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Biofilm formation under high shear stress increases resilience to chemical and mechanical challenges

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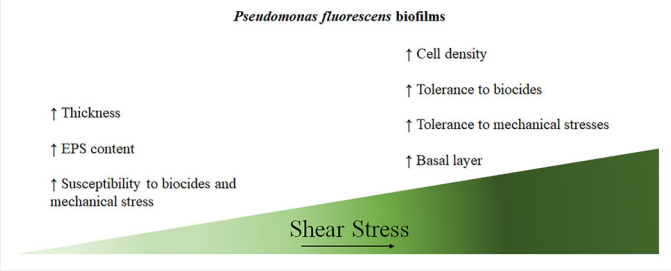
ABSTRACT

The effect that the hydrodynamic conditions under which biofilms are formed has on their persistence is still unknown. This study assessed the behaviour of *Pseudomonas fluorescens* biofilms, formed on stainless steel under different shear stress (τ_w) conditions (1, 2 and 4 Pa), to chemical (benzalkonium chloride – BAC, glutaraldehyde – GLUT and sodium hypochlorite – SHC) and mechanical (20 Pa) treatments (alone and combined). The biofilms formed under different τ_w showed different structural characteristics. Those formed under a higher τ_w were invariably more tolerant to chemical and mechanical stresses. SHC was the biocide which caused the highest biofilm killing and removal, followed by BAC. The sequential exposure to biocides and mechanical stress was found to be insufficient for effective biofilm control. A basal layer containing biofilm cells mostly in a viable state remained on the surface of the cylinders, particularly for the 2 and 4 Pa-generated biofilms.

GRAPHICAL ABSTRACT



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Biocide tolerance; biofilm resilience; mechanical removal; rotating cylinder reactor; shear stress

Introduction

Biofilms constitute a protected growth modality that allows the colonizing bacteria to survive under hostile environments. Their development, structure and population dynamics are strongly influenced by the hydrodynamic conditions under which they are formed (León Ohl et al. 2004; Simões et al. 2008; Picioreanu et al. 2009; Jones and Buie 2019; Krsmanovic et al. 2021). Bacteria can sense shear and respond by initiating biofilm formation (Rodesney et al. 2017). The moving fluid in movement applies forces to the biofilm structure in many directions, which leads to several outcomes including viscoelastic deformation, rolling and development of streamers, oscillatory movements and detachment (Stewart 2012). There are two main ways by which the hydrodynamic stress is known to affect cell behaviour: (1) shear stress (τ_w – the tangential force per unit area that is exerted by the moving fluid on the surface) effects – associated with the application of a force that causes cell motion, and (2) mass transfer effects – involving the transport of dissolved solutes (Stewart 2012).

Control of microbial growth is required in industrial systems where wet surfaces provide favourable conditions for microbial proliferation. A disinfection process aims to reduce the surface population of viable microorganisms through the use of biocides after cleaning and to prevent microbial growth on surfaces before restarting a production cycle (Simões et al. 2010). Biocidal efficacy determination before an industrial application is often performed and validated in suspension tests with ready-to-use dilutions (EN-

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1276 2009). This standardized approach helps to understand why chemical treatments alone are insufficient for biofilm control. Therefore, biocides used in industrial disinfection can kill the free-living microorganisms, but fail to kill cells colonising a surface (biofilm) (Simões et al. 2005; Lemos et al. 2015). The limited effects of chemical treatments are of great concern as the residual microorganisms left on the equipment surfaces after biocide treatment can promote rapid biofilm re-establishment. Therefore, chemical agents and mechanical forces are parameters often used simultaneously in industrial biofilm control (Simões et al. 2009; Simões and Simões 2013). For instance, the efficiency of processes like clean-in-place (CIP) often used to control microbial growth and biofilm development in industrial settings can be improved by the use of high τ_w and turbulent flows (Li et al. 2019). However, the influence of current biocides on biofilm removal is still unclear as well as the role of the hydrodynamic conditions under which biofilms are formed on their control. Therefore, this study assessed the effectiveness of three biocides, benzalkonium chloride (BAC), glutaraldehyde (GLUT) and sodium hypochlorite (SHC), on the control of biofilms formed under different τ_w. The selected biocides belong to distinct chemical classes – quaternary ammonium compounds (BAC), aldehydes (GLUT) and halogen-based oxidizers (SHC), commonly used for industrial disinfection (Lin et al. 2020). Biofilms were formed on stainless steel (SS) cylinders under three distinct τ_w to ascertain the impact of the hydrodynamic conditions under which biofilms were formed on their tolerance to chemical and mechanical stresses.

Materials and methods

Microorganism, culture conditions and reagents

Pseudomonas fluorescens ATCC 13525^T was used throughout this study. This strain has already been used as model for biofilm control testing (Simões et al. 2005, 2009; Fernandes et al. 2020). Bacterial cells were grown overnight using a synthetic nutrient medium, with glucose as the main carbon source (glucose 5 g l^{-1}, peptone 2.5 g l^{-1}, yeast extract 1.25 g l^{-1} and 0.2 M phosphate buffer at pH 7) (Simões et al. 2009) at 25 ± 3 °C and under agitation (120 rpm) in an orbital incubator (New Brunswick Scientific, I26, USA). All growth medium compounds were purchased from Merck (VWR, Portugal). Cells were harvested by centrifugation (Eppendorf centrifuge 5810R) at 3,777 ×*g*, 5 min, washed twice with 0.2 M phosphate saline buffer (PBS; 8 g l^{-1} of NaCl – Labkem, Spain, 0.2 g l^{-1} of KCl – VWR, Belgium, 1.44 g l^{-1} of Na_2HPO_4 and 0.24 g l^{-1} of KH_2PO – Chem-Lab NV, Belgium; pH 7) and resuspended in the same buffer (for antimicrobial activity assessment against planktonic cells) or the appropriate medium (for biofilm formation) to achieve the bacterial concentration required for further experiments.

BAC (PubChem CID: 8753), GLUT (PubChem CID: 3485), SHC (PubChem CID: 23665760) and biocide neutralizing components were purchased from Sigma-Aldrich (Portugal). Solutions of biocides were prepared in sterile distilled water (DW). Biocide neutralization was done using the universal neutralizer (lecithin 3 g l^{-1}, polysorbate 30 g l^{-1}, sodium thiosulphate 5 g l^{-1}, L-histidin 1 g l^{-1}, saponin g l^{-1} in 1% phosphate buffer 0.25 M pH 7.2) for 10 min (EN-1276 2009). The universal neutralizer was validated in terms of neutralization efficacy against the biocides tested and for the absence of antimicrobial activity, as requested by the European Standard EN-1276 (2009).

Antimicrobial activity against planktonic bacteria

The antimicrobial activity against planktonic *P. fluorescens* was performed according to the European Standard EN 1276 (2009) to determine the minimum bactericidal concentration (MBC) for each biocide. Briefly, overnight grown cultures were centrifuged at 3,777 ×*g* for 5 min and washed once with PBS (pH 7). The bacterial pellets were resuspended in PBS and adjusted to 10^8 cells ml^{-1} before biocide exposure. Afterwards, cell suspensions were exposed to different concentrations of biocides for 30 min. After exposure, biocide neutralization was performed using the universal neutralizer and the number of viable cells was determined using the Live/Dead *Bac*Light bacterial viability kit (Invitrogen) as described by Borges et al. (2013). *Bac*Light is composed of two nucleic acid-binding stains: SYTO 9^{TM} and propidium iodide. SYTO 9^{TM} penetrates all bacterial membranes and stains the cells green, while propidium iodide only penetrates cells with damaged membranes, and the combination of the two stains produces red fluorescing cells. The MBC was considered as the minimum biocide concentration where all the cells coloured red (stained with propidium iodide).

For microscopic analysis, bacteria were diluted to an adequate concentration (to have 30–250 cells per microscopic field), being thereafter microfiltered through a Nucleopore® (Whatman, Middlesex, UK) black polycarbonate membrane (pore size 0.22 µm), stained with 250 µl of SYTO 9^{TM} solution and 250 µl of propidium iodide solution from the Live/Dead kit,

and left in the dark for 10 min. A microscope (AXIOSKOP; Zeiss, Göttingen, Germany) fitted with fluorescence illumination and a 100× oil immersion fluorescence objective was used to visualize the stained cells. The optical filter combination consisted of a 515–560 nm excitation filter, in combination with a dichromatic mirror at 580 nm and a suppression filter at 590 nm. Bacterial images were digitally recorded as micrographs using a microscope camera (AxioCam HRC; Zeiss) for further cell enumeration.

Biofilm formation

A rotating cylinder reactor was used to grow biofilms on SS cylinders inserted in a 5 l glass vessel and maintained under specific conditions of τ_w through the use of shaft-drive agitators (Heidolph, Germany). AISI316 SS cylinders with a surface area of 34.6 cm^2 (diameter = 2.2 cm; length = 5.0 cm) were used. Before biofilm formation, SS cylinders were cleaned and disinfected according to Gomes et al. (2018a, 2021). Three shear stress conditions were tested in independent experiments: 1, 2 and 4 Pa. In every experiment, three SS cylinders were used for biofilm formation. The 5 l vessel was inoculated with 250 ml of 10^8 cells ml^{-1} of *P. fluorescens* in the stationary growth phase, being afterwards continuously fed with sterile diluted synthetic nutrient medium (0.05 g l^{-1} glucose, 0.025 g l^{-1} peptone, 0.0125 g l^{-1} yeast extract and 0.2 M phosphate buffer at pH 7) at a constant flow rate (1.5 l h^{-1}). These conditions enforce bacterial adhesion instead of planktonic growth. The biofilms were allowed to grow for five days to obtain significant biomass for further tests. The biofilms on the SS cylinders were carefully immersed in 0.2 M PBS at pH 7 to remove loosely attached cells. Then, the biofilms were characterized and tested for their tolerance to chemical and mechanical treatments.

Biofilm characterization

Biofilms were characterized in terms of thickness, wet and dry mass, density, cell density and protein and extracellular polysaccharide content – structural characterization. Moreover, biofilms cells were characterized in terms of susceptibility to biocides – the minimum bactericidal concentration of biofilm cells.

Structural characterization

Biofilm thickness was immediately assessed after sampling using a needle connected to a digital micrometer (VS-30H, Mitsubishi Kasei Corporation, Tokyo, Japan). Wet biofilm mass was obtained by measuring the weight of the cylinder with and without biofilm. Biofilm density was assessed as the ratio between the biofilm dry mass and thickness. For further experiments, the biofilms were removed from the cylinders using a SS scraper and resuspended in 10 ml of extraction buffer (0.76 g l^{-1} $Na_3PO_4.H_2O$, 0.36 g l^{-1} $Na_2HPO_4.H_2O$, 0.53 g l^{-1} NaCl, 0.08 g l^{-1} KCl). Afterwards, the biofilm suspensions were homogenized by vortexing (30 s, 100%) as described by Simões et al. (2005, 2009).

The total extracellular polymeric substances (EPS) (in terms of proteins and polysaccharide content) were quantified after extraction with Dowex® Marathon© resin (NA^+ form, strongly acidic, 20–50 mesh, Sigma) according to Frølund et al. (1996). Briefly, Dowex resin was added to the biofilm suspension in a 25 ml beaker and the extraction took place for 4 h at 400 rpm and 4 °C. The extracellular components present in the supernatant were separated from cells through centrifugation (3,777 ×g, 10 min). The biofilm proteins were determined by the Lowry modified method (Sigma) using bovine serum albumin as standard (Lowry et al. 1951; Peterson 1979). The polysaccharides were quantified using the phenol-sulphuric method using glucose as standard (DuBois et al. 1956).

The dry biofilm mass was assessed by the determination of the total volatile solids which are equivalent to the amount of biological mass. Homogenized biofilm suspensions were placed in a furnace at 550 ± 5 °C for 2 h (APHA 1989). The biofilm mass accumulated was expressed in mg of biofilm cm^{-2} of the surface area of the cylinder.

The number of biofilm cells was assessed after staining with the Live/Dead BacLight bacterial viability kit as described above for planktonic studies.

Three independent experiments were performed for biofilm characterization.

The minimum bactericidal concentration of biofilm cells

The five day-old biofilms formed under the three distinct τ_w were removed from the cylinders using a SS scraper and resuspended in an extraction buffer. After extraction of EPS, cell suspensions were separated into two fractions. One fraction was adjusted to 10^8 cells ml^{-1} and used immediately to assess the MBC of the selected biocides, as performed for the planktonic cells. The other fraction was transferred to 20 ml of fresh synthetic medium and put in an orbital shaker (120 rpm, 27 °C) for 24 h. After that incubation

period, the cells were centrifuged (3,777 ×g, 5 min), adjusted to a cell density of 10^8 cells ml^{-1} and used to assess the biocide MBC as described above. At least, five independent experiments were performed for each condition tested.

Biofilm chemical removal

The cylinders plus biofilm were immersed in 300 ml glass flasks containing biocide solution prepared in DW at a final concentration corresponding to 10 × MBC of planktonic cells. The control (untreated biofilm) had an equivalent volume of DW added to it instead of biocide. Biofilm exposure to biocide solutions was carried out at the τ_w (1, 2 or 4 Pa) under which the biofilms were formed, for 30 min (Lemos et al. 2015). After treatment, both biocide solutions plus biofilm detached cells and the remaining biofilm on the SS cylinders were exposed to the biocide neutralizer for 10 min. Biofilm removed bacteria were further harvested by centrifugation at 3,777 ×*g* for 5 min and resuspended in DW to determine the numbers of total and viable cells using the Live/Dead BacLight bacterial viability kit as described above. The cylinders plus biofilm were also used for further experiments on mechanical removal. Three independent experiments were performed for each condition tested.

Biofilm mechanical removal

The cylinders plus biofilm after biocide treatment were exposed to a shear stress of 20 Pa for 1 min to assess the impact of mechanical stress on the removal of the biofilm remaining after the chemical treatment. Experiments were also performed with biofilms not exposed to the biocides, to understand the role of mechanical stress alone. Bacteria removed from the biofilms were characterized in terms of their total and viable number using the Live/Dead BacLight bacterial viability kit. The biofilm remaining from the 20 Pa exposure was removed from the cylinders using a SS scraper and resuspended in DW for the determination of total and viable bacteria. Three independent experiments were performed for each condition tested.

Computational fluid dynamic analysis

Computational fluid dynamic (CFD) analysis was developed in ANSYS Fluent 20. A mesh was created for the working volume (300 ml glass flask with dimensions of 11 cm in length and a diameter of 7.8 cm – the same as for the flasks used for biofilm exposure to biocides), walls, and cylinder (located precisely in the middle of the glass flask) in the form of tetrahedral and hexahedral meshes. The cylinder had a more refined mesh in comparison to the other structures to improve the accuracy of the solution. The mesh had a total of 73,896 nodes and 429,994 elements. The simulations were carried out by creating a moving wall in the cylinder under 1, 2 and 4 Pa of wall τ_w. All the simulations were conducted in the steady state condition, using a second order upwind method and the SIMPLE algorithm was used for pressure-velocity coupling. When the residuals of the numerical solution were reduced to 10^{-4}, the simulation stopped. Using the k-ε model (Ahmed et al. 2012; Benaissa et al. 2012), it was possible to reach a solution with high numerical stability and low computational efforts, while also understanding the mixing conditions and the τ_w on the cylinder surface (Zeng et al. 2016).

Statistical analysis

Data were analyzed by the nonparametric Kruskal–Wallis test using the statistical software SPSS 20.0 (Statistical Package for the Social Sciences). Statistical calculations were based on a confidence level $\geq$ 95%, assuming a significance level for the separation set at $p < 0.05$.

Results and discussion

Hydrodynamic conditions are mainly related to two phenomena in the biofilm development process: mass transport and mechanical stress. For instance, high fluid velocities are associated with increasing mass transport phenomena from the bulk to the surface (Fernandes et al. 2021), and mechanical stress induces biofilm erosion and sloughing (Simões et al. 2010). The consequences from the fluid movement to the biofilm colonizing cells are diverse, i.e. cell viability, growth, metabolism, diversity, quorum sensing – as a consequence of the τ_w, reaction-diffusion phenomena and stochastic events (Lange et al. 2001; Liu and Tay 2002; Kirisits et al. 2007; Rochex et al. 2008; Krsmanovic et al. 2021).

In the current study, the structure of the *P. fluorescens* biofilms was found to be dependent on the τ_w under which they were formed (Figure 1). A τ_w of 1 Pa formed sparse biofilms scattered on the SS surface while increasing the τ_w increased surface coverage. This was particularly noticeable when comparing biofilms formed under 1 Pa with those generated under 4 Pa. Table 1 shows the influence of τ_w on the characteristics of *P. fluorescens* biofilms. Under lower

Figure 1. Visual inspections of SS cylinders without biofilm (a) and covered with 5-day old biofilms formed under a τ_w of 1 (b), 2 (c) and 4 (d) Pa. Cylinder dimensions: diameter = 2.2 cm; length = 5.0 cm.

Table 1. Structural characteristics of *P. fluorescens* biofilms developed on SS cylinders under a wall τ_w of 1, 2 and 4 Pa.

τ_w (Pa)	1	2	4
Thickness (μm)	338 ± 21*	260 ± 39*	118 ± 11*
Biovolume (g cm^{-2})	1.35 ± 0.11*	2.16 ± 0.18*	5.37 ± 0.21*
Cell density (log cells cm^{-2})	7.83 ± 0.62*	8.16 ± 0.41*	8.58 ± 0.55*
Dry mass (mg cm^{-2})	0.455 ± 0.08*	0.561 ± 0.05*	0.634 ± 0.06*
Water content (% of total biofilm mass)	98.8 ± 3.1	95.6 ± 5.6	93.1 ± 1.6
Extracellular polysaccharides (% of total biofilm polysaccharides)	81.5 ± 11*	50.5 ± 8.8*	38.3 ± 14*
Extracellular proteins (% of total biofilm proteins)	79.6 ± 16*	41.9 ± 14*	22.7 ± 6.2*

*Indicate that statistically significant differences were found among different shear stresses ($p < 0.05$). Values are the means ± standard deviations of three independent experiments.

τ_w the biofilms formed were thicker and fluffy, with a lower biovolume, but a higher content of water and extracellular polysaccharides and proteins than those formed under a higher τ_w. Increasing the τ_w increased surface colonisation in terms of cell density and mass, and significantly decreased the production of EPS. These differences were significant ($p < 0.05$) when comparing the values of thickness, biovolume, cell density, dry mass and extracellular polysaccharides and proteins between each of the three τ_w tested. These results demonstrate that the characteristics of biofilms formed on the SS cylindrical surfaces were influenced by the τ_w under which they were formed. The influence of the hydrodynamic conditions on biofilm formation has previously been demonstrated in diverse systems operating under linear flow (Stoodley et al. 1998; Pereira et al. 2002; Simões et al. 2007; Simões et al. 2008). In particular, the results from Simões et al. (2007) corroborate this study. It was reported that the *P. fluorescens* strain used in the present study formed biofilms in a flow cell reactor (with a half-pipe section where biofilms formed on flat surfaces) and a higher Reynolds number formed denser biofilms with reduced content of EPS (Simões et al. (2007). The work of Jones and Buie (2019) further helps in the understanding of the higher cell density found in the present study for the 2 Pa and 4 Pa-generated biofilms, where increased τ_w reduced biofilm development time while increasing its metabolic rate. In accordance with the present results, Paul et al. (2012) also reported an increase in the biofilm density for higher τ_w. Saur et al. (2017) found that that high τ_w increased the number of attached bacteria but also altered their spatial distribution on the substratum surface. Other studies reveal notable evidence for the role of hydrodynamics in shaping biofilm morphology, composition and dynamics (Stoodley et al. 2002; Risse-Buhl et al. 2017). However, it seems consensual that the existence of τ_w promotes thicker and denser biofilm growth when compared with static environments (Krsmanovic et al. 2021). However, the role of hydrodynamic conditions

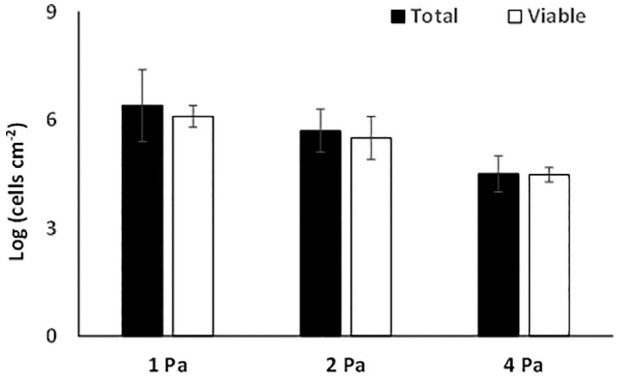

Figure 2. Total and viable cells removed from biofilms developed under the distinct τ_w after exposure to 20 Pa for 1 min. Values are the means ± standard deviations (SDs) of three independent experiments.

on biofilm formation and behaviour is highly dependent on the microorganisms used and the process conditions (i.e. time, type and characteristics of surface material, type and level of nutrients, etc) (Stoodley et al. 2002; Simões et al. 2010).

The development of effective biofilm control strategies depends on having a clear understanding of the parameters and mechanisms underlying biofilm development and behaviour. Although the role of the hydrodynamic conditions on the mechanical properties and composition of biofilms is recognized (Krsmanovic et al. 2021), knowledge on the impact of hydrodynamics in biofilm control remains to be understood. Therefore, in the present work biofilms developed under different hydrodynamic conditions (τ_w of 1, 2 and 4 Pa) were exposed to a τ_w of 20 Pa (Figure 2) to understand the impact of an increase in τ_w in biofilm removal and viability. The results demonstrate that different hydrodynamic conditions during biofilms formation affected biofilm resistance to removal ($p < 0.05$). An increase in τ_w for biofilm formation increased their resistance to removal. However, no effects of biofilm exposure to 20 Pa were observed in the viability of *P. fluorescens*, regardless of the τ_w under which the biofilms were formed ($p > 0.05$). Considering the results of Horn et al. (2003), such modest biofilm removal results are not surprising. These authors reported that biofilms can resist local τ_w values up to a factor of 20 compared with the mean local τ_w acting on the complete biofilm surface. The results further propose that the cell density in the 4 Pa-generated biofilms accounts more significantly for biofilm resilience, when exposed to 20 Pa, than the higher content of extracellular proteins and polysaccharides found on these 1 Pa and 2 Pa-generated (Table 1).

To work out the effects of hydrodynamic conditions for biofilm formation on chemical treatment, the biofilms were exposed to three biocides (BAC, GLUT and SHC). These biocides belong to distinct chemical classes and are known to exert antimicrobial activity through distinct mechanisms (McDonnell and Russell 1999). First, the MBC values of the selected biocides against *P. fluorescens* in suspension following the EN-1276 (2009) protocol were determined. Then, these MBC values were taken as reference for biofilm experiments, where 10 × MBC was used. It was found that the lowest MBC was displayed by SHC (50 mg l^{-1}) followed by BAC (65 mg l^{-1}) and GLUT (160 mg l^{-1}).

A 30 min exposure of biofilms to the biocides at 10 × MBC caused the removal of distinct amounts of cells to the bulk phase (Figure 3). SHC was the most effective biocide in promoting biofilm dispersal and cell killing effects, in contrast with GLUT ($p < 0.05$). Analysis of the viability of *P. fluorescens* biofilm released cells shows that all biocides caused total killing when biofilms were formed under 1 Pa and 2 Pa. The 4 Pa-generated biofilms were the most resistant to removal and a significant number of viable cells were detected in the bulk phase, after exposure to the biocides tested, particularly BAC and SHC ($p > 0.05$). This result suggests that standardized disinfection plans aimed at effective biofilm control will certainly fail if these were developed using cells in the planktonic state or biofilms developed under process conditions far from those encountered in the real environment. In addition, the use of adequate biofilm reactors is a critical aspect of biofilm studies. While microdevices are becoming attractive for biofilm formation and analysis due to their high-throughput status, it is unclear whether they can provide conditions mimicking those found in a real scenario (particularly on industrial surfaces) (Gomes et al. 2014; 2018b). The biofilm formation process due to sedimentation phenomena will likely prevail over convective and diffusive transport when using microdevices. The relative volume of cells per volume of biofilm reactor is another critical aspect when trying to simulate a specific real scenario. Conversely, the system used in the current study is advantageous for biofilm formation and testing as it allows the formation of large amounts of biofilm on cylindrical surfaces and the biocide treatment occurs mostly under the τ_w planed. These conditions avoid the existence of loosely adhering cells, typical from static systems and sediment-based biofilms (Busscher and van der Mei 2006).

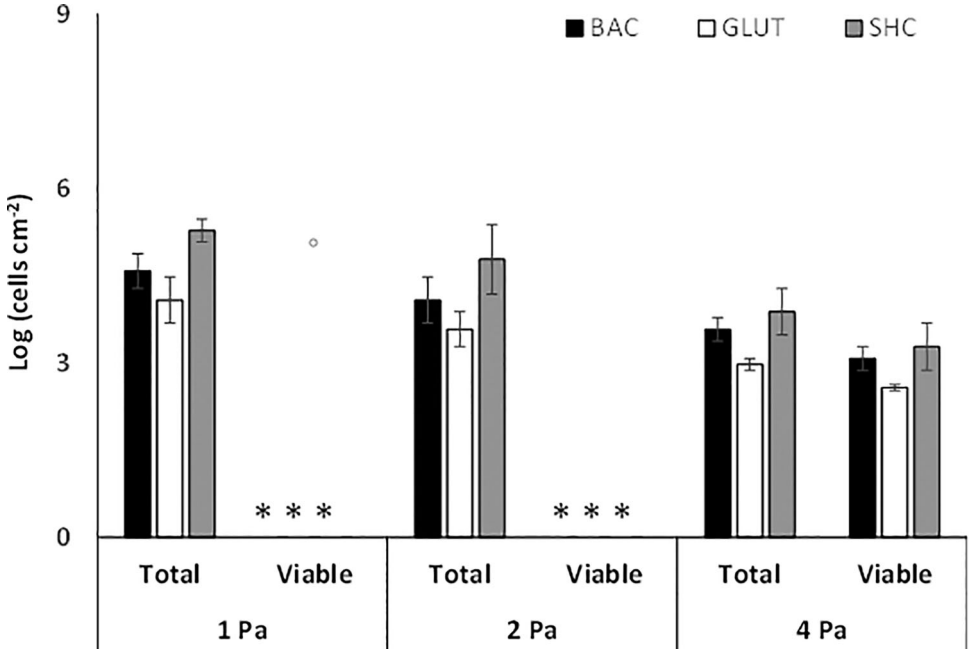

Figure 3. Total and viable cells removed from biofilms developed under the distinct τ_w after exposure to the selected biocides at 10 × MBC. *No viable cells detected. Values are the means ± SDs of three independent experiments.

The MBC of the biocides against biofilm cells was assessed to understand whether biofilm formation under a distinct τ_w affects biocide tolerance. Table 2 shows marked differences in the MBC values, being cells from biofilms formed under higher τ_w and more tolerant to the biocides. It should be noted that, in general, the MBC values are considerably higher than the in-use concentrations of the selected biocides for industrial disinfection (Ferreira et al. 2010; Meireles et al. 2018). This reinforces the inadequacy of planktonic testing methods for evaluating antimicrobial agents to be used as a means of controlling biofilms. In addition, higher heterogeneity in the MBC values was observed when using cells from biofilms formed under 4 Pa, followed by these from 2 Pa-generated biofilms. This behaviour of biofilm cells corroborates the 'insurance hypothesis' of Boles et al. (2004), where inherent biofilm diversity can safeguard the community under adverse conditions. A more drastic physiological adaptation seems to account for biofilm resistance to killing and removal under higher τ_w. The putative presence of persister cells in the biofilms tested cannot be disregarded, particularly for the 2 Pa and 4 Pa-generated ones. Although the presence of persister cells has been mostly studied for antibiotic exposed biofilms, there are reports of the occurrence of persister cells following biocide exposure (Simões et al. 2011; Sanchez-Vizuete et al. 2015).

Lastly, the biofilm cells, in the absence of EPS, were grown planktonically and the MBC values were determined (Table 3). The values obtained were found to be close to those from exclusive planktonic growth. This switch in behaviour when biofilm cells were cultivated in suspension highlights their plasticity and reinforces the persister hypothesis, where biocide susceptibility becomes close to that of the parent population. The persister cells are not believed to be mutants (Lewis 2010).

Biocide-treated biofilms (30 min exposure to biocides at 10 × MBC) were further submitted to 20 Pa of τ_w and the number of total and viable cells

Table 2. Range of MBC values in mg l^{-1} (from five independent experiments) of the selected biocides against *P. fluorescens* biofilm cells (after EPS extraction) formed under 1, 2 and 4 Pa.

	τ_w (Pa)		
Biocide	1	2	4
BAC	[75–110]	[95–135]	[110–155]
GLUT	[180–235]	[200–260]	[210–280]
SHC	[65–85]	[85–110]	[100–140]

Table 3. Range of MBC values in mg l^{-1} (from five independent experiments) of the selected biocides against *P. fluorescens* biofilm cells (after EPS extraction) formed under 1, 2 and 4 Pa and cultivated planktonically for 24 h.

	τ_w (Pa)		
Biocide	1	2	4
BAC	[70–85]	[70–80]	[65–90]
GLUT	[160–180]	[155–190]	[160–180]
SHC	[60–80]	[65–80]	[70–90]

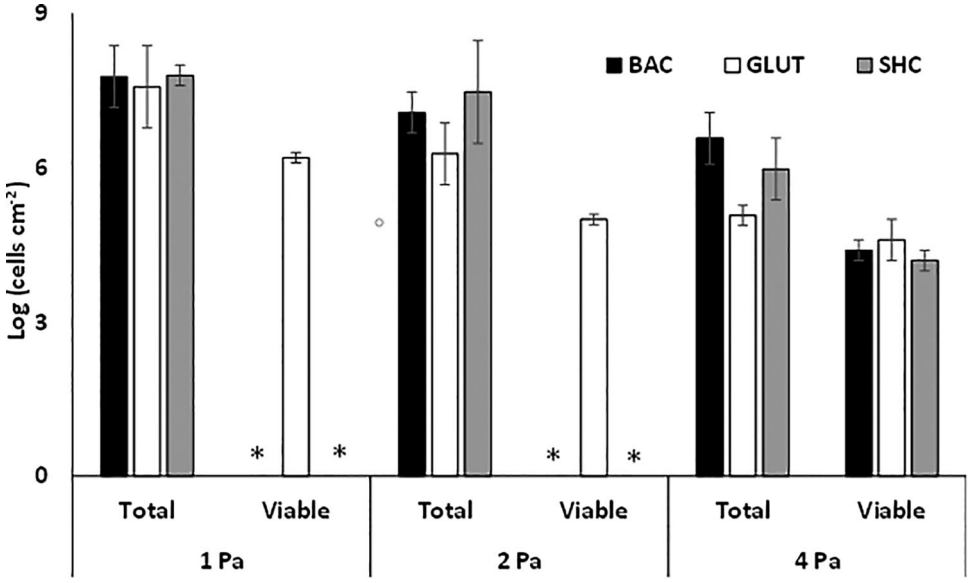

Figure 4. Total and viable cells removed from biofilms developed under the distinct τ_w after sequential exposure to the selected biocides at 10 × MBC and a τ_w of 20 Pa. *No viable cells detected. Values are the means ± SDs of three independent experiments.

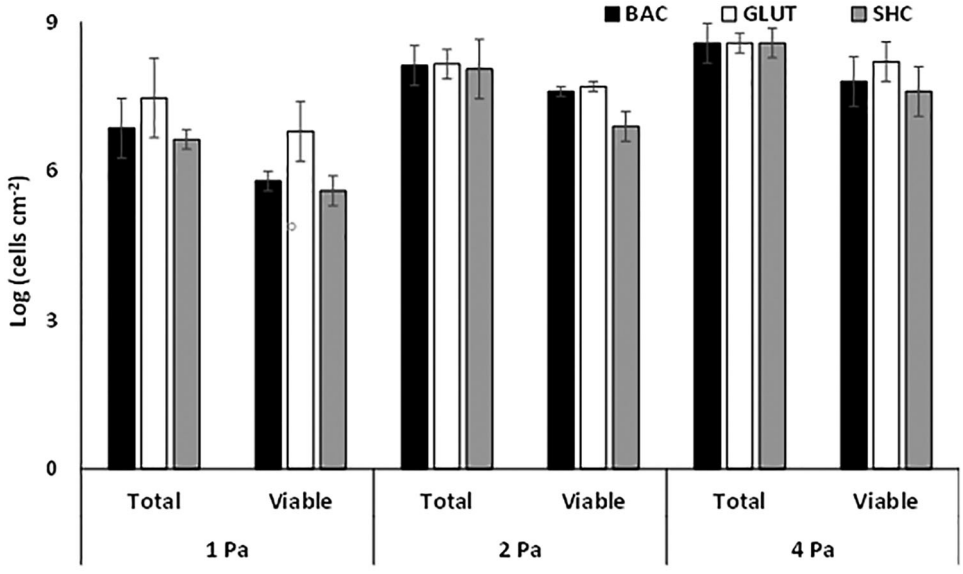

Figure 5. Total and viable cells remaining on the surface of SS cylinders with biofilms developed under the distinct τ_w after sequential exposure to the selected biocides at 10 × MBC and a τ_w of 20 Pa. Values are the means ± SDs of a minimum of three independent experiments.

removed was determined (Figure 4). An increase in biofilm removal was observed from that mechanical treatment, particularly for BAC and SHC pre-exposed biofilms and, in comparison with the use of mechanical stress alone (Figure 2). The comparison between Figures 2 and 4 reveals that GLUT had no effects on biofilm removal ($p > 0.05$). The present results further highlight the role of increasing τ_w under which biofilms are formed on their resilience and tolerance to biocide exposure. While GLUT was ineffective in killing the overall biofilm cells removed, BAC and SHC were only effective against 1 and 2 Pa-generated biofilm released cells ($p < 0.05$). The numbers of total and viable biofilm cells remaining on the surface after both chemical (30 min exposure to biocides at 10 × MBC) and mechanical (20 Pa of τ_w for 1 min) treatments are shown in Figure 5. Biofilms formed under 1 Pa had lower amounts of remaining adhered cells, while the highest numbers were observed for the 4 Pa-generated biofilms ($p < 0.05$). GLUT is highlighted as the less effective biocide, with the following numbers of adhered cells remaining (log cells cm^{-2}):

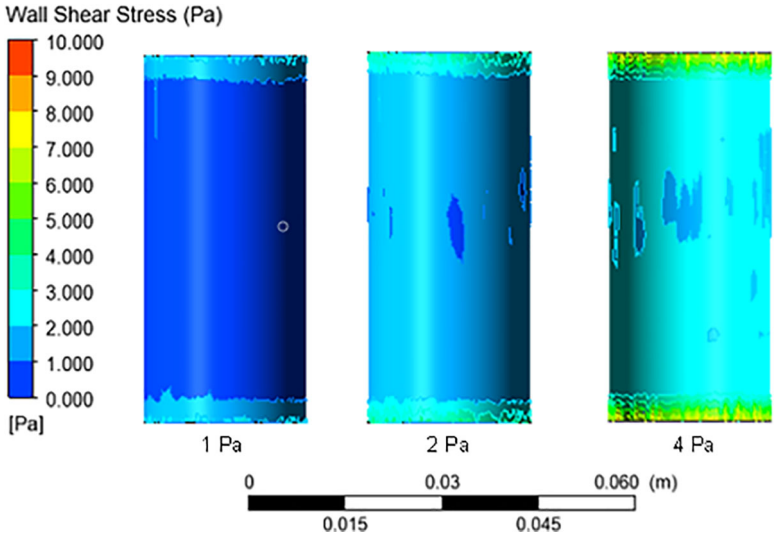

Figure 6. CFD analysis of SS cylinders in a 300 ml flask used for biocide exposure under the three τ_w.

7.5 (1 Pa), 8.2 (2 Pa) and 8.6 (4 Pa). The lowest values were found after SHC exposure: 6.6 (1 Pa), 8.0 (2 Pa) and 8.5 log cells cm^{-2} (4 Pa). In terms of viability most of the adhered cells were in a viable state. The most significant difference between total and remaining viable cells was observed for biofilms formed under 1 Pa and exposed to SHC and BAC (a reduction of 1 log cells cm^{-2}). Biofilms formed under 4 Pa and exposed to GLUT provided the closest amount of total and viable cells (a reduction of 0.4 log cells cm^{-2}). However, the differences between the numbers of total and viable cells are not significant for the biofilms formed under the distinct τ_w and exposed to the same biocide ($p > 0.05$). These results clearly demonstrate the existence of a biofilm basal layer highly tolerant to killing and removal by chemical and mechanical strategies. Such a highly resilient biofilm layer was described in previous studies (Simões et al. 2009; Paul et al. 2012; Lemos et al. 2015; Fanesi et al. 2021). In particular, Paul et al. (2012) showed the presence of a compact biofilm basal layer that resisted shear stresses as high as 13 Pa, where above the basal layer the cohesion was lower and depended on the τ_w applied during biofilm development. Lieleg et al. (2011) also reported that *P. aeruginosa* biofilms show self-healing capabilities, and their mechanical properties remained largely unaffected when exposed to a diversity of chemical molecules, including antibiotics, or large shear stresses. The present study demonstrates that under the set of conditions tested, the dimension and resilience of the basal layer seems to increase with the τ_w under which biofilms were developed. Moreover, the basal layer exists even after exposure to 20 Pa and is highly tolerant to biocidal effects.

CFD studies were performed to understand the τ_w profile on the surface of the cylinders (Figure 6), taking into account that the τ_w will not be perfectly distributed across the surface of the cylinder. For the three hydrodynamic conditions tested there was a higher τ_w zone present in each extremity of the cylinder, an effect apparently unavoidable taking into account the study of Ochoa et al. (2007). However, the most significant surface area was exposed to uniform τ_w values and minor gradients were observed for the conditions tested, highlighting that biofilm exposure to biocides occurred mostly under the planned hydrodynamic conditions.

Conclusions

Generalization regarding biofilm disinfection is inadequate. The hydrodynamic conditions under which biofilms are formed impacts their structure and composition as well as their tolerance to biocides and mechanical stress. In particular, the use of higher τ_w resulted in *P. fluorescens* forming cell-denser and EPS poor biofilms. Biofilm resilience to chemical and mechanical stress increased with the increase in the τ_w under which biofilm was formed. Regarding disinfection testing, BAC and SHC only caused the effective killing of cells dispersed from 1 Pa and 2 Pa-generated biofilms, while a significant number of cells removed from 4 Pa-generated remained in a viable state. The application of biocides at 10 × MBC followed by a τ_w of 20 Pa did not cause effective biofilm removal from the SS surface. A basal layer, highly colonized by viable *P. fluorescens*, was observed for the several conditions tested. The cellular density of this layer increased with an increase in the τ_w under

which the biofilms were formed. The MBC of cells (without EPS) was higher for the biofilms formed under higher τ_w. However, these cells were also able to switch to a state with a susceptibility similar to that of planktonic cells (non-biofilm related), particularly when biofilms were formed under higher τ_w.

Overall, this work highlights the importance of hydrodynamic conditions on biofilm resilience to chemical and mechanical control strategies, further emphasizing the need for the development of adequate biofilm-based tests for biocidal validation.

Disclosure statement

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