

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Introduction

The presence of microorganisms in biofilms formed along drinking water distribution systems (DWDS) is frequent and usually does not present a significant risk for consumers as long as pathogens are not entrapped in the biofilm and no critical cell release to the bulk phase happens. Biofilms in DWDS may harbor pathogens (WHO 2011; van der Wielen and van der Kooij 2013; Qin et al. 2017) that may be causative agents of waterborne diseases (Gulati and Ghosh 2017). Biofilm development in DWDS is dependent on several factors, particularly nutrient availability, temperature, the characteristics of the adhesion surface, hydrodynamic conditions and the presence/concentration of disinfectants (Inkinen et al. 2018). Materials typically used in DWDS, particularly in household and hospital plumbing, are comprised of plastics (for example, polyvinyl chloride-PVC, chlorinated PVC, polypropylene, polyethylene-PE, and polybutylene) and metallics (copper, galvanized iron, cast iron, galvanized steel) (WHO 2006). Several studies have investigated the influence of pipe materials on biofilm development in DWDS. However, the conclusions are controversial. For example, Lehtola et al. (2004) found that biofilm development on PE was higher than on copper pipes. For longer periods (> 200 days) the biofilms formed on copper pipes was not significantly different from that formed on PE (Lehtola et al. 2004). Niquette et al. (2000) observed lower biofilm formation on plastic pipes (PVC and PE) and higher formation on gray iron. Cloete et al. (2003) observed higher biofilm formation on PVC compared to galvanized steel. More recently, Assaidi et al. (2018) observed higher *Legionella pneumophila* colonization in 45 d old biofilms formed on galvanized steel surfaces than on stainless steel, copper and plastics (PVC, cross-linked PE and polypropylene random copolymer). The variety of results observed among different studies may be related to other factors influencing biofilm formation, such as water velocity, the type of microorganisms, pipe corrosion, the incubation period, the temperature or the nutrient/disinfectant availability and the characteristics of the material, such as roughness and the hydrophobic/hydrophilic balance (Eboigbodin et al. 2008).

In some European countries and in the USA, the use of plastic pipes in recent buildings has been preferred over the use of copper (Cooper and Hanlon 2010; Naismith et al. 2017; Innovation 2019). Although copper is more expensive than plastics and

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other metallic pipes it is generally more resistant to corrosion, has no significant effects on the water quality, is easy to install and has antimicrobial properties (Żabnieńska-Góra and Dudkiewicz 2018). The antimicrobial properties of copper materials may have an important action in preventing the build-up of biofilms in DWDS and the interest in its use is re-emerging (Gulati and Ghosh 2017; Rhoads et al. 2017; Assaidi et al. 2018; Inkinen et al. 2018; Khan et al. 2019). Rhoads et al. (2017) observed an important role of copper in *L. pneumophila* biofilm control in a stagnant DWDS. Khan et al. (2019) observed that chlorine resistance increased when 5 d old biofilms were encountered on PVC and copper pipes. Other authors (Gulati and Ghosh 2017; Assaidi et al. 2018), studied biofilm formation on different metallic and plastic materials for 72 h and 45 d, respectively, and observed relevant effects from copper use in the prevention of *Sphingomonas paucimobilis* and *L. pneumophila* biofilms.

Different strategies are currently applied in DWDS to guarantee that the water is delivered under the desired quality standards. These include filtration processes, UV disinfection and the use of disinfectants and oxidants before and during water transport, as well as all the complementary maintenance and monitoring processes along the system (Simões and Simões 2013). For instance, the use of residual concentrations of disinfectant along the DWDS is an important strategy to control microbial proliferation, particularly in the bulk phase. Nevertheless, the efficiency of chlorine in preventing biofilm formation in plumbing and DW systems is limited (Simões and Simões 2013). Furthermore, residual disinfectant concentrations are difficult to maintain. Water age (i.e. the time it takes for water to travel from the source to consumers tap), organic matter, reactive plumbing (such as copper, stainless steel and ductile iron), a high surface to volume ratio and warm temperatures cause chlorine decay (Nguyen et al. 2012; Rhoads et al. 2016, 2017; Zhang et al. 2017). Pipe flushing is another strategy commonly used in DWDS for cleaning purposes, acting by removing sediments, contaminants and biofilms. Flushing consists of running water at high flow through pipes to replace water and to clean surfaces in DWDS or plumbing systems. For example, this strategy can be used in plumbing systems in cases of significant water contamination, in order to remove the contaminated water and restore safe drinking water in buildings, preventing exposure to chemical and/or biological contamination (Casteloes et al. 2015; Ragain et al. 2019). However, the protocols used are controversial, and several adjustments in flushing time have been studied in order to avoid an increase in exposure to contaminations, if short time flushing is applied (Casteloes et al. 2015; Ragain et al. 2019). Moreover, the effects of flushing in biofilm control remain to be adequately addressed.

This study aimed to assess copper alloys as cheaper alternatives to elemental copper materials in biofilm prevention and as a complementary strategy to overcome the limitations of chlorine decay in DWDS. *Stenotrophomonas maltophilia* was used as a model bacterium for biofilm formation. *S. maltophilia* species are recognized as opportunistic multi-drug resistant (Rizek et al. 2018). Strains of this species are commonly found in drinking water and their presence in tap water may have significant public health implications (Zanetti et al. 2014; Dai et al. 2017; Destiani and Templeton 2018; Bae et al. 2019). A rotating cylinder reactor (RCR) was used for biofilm formation and for further testing of mechanical stress and chlorine exposure. The RCR allows biofilm testing under a realism-based approach, using curved surfaces, typical of pipes, and hydrodynamic conditions similar to those found in DWDS. Mimicking the conditions found in DWDS has significant importance for validating the action of the selected copper alloys for putative practical application. The RCR has already been used for biofilm testing, allowing the formation of homogeneous biofilms under controlled conditions (Simões et al. 2005; Lemos et al. 2015; Gomes et al. 2018). The membrane integrity of the biofilm released cells was further evaluated to understand the impact of each condition tested on the microbiological status of the bulk phase.

Materials and methods

Bacteria and culture conditions

Stenotrophomonas maltophilia isolated from a DWDS (Simões et al. 2007) was used in this study. One colony of *S. maltophilia* was picked from R2A agar medium to inoculate 1 l flasks containing 250 ml of R2A broth, which was incubated overnight at 25 °C and under agitation (120 rpm).

Surface materials for biofilm formation

Four surface materials with different copper content were used: stainless steel (SS) (with 0% copper was used as a negative control); elemental copper (with 100% Cu was used as a positive control); 96% copper alloy; and 57% copper alloy. The composition of each

Table 1. Elemental composition of the surface materials used for biofilm formation.

Material (US Standard)	Material composition (%)						
	Cu	Fe	Ni	Mn	Zn	Cr	Other
AISI 316	–	62	10-14	2	–	16-18	C, Si, P, S, Cr Mo, N
C11000	100	–	–	–	–	–	–
C18000	96	–	4	–	–	–	–
C38500	57	5	–	–	39	–	Pb (3 %)

*Data provided by the suppliers: Universal AFIR, Porto, Portugal and Neves & Neves, Trofa, Portugal.

surface material is presented in Table 1. Three cylinders from each material were used for biofilm formation with a sampling area of 39.27 cm^2 ($d = 2.5$ cm; $l = 5$ cm) per cylinder. Before biofilm formation, the cylinders were degreased with absolute ethanol (Panreac Applichem, Darmstadt, Germany). To remove surface oxides, each cylinder was exposed for 2 min to HCl (39% sp gr 1.19 from Fisher, Leicestershire, UK) diluted two times in water. This step was repeated at least twice, until a colourless final washing solution was obtained. To conclude the oxide removal, coupons were washed in distilled water, dried with a paper towel and abraded with abrasive paper P1000 until a homogeneous appearance was achieved according to a standard protocol (ASTM 1999) and further validated by analysing through spectral domain optical coherence tomography (THORLABS GmbH, Lübeck, Germany) (Supplementary material, Figure S1). Afterwards, the cylinders were rinsed thoroughly with distillated water and dried with paper. After this process, the cylinders were exposed to ultraviolet light for 45 min for further disinfection.

Biofilm formation

A rotating cylinder reactor (RCR) was used for biofilm formation under conditions mimicking DWDS. The RCR (Figure 1) was composed of a 5 l vessel where three cylinders were immersed in a bacterial suspension. The cylinders were connected with a synchronizing belt, guaranteeing that they were continuously rotating at the same speed and direction through an overhead stirrer. The 5 l vessel was inoculated with 500 ml of bacterial suspension prepared as described previously and diluted to a final volume of 5 l in sterile synthetic tap water (STW, prepared as described by Gomes et al. 2018), to a final concentration of 2×10^7 cells ml^{-1}. The RCR operated under batch conditions for 2 h in order to promote initial bacterial adhesion. Afterwards, the RCR was continuously fed with 10 times diluted R2A broth at 0.5 l h^{-1}, ensuring a constant dilution rate of 0.1 h^{-1}. The RCR

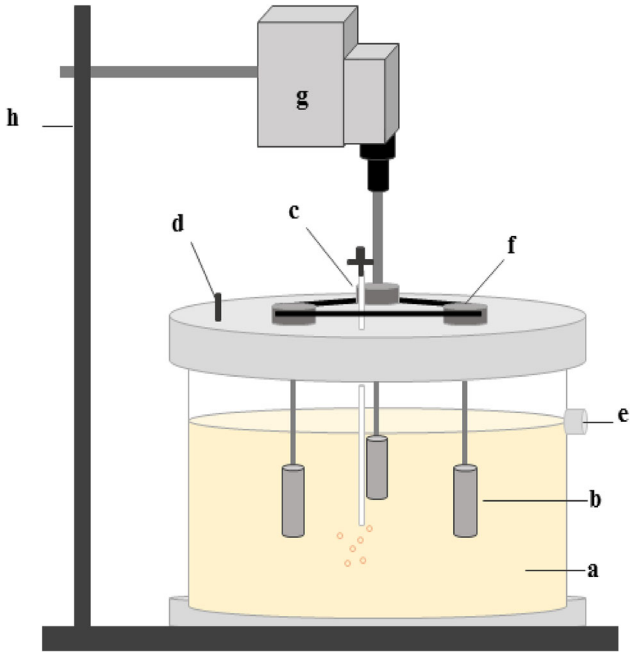

Figure 1. Schematic representation of the rotating cylinder reactor system. **a** – 5 l vessel with bacterial suspension; **b** – rotating cylinders for biofilm formation; **c** – inlet of filter sterilized air; **d** – inlet of diluted R2A broth; **e** – outlet of wastes (bacterial suspension and medium), **f** – reactor lid with synchronizing belt; **g** – stirring overhead; **h** – support for stirring system.

operated with three cylinders made from the same material, one cylinder being used as a control (no exposure to the selected chemical and mechanical treatments) and the two remaining cylinders to assess the treatment efficacy (the same treatment was applied to both cylinders in each assay). Biofilms were formed for 7 days in order to obtain mature biofilms (Simões et al. 2005), under constant rotation speed, corresponding to a liquid flow flow of 0.1 m s^{-1} on the cylinder surface (equivalent to 0.1 Pa of shear stress), similar to the typical water velocity in plumbing systems (Husband and Boxall 2011; Neilands et al. 2012; Ragain et al. 2019). The shear stress (τ_W) on the cylinder surface was assessed according to Equation 1 (Altman et al. 2009):

$$f = \frac{2\tau_W}{\rho V^2} \quad (1)$$

where f is the fanning factor, ρ is the fluid density (1,000 kg m^{-3}) and V is the fluid velocity (m s^{-1}).

The linear velocity of the fluid (0.1 m s^{-1}) on the cylinder surface is given by Equation 2, where N is the rotational speed of the cylinder (rpm) and D is the cylinder diameter (0.025 m):

$$V = N\pi D \quad (2)$$

The fanning factor is assessed according to Equation 3 (Gabe and Walsh 1983):

$$f = 0.158 Re_a^2 \quad (3)$$

where the Reynolds number of agitation (Re_a) is obtained from Equation 4 (Mancilla et al. 2019):

$$Re_a = \frac{D^2 N \rho}{\mu} \quad (4)$$

where μ is the fluid dynamic viscosity (0.001 kg m^{-1} s^{-1}).

After biofilm formation for 7 d each cylinder was removed from the RCR and carefully washed in saline water (0.85% NaCl) in order to remove non-adhered bacteria. Then, one cylinder was used for biofilm characterization and the other two cylinders were exposed to one of the treatments tested: sodium hypochlorite (NaOCl) at 10 mg l^{-1} of free chlorine for 10 min; high shear stress exposure (10 Pa for 30 s, equivalent to a flushing situation), or a combination of both chemical and mechanical treatments. The assay was performed at least three times for each material and for each treatment, with duplicates.

Biofilm characterization

Biofilms formed in the RCR were characterized in terms of wet and dry mass, cell density, culturability and content of organic deposits (proteins and polysaccharides). The biofilms were removed from the cylinder surfaces by scraping four times for 1 min using a stainless steel scraper and resuspended in 20 ml followed by rinsing four times with 5 ml per rise of extraction buffer (0.76 g l^{-1} $Na_3PO_4 \cdot H_2O$, 0.36 g l^{-1} $Na_2HPO_4 \cdot H_2O$, 0.53 g l^{-1} NaCl, 0.08 g l^{-1} KCl) (Frølund et al. 1996). The suspension was further homogenized for 2 min by vortexing (VV3 model, VWR). The scraping efficiency was evaluated by performing the procedure once more and the suspension collected was characterized for the presence of cells by fluorescence microscopy, after staining with 4′,6-diamidino-2-phenylindole (DAPI), according to Gomes et al. (2016). A LEICA DM LB2 epifluorescence microscope connected to a Leica DFC300 FX camera (Leica Microsystems Ltd, Heerbrugg) was used to visualize the stained samples. The results demonstrated that < 0.0001% (regardless of the surface material used) of the total biofilm cells remained adhered on the cylinder surface (data not shown).

Biomass quantification

The wet mass was obtained by the difference between the mass of the cylinder with biofilm (before scraping) and the mass of the cylinder without biofilm (after scraping). The dry biofilm mass was assessed by the total volatile solids (TVS), with a detection limit of 0.25 mg cm^{-2}. A volume of 10 ml of the homogenized biofilm suspension was placed in crucibles and dried for 24 h at 105 °C. After that, the crucibles were weighed and placed in a furnace at 550 °C for 2 h and weighed again in order to assess the TVS (APHA 1989). The water content was estimated as the difference between the wet mass and the dry mass.

Cellular density

The biofilm cellular density was evaluated in terms of culturable, total and intact membrane bacteria. To assess culturable bacteria, serial dilutions of biofilm suspensions were prepared and plated on R2A agar plates. The plates were incubated at 25 °C for 48 h before CFU enumeration. For the enumeration of total cells and those with no damaged membrane, 500 µl of biofilm suspension were filtered through a 0.22 µm black polycarbonate membrane Nucleopore® (Whatman, Middlesex, UK). The membrane was stained with the Live/Dead BacLight bacterial viability kit (Invitrogen Life Technologies, Alfagene). Volumes of 250 µl of SYTO 9™ and 50 µl of propidium iodide (PI) were added to the black polycarbonate membrane and left in the dark for 10 min. The samples were analyzed using a LEICA DM LB2 epifluorescence microscope connected to a Leica DFC300 FX camera (Leica Microsystems Ltd, Heerbrugg). The optical filter combination for optimal viewing of the stained preparations consisted of a 515–560 nm excitation filter combined with a dichromatic mirror at 580 nm and a suppression filter at 590 nm. Bacteria with intact membranes (green stained) and the total (sum of green and red stained) number of cells were assessed from counts of a minimum of 20 fields of view. The detection limit of the method is 4.8 log cells cm^{-2}.

Quantification of organic deposits - proteins and polysaccharides

A biofilm suspension was used for extraction of organic deposits, including the extracellular polymeric substances (EPS). For that, a Dowex® Marathon © resin (Na^+ form, strongly acidic, 20–50 mesh, Sigma-Aldrich, Steinheim, Germany) was inserted in the biofilm suspension and the extraction took place at 4° C for 4 h and under constant agitation (400 rpm) (Frølund et al. 1996; Redmile-Gordon et al. 2014; Lemos et al. 2015). This resin is used to remove cations from the EPS matrix, breaking up the aggregates and causing EPS release. After the extraction, the suspension was centrifuged at 3700 g for 5 min in order

Table 2. Characterization of the biofilms formed on selected materials with different copper content.

	Material / copper content (%)			
	0 (SS)	57	96	100
Wet mass (mg cm^{-2})	120 ± 26	78.0 ± 4.5*	90.0 ± 14	92.0 ± 23
Dry mass (mg cm^{-2})	4.50 ± 1.6	2.71 ± 0.76	2.61 ± 0.10	3.24 ± 1.3
Water content (%)	96.8 ± 1.3	96.5 ± 2.89	96.6 ± 0.51	97.2 ± 0.50
Organic deposits - proteins (mg g^{-1} biofilm)	16.4 ± 3.4	5.08 ± 1.50*	7.41 ± 2.9*	4.62 ± 1.6*
Organic deposits - polysaccharides (mg g^{-1} biofilm)	143 ± 33	142 ± 26	113 ± 58	n.d
Biofilm culturability (log CFU cm^{-2})	7.04 ± 0.67	6.53 ± 0.73	5.55 ± 0.75*	5.00 ± 0.93*
Biofilm viability (log non-damaged cells cm^{-2})	7.08 ± 0.36	6.77 ± 0.54	6.77 ± 0.85	6.14 ± 1.1
Biofilm cellular density (log cells cm^{-2})	7.78 ± 0.22	7.84 ± 0.06	7.78 ± 0.27	7.59 ± 0.41

- n.d. – non-detected (polysaccharides below the quantification limit – 5 mg l^{-1})
* $p < 0.05$ – statistically significant different from the control (0% copper)

to harvest the organic deposits present in the supernatant. The total amounts of proteins and polysaccharides were assessed, as these are the major macromolecules present in organic deposits of microbial origin (Decho and Gutierrez 2017). The polysaccharides were quantified according to the phenol-sulfuric method (Dubois et al. 1951) using glucose as standard. The quantification of proteins in the organic deposits was performed by the Bradford microassay (Sedmak and Grossberg 1977), using bovine serum albumin as standard. The detection limit of the used methods was 5 mg l^{-1}.

Biofilm treatment with chlorine

Sodium hypochlorite from Sigma-Aldrich (Steinheim, Germany) was used to prepare a solution with 10 mg l^{-1} of free chlorine in STW. Free chlorine concentrations were adjusted using a photometer from Hanna Instruments using the N,N-diethyl-p-phenylenediamine (DPD) method (test kit from Hanna Instruments, Woonsocket, USA). Cylinders containing biofilms were washed in sterile STW to remove weakly adhered cells. Then, biofilms were exposed to 200 ml of chlorine solution at 10 mg l^{-1}, for 10 min, in a 250 ml glass beaker, under constant rotation speed (76 rpm – equivalent to the rotation speed during biofilm formation) corresponding to a linear velocity of 0.1 m s^{-1} (Equation 2). After exposure, the cylinders were immersed in a solution of sodium thiosuphate at 0.50% (wt/v) for 10 min in order to neutralize the residual chlorine. Afterwards, the biofilm was scraped and resuspended in extraction buffer as described previously. The sample was analyzed in terms of culturability, the presence of cells with non-damaged membrane and total cell number, as described previously. The chlorine solution used for biofilm treatment was also analyzed in terms of cell membrane integrity in order to characterize the bacterial status in the bulk phase, i.e. the damage of biofilm released bacteria.

The reduction in the culturability and membrane integrity were assessed as the difference between the log CFU cm^{-2} or log cells cm^{-2} before treatment and the log CFU cm^{-2} or log cells cm^{-2} after treatment, respectively.

Biofilm treatment by hydrodynamic stress

Cylinders containing biofilms were immersed in a beaker containing 200 ml of STW to remove weakly adhered cells. Then, the biofilms were transferred to 200 ml of sterile STW in a 250 ml glass beaker for mechanical treatment. For that, the rotation speed under which the biofilm was exposed was increased in order to guarantee a linear velocity on the cylinder surface of 1.5 m s^{-1} (10 Pa, based on Equations 1–4) for 30 s, mimicking specific situations of flushing in DWDS for removal of deposits and biofilms (Ellison 2003). After exposure, the cylinders were immersed in STW in order to remove non-adhered cells. The biofilm was scraped and resuspended in extraction buffer, as described previously and further analyzed in terms of culturability and the numbers of total and damaged cells. The STW solution used for biofilm treatment was also analyzed in terms of numbers of damaged cells.

Combined treatments in biofilm control

Cylinders containing biofilms were also treated with the combination of both chemical and mechanical treatments. The chemical treatment was applied followed by mechanical treatment, according to the conditions described previously. The STW used for biofilm treatment was also analyzed in terms of the number of damaged cells in order to evaluate biofilm removal, cell numbers and their viability in the bulk phase.

Statistical analysis

Statistical analysis of the results was performed using the software IBM® SPSS® Statistics (Statistical

Package for the Social Sciences) version 25.0. One-way analysis of variance (ANOVA) was applied and the comparisons between and within experimental groups were carried out using the Tukey test. Statistical calculations were based on a confidence level ≥ 95% ($p < 0.05$) which was considered statistically significant.

Results

Biofilm formation on copper materials

Biofilms formed on four different surface materials were characterized in terms of mass, water content, proteins and polysaccharides from organic deposits, and cell density (Table 2). The biofilms were composed mostly of water (>96%). The dry mass and water content were similar for all the biofilms, regardless of surface material under which they were formed ($p > 0.05$). The biofilms formed on copper materials had lower wet mass than those formed on SS (0% copper), making the differences more significant when using the 57% copper alloy ($p < 0.05$).

Regarding the content of extracellular organic deposits, it was observed that biofilms formed on copper materials had lower levels of proteins than those formed on SS ($p > 0.05$). The use of elemental copper surfaces also allowed biofilm formation with a low content of polysaccharides in the extracellular organic deposits. No significant differences were observed when comparing the content of polysaccharides in the organic deposits of biofilms formed on SS and on 57 and 96% copper alloys ($p > 0.05$). Biofilm cell density and the number of non-damaged cells were found to be similar, regardless of the surface material used ($p > 0.05$). In terms of biofilm culturability, a decrease was observed with an increase in the surface copper content. Materials with 100 and 96% copper caused reductions of 2 and 1.4 log CFU cm^{-2}, respectively ($p < 0.05$).

Biofilm control by chlorine and shear stress

Chemical treatment with chlorine

Biofilm culturability after treatment with 10 mg l^{-1} of free chlorine for 10 min was lower (Figure 2) when using 100 and 96% copper surfaces ($p < 0.05$). No significant differences were observed in the culturability of biofilms formed on 57% copper alloy compared to SS ($p > 0.05$). However, CFU reduction due to NaOCl exposure (in comparison with the non-NaOCl treated biofilms formed on the same material) was only significant when using the 96% copper alloy ($p < 0.05$). Also, the number of non-damaged *S. maltophilia* cells in biofilms formed on the 96% copper alloy followed the same trend as observed for biofilm culturability (Figure 3) i.e. the number of non-damaged *S. maltophilia* cells was lower on the 96% copper alloy than on SS as well as in the non-NaOCl treated biofilms formed on the 96% copper alloy ($p < 0.05$). Treatment

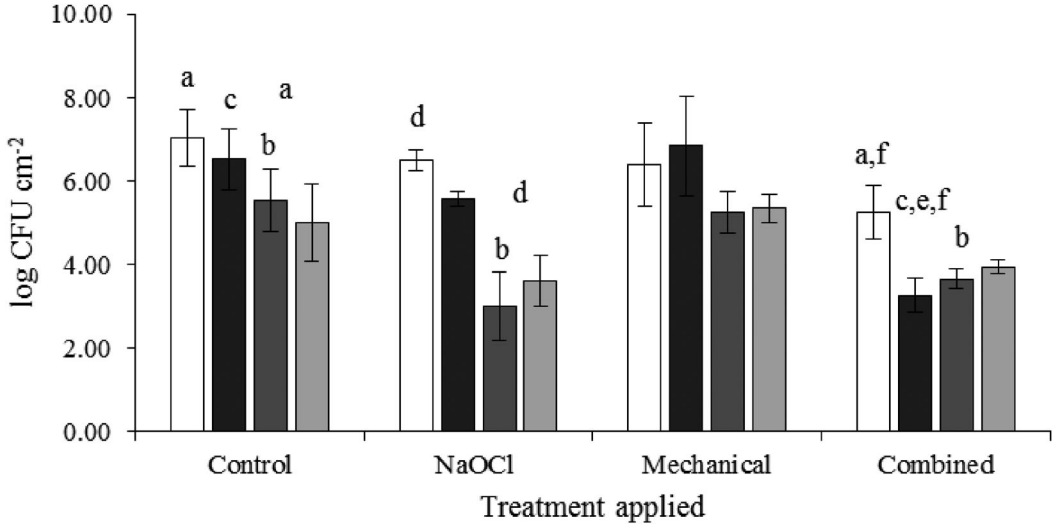

Figure 2. Culturability of biofilms formed on surface materials with different copper content before and after exposure to chemical or/and mechanical treatments. Control – biofilms not exposed to any treatment; NaOCl – biofilms exposed to 10 mg l^{-1} of free chlorine for 10 min; mechanical – biofilms exposed to 10 Pa (fluid velocity 1.5 m s^{-1}) for 30 s; combined – biofilms exposed to both chemical and mechanical treatments. □ – Material with no copper in its composition (0% copper); ■ – material with 57% copper content; ■ – material with 96% copper content; ■ – elemental copper (100% copper). a, b, c, d, e represent conditions with statistically significant differences (confidence level ≥ 95%) in comparison with each control (the control value is the first condition signaled with the specific letter).

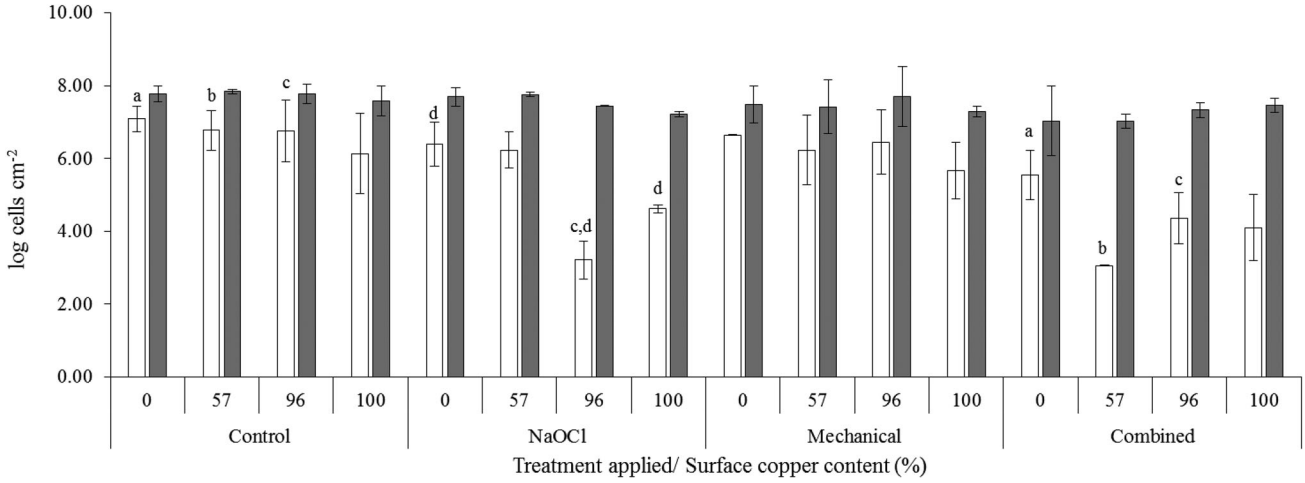

Figure 3. Cellular density (□ – non-damaged cells and ■ – total cells) of biofilms formed on surface materials with different copper content before and after exposure to chemical or/and mechanical treatments. Control – biofilms not exposed to any treatment; NaOCl – biofilms exposed to 10 mg l^{-1} of free chlorine for 10 min; mechanical – biofilms exposed to 10 Pa (fluid velocity 1.5 m s^{-1}) for 30 s; combined – biofilms exposed to both chemical and mechanical treatments. a, b, c, d represent conditions with statistically significant differences (confidence level ≥ 95%) in comparison with each control (the control value is the first condition signaled with the specific letter).

with chlorine significantly did not affect the numbers of non-damaged membrane and culturable *S. maltophilia* cells when biofilms were formed on SS or on 100 and 57% copper surfaces ($p > 0.05$) compared with non-NaOCl treated biofilms formed on the same materials.

Mechanical treatment by exposure to high shear stress

The exposure to 10 Pa shear stress, corresponding to a fluid velocity of 1.5 m s^{-1} for 30 s caused no significant biofilm removal ($p > 0.05$). This treatment had no effects on *S. maltophilia* biofilm culturability (Figure 2) or in membrane integrity (Figure 3), regardless of the surface material tested ($p > 0.05$).

Combined treatment – exposure to chlorine and high shear stress

The combination of chemical and mechanical treatments did not reduce biofilm culturability (Figure 2) when these were formed on elemental copper (100%) and compared with the behaviour of biofilms formed on the same material but not exposed to the combined treatment ($p > 0.05$). However, the CFU reduction caused by the combination of treatments was only higher than the reduction caused by chlorine treatment alone for biofilms formed on the 57% copper alloy ($p < 0.05$). It was also possible to observe a reduction in the numbers of cells with non-damaged membranes caused by the combined treatments for biofilms formed on SS (Figure 3) ($p < 0.05$). However, higher amounts of *S. maltophilia* cells with damaged membrane were observed when 96 and 57% copper alloys were used ($p < 0.05$).

Bulk phase analysis after biofilm treatment

Biofilm released cells (the total and those with non-damaged membrane) were quantified in order to understand the state of these cells following the chemical and mechanical treatment. The total number of cells in the bulk water (Figure 4) was not dependent on the surface material or the treatment applied ($p > 0.05$). Nevertheless, significant differences were observed in terms of non-damaged cells released from the chemically and/or mechanically treated biofilms (Figure 4). The number of non-damaged cells in the bulk phase after chlorine treatment was lower when materials with 100 and 57% copper content were used (4.1 and 4.4 log non-damaged cells cm^{-2}, respectively) compared with SS (5.4 log non-damaged cells cm^{-2}) ($p < 0.05$). The damage to the membranes of *S. maltophilia* cells released from biofilms formed on the 96% copper alloy was not statistically different from that observed for biofilms formed on SS ($p > 0.05$). The use of copper materials did not affect the number of non-damaged cells detected in the bulk water after mechanical treatment ($p > 0.05$). The comparison between the effects of the chemical and mechanical treatments on the numbers of non-damaged cells in the bulk phase shows higher numbers after the mechanical treatment ($p < 0.05$). The combination of treatments did not reduce the number of the released bacteria with intact membranes in comparison with NaOCl alone ($p > 0.05$). In general, higher numbers

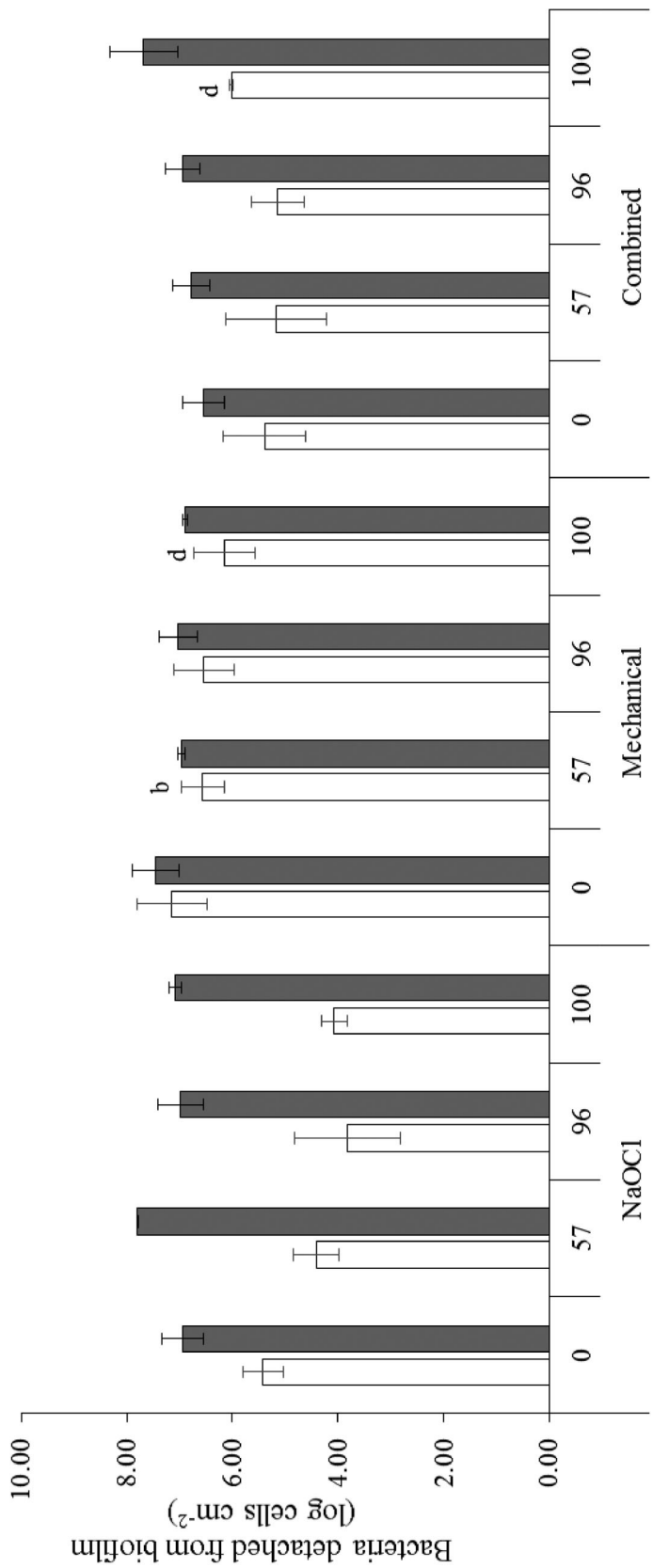

Figure 4. Cells removed from the cylinder's surface after each treatment. Control – biofilms not exposed to any treatment; NaOCl – biofilms exposed to 10 mg l^{-1} of free chlorine for 10 min; mechanical – biofilms exposed to 10 Pa (fluid velocity 1.5 m s^{-1}) for 30 s; combined – biofilms exposed to both chemical and mechanical treatments. □ – non-damaged cells; ■ – total cells. a, b, c, d represent conditions with statistically significant differences (confidence level ≥ 95%) in comparison to each control (the control value is the first condition indicated with the specific letter).

of non-damaged bacteria were released after the mechanical treatment (for all the materials) and after the combination of chemical and mechanical treatments in biofilms formed on elemental copper than after the chemical treatment ($p < 0.05$). For biofilms formed on surface materials with 0, 57 and 96% of copper, the number of non-damaged bacteria released from biofilms treated with the combination of mechanical and chemical stresses was similar to those released after the chemical treatment ($p > 0.05$).

Discussion

Biofilm formation in DWDS typically changes the aesthetic characteristics of the delivered water, accelerates pipe corrosion, and is a potential causative agent of waterborne diseases due to the presence of pathogens (Simões and Simões 2013). Therefore, the existence of efficient strategies to control biofilm development in DWDS is of the utmost importance. Current disinfection strategies are unable to prevent or eradicate biofilms (Simões and Simões 2013). The use of materials with antimicrobial properties requires further research as they may play a crucial role in preventing biofilm control. Copper has been applied in plumbing systems but it is losing attractiveness for plastic pipes, mainly due to its expensive cost. However, copper has known antimicrobial characteristics that may have a significant impact on biofilm control. The use of copper alloys, with reduced copper content, could help to reduce the costs from material acquisition while having the potential advantage of exerting antimicrobial activity. Several studies describing disinfection strategies were performed using bacteria in the planktonic state but these are inadequate to represent the behavior of those found adhered on surfaces (Mir et al. 1997; Khan et al. 2017; Köhler et al. 2018; Forbes et al. 2019; Samir et al. 2019). Several other works studied DW biofilms formed using reactors operating under conditions far from those found in DWDS, particularly the annular reactor (Chang and Craik 2012), the rotating disc reactor (Murga et al. 2001; Pelleïeux et al. 2012), and the CDC biofilm reactor (Park and Hu 2010; Abe et al. 2011; Armbruster et al. 2012). In these reactors the sampling area is modest and biofilms are formed on flat surfaces, which do not mimic the curved geometry of the pipes. The use of coupons typically engineered to be flat can cause flow disturbance if the surface is not flushed with the coupon holder, affecting the hydrodynamic conditions under which the biofilms are formed. Therefore, in the present work a RCR is used, with a considerably high sampling area and using entire cylinders for biofilm formation in order to reduce the influence on the flow pattern and increase the similarity with curved pipes.

The present study showed that elemental copper and copper alloys influenced biofilm formation. The 57% copper alloy reduced the dry mass of biofilms compared with those formed on SS. Though the use of 57% copper alloy did not influence biofilm cell density, it reduced the protein content in the organic deposits. The content of polysaccharides in the organic deposits was also reduced when elemental copper was used. Previous studies reported that copper nanoparticles or copper ions may influence EPS formation. For example, Chari et al. (2017) observed that exposure to copper nanoparticles (CuNP) was responsible for a reduction in EPS production by aquaculture pathogens (*Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Aeromonas hydrophila*). Tabrez Khan et al. (2013) also observed a decrease in EPS production after exposure to CuNP at 50 g l^{-1} for 16 h, observing that copper had a greater influence on protein production than on polysaccharide production. Contradicting the present results, Katner et al. (2018) and Miao et al. (2017) found that exposure to copper was associated with an increase in EPS production by anaerobic ammonia-oxidizing bacteria and by wastewater activated sludge, respectively. Therefore, the effects of copper exposure on EPS production is non-consensual. However, no previous data exist on the influence of alloys with diverse copper content on biofilm EPS production/organic matter deposition or on the effects of metal mixtures. The possible potentiation and antagonistic effects of metals in EPS production have not been explored and cannot be disregarded when alloys are used. The significant results obtained for biofilms formed on 57% copper alloy may be due to a synergic effect with other metals presented in the alloy. Some studies have described the effects of metals on EPS production, demonstrating that other alloy elements may have an impact on bacterial behavior. For example, Pal and Paul (2013) reported a decrease in EPS production with an increase in nickel concentration. On the other hand, Redmile-Gordon and Chen (2017) found that zinc may stimulate EPS production.

The use of a residual disinfectant concentration along the DWDS is an important strategy to minimize biofilm regrowth and avoid waterborne diseases (Al-Jasser 2007). Chlorine is one of the most commonly used disinfectants in DWDS and a concentration of between 0.2 and 1 mg l^{-1} should be kept at

the delivery point (WHO 2011). In plumbing systems, the chlorine concentration decreases with time and control of the free chlorine concentration has been found to be critical (Zheng et al. 2015). Chlorine decay along DWDS and the plumbing systems depends on several factors, *viz*. temperature, the pipe material and age, biofilm and corrosion products, the flow regime, the organic matter content and the initial chlorine concentration (Kim and Kim 2017). The results from the present study indicate that copper materials are an attractive complementary strategy to overcome the problems of chlorine decay. The number of CFU from biofilms formed on materials with 96 and 100% copper was lower than those of biofilms formed on SS and treated with chlorine. The CFU of non-treated biofilms formed on 100% copper were similar to those observed in biofilms formed on SS after treatment with the combination of chlorine and shear stress. The use of 96 and 100% copper materials was demonstrated to be more effective in *S. maltophilia* biofilm control than the use of chlorine against biofilms formed on SS. This corroborates the findings of Zhou et al. (2009) where biofilm CFU on copper surfaces in the absence of chlorine was lower than the CFU levels of biofilms formed on SS exposed to 0.6 mg l^{-1} of chlorine.

A shock treatment that could be applied in situations of critical DW contamination was also simulated (free chlorine at 10 mg l^{-1} for 10 min and an increased shear stress – 1.5 m s^{-1} – for 30 s) (EPA 2010; Van Nevel et al. 2017). The results demonstrated that 10 mg l^{-1} of chlorine was inefficient for *S. maltophilia* biofilm control. The higher biofilm CFU reduction caused by chlorine was observed on 96% copper alloy. However, it is important to highlight that the chlorine did not reduce the CFU of *S. maltophilia* in biofilms grown on 0, 57 and 100% copper. Other studies have also reported the modest effects of chlorine in biofilm control. Buse et al. (2019) achieved log reductions of 2, 3 or 4 in *L. pneumophilia* biofilms for concentration × time (Ct) values of 13, 51 and 88 mg min l^{-1}, respectively. Gomes et al. (2018) also found that chlorine was inefficient against *S. maltophilia* biofilms, obtaining 35% removal after exposure to extreme chlorine conditions (175 mg l^{-1} of NaOCl for 30 min – Ct of 5,250 mg min l^{-1}).

Pipe flushing and the use of high chlorine doses are important strategies commonly used to control critical contaminations. However, these strategies may be responsible for the detachment of small portions of biofilm into the transported water that can reach the consumers̀ tap. Therefore, it is important to characterize the bacteria in the bulk phase in order to evaluate whether significant numbers of viable/non-damaged bacteria would be dispersed as result of treatment failure. Flushing was not found to be effective in the control of biofilms formed on the diverse surface materials tested. Despite no significant reduction in biofilm cellular density being observed during the flushing treatment, it is important to note the relevant abundance of non-damaged bacteria detected in the bulk water (6.1–7.1 log cells cm^{-2}). El-Chakhtoura et al. (2018) recently described the increase in CFU abundance and Shannon diversity in transported water after flushing treatment.

The number of non-damaged cells in transported water after chlorine treatment seems to be dependent on the materials used for biofilm formation. The use of copper materials reduced the number of non-damaged bacteria in the bulk phase compared to SS. This suggests that copper materials can be an adequate choice for plumbing systems. Their use reduced the levels of non-damaged bacteria detached from biofilms to the bulk phase, minimizing the microbiological risks from exposure to contaminated water. It is important to emphasize that the recommended shock treatments (a Ct value of 100 mg min l^{-1} (EPA 2010)) for critical levels of microbiological contamination only caused CFU reductions of 0.5 (SS); 1.4 (57% copper alloy); 2.5 (96% copper alloy); 0.9 (100% copper) log CFU cm^{-2}.

The combination of hydrodynamic stress and chlorination had no advantage in comparison to chlorination alone. The only exception was found for biofilms formed on 57% copper alloy where a high reduction in the number of non-damaged cells was observed from the combined treatment. Lemos et al. (2015) also found that the combination of chemical and mechanical treatments was not effective in biofilm removal when they were formed at 0.12 Pa and 0.17 Pa (a hydrodynamic stress similar to that tested in the present work).

Conclusions

The formation of biofilms by a pure strain of *S. maltophilia* (7 d old) was not significantly influenced by the use of copper surfaces in comparison with SS. Many biofilm characteristics, particularly the cell density, was not affected by the surface type. However, the benefits from the use of copper surfaces rely on the efficiency to disinfect. The selected copper materials demonstrated better performance in biofilm prevention/control than chlorine as a higher CFU

reduction was obtained using copper materials than from the use of chlorine against biofilms formed on SS. The application of shock treatments (10 mg l^{-1} of free chlorine for 10 min and/or high shear stress – 1.5 m s^{-1} for 30 s) was not effective in biofilm inactivation or removal. Biofilm disinfection was dependent on the material used for biofilm formation. Chlorine treatment did not reduce biofilm culturability when formed on surface materials with 0, 57, and 100% copper; a culturability reduction due to chlorine treatment was only observed in S. *maltophilia* biofilms formed on 96% copper alloy. Flushing was also not effective in biofilm control and the combination of both chemical and mechanical treatments (free chlorine at 10 mg l^{-1} for 10 min and a fluid velocity of 1.5 m s^{-1} for 30 s) only had a positive impact in the control of biofilms formed on 57% copper alloy. Therefore, the strategies (residual chlorine and flushing) commonly used to control critical levels of contaminations in DWDS were not effective in biofilm control. However, the results demonstrated that copper alloys reduced the number of *S. maltophilia* cells with non-damaged membrane in the transported water.

Disclosure statement

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