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The effect of shear stress on the formation and removal of *Bacillus cereus* biofilms

Madalena Lemos, Filipe Mergulhão, Luís Melo, Manuel Simões *

LEPABE, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, s/n, 4200-465 Porto, Portugal

A b s t r a c t

The influence of the shear stress (r_w) under which biofilms were formed was assessed on their susceptibility to removal when exposed to chemical and mechanical stresses. A rotating cylinder reactor was used to form biofilms, allowing the simulation of r_w conditions similar to those found in industrial settings, particularly in areas with low r_w like elbows, corners, valves and dead zones. *Bacillus cereus* was used as a model bacterium for biofilm formation. Biofilms were formed on AISI316 stainless steel cylinders under different r_w (estimated at 0.02, 0.12 and 0.17 Pa) for 7 days. Some phenotypic characteristics, including thickness, biomass production, cellular density and extracellular proteins and polysaccharides content were assessed. Biofilm density was found to increase significantly with r_w while the thickness decreased. Also, biofilms formed at 0.02 Pa had lowest biomass content, cell density and extracellular polysaccharide content. Those characteristics were not statistically different for the biofilms formed under 0.12 and 0.17 Pa.

Ex situ tests were performed by treating the biofilms with the biocide benzyldimethyldodecyl ammonium chloride (BDMDAC), followed by exposure to increasing r_w conditions, up to 1.84 Pa (whereas the maximum r_w used during growth was 0.17 Pa). The biofilms formed under low r_w were more resistant to removal caused by the BDMDAC action alone. Those formed under higher r_w were more resistant to the mechanical and the combined chemical and mechanical treatments. The amount of biofilm remaining on the cylinders, after both treatments was statistically similar for biofilms formed under 0.12 and 0.17 Pa. The resistance of biofilms to removal by mechanical treatment (alone and combined with BDMDAC) was related to the amount of matrix polysaccharides. However, none of the methods investigated were able to remove all the biofilm from the cylinders.

1. Introduction

There is a lack of efficient strategies to clean stagnant zones in industrial plants (Brooks and Flint, 2008). Crevices, corners, dead zones, valves or areas where the mixing rate is low are almost inevitable. Stagnation promotes bacterial accumulation, ultimately leading to biofouling (Manuel et al., 2010). Biofouling is a damaging problem, affecting the energetic efficiency of industrial processes, causing corrosion of the surfaces, decreasing product quality and eventually promoting the spread of pathogens and resistant infectious diseases (Costerton et al., 1999; Ludensky, 2003; Srey et al., 2013). In industrial settings, surface disinfection is usually focused on the use of biocides, aiming to inactivate the microorganisms (Cloete et al., 1998; Faille et al., 2013). Since biofilms are complex biological structures adhered to a sur- face, these strategies often fail, as the removal of biomass is neglected. Hence, cleaning the biomass from the surfaces is fundamental for controlling biofilm development (Flemming, 2011).

In the biofilm formation process, the hydrodynamic conditions define the transport of the cells, oxygen and nutrients from the bulk fluid to the microbial film (Bryers and Characklis, 1981; Simões et al., 2007b; Stoodley et al., 1999). In fact, the overall flow conditions affect the biofilm structure, as they influence the bulk liquid velocity and shear stress exerted on the surface (Cao and Alaerts, 1995; Peyton, 1996; Vieira and Melo, 1999). The substrate loading rate also affects strongly the transport of the substrate to the cells, therefore influencing their metabolic growth, the production of extracellular polymeric substance (EPS) and ultimately the structure of the biofilm (Vieira et al., 1993).

Diverse studies have demonstrated the influence of hydrodynamic conditions on biofilm behaviour. Douterelo et al. (2013) examined biofilms from drinking water distribution systems and found that the hydraulic regime influenced the bacterial composition and community structure. They also observed that flushing (sudden flow of fluid at high shear stresses inside the system) did not succeed in total biofilm removal but altered the biofilm microbial community. Paul et al. (2012) studied the influence of the substrate and hydrodynamic conditions on biofilm formation and erosion, measuring biofilm thickness and density. Their results showed that increasing the shear stress experienced during growth resulted in biofilms with lower thickness and mass, and higher volumetric density, compared with low shear stress conditions. The authors also found that the biofilms presented stratified cohesion: exposure to shear stress ≤ 2 Pa caused detachment while shear stress >2 Pa caused compression of the biofilm. The effect of environmental conditions on biofilm formation, their structure, composition and physical proper- ties have been reported by Cloete et

al. (2003), Derlon et al. (2008) and Rochex et al. (2008).

The aim of this study was to investigate the influence of the shear stress under which biofilms were formed on their resistance to removal by chemical and mechanical treatments. A rotating cylinder reactor (Simões et al., 2005) was used to form biofilms on stainless steel cylinders at low shear stresses mimicking conditions found in engineered systems. The low shear stresses simulated with this reactor are often found in elbows, valves, dead zones, corners and in sudden pipe expansions (Jensen and Friis, 2005; Lelièvre et al., 2002). Also, typical shear stress values found in drinking water distribution systems are in the range of those used in this study (Gomes et al., 2014).

The combination of mechanical action and chemical treatment was used to challenge biofilms formed by *Bacillus cereus*.

B. cereus is an industrial contaminant and a public health hazard widespread in nature and frequently isolated from dairy products and equipment (Blel et al., 2008; Faille et al., 2014; Lee et al., 2010; Nam et al., 2014; Peng et al., 2002).

2. Materials and methods

2.1. Bacteria and culture conditions

Biofilms were formed by a *B. cereus* strain, previously isolated from a disinfectant solution and identified by 16S rRNA gene sequencing (Simões et al., 2007a). The bacterial growth conditions were 27 ± 1 °C, pH 7 and glucose as the carbon source (Simões et al., 2005).

The bacterium planktonic culture grew in a sterile concentrated nutrient medium (CNM) consisting of 5 g L^{-1} of glucose, 2.5 g L^{-1} of peptone and 1.25 g L^{-1} of yeast extract, in 0.2 M phosphate buffer (KH_2PO_4; Na_2HPO_4) at pH 7. For biofilm formation, a sterile diluted nutrient medium (DNM), which is a 1:100 dilution of the CNM in the same phosphate buffer (PB) was used.

2.2. Antimicrobial chemical

The antimicrobial compound used to challenge the biofilms was benzyldimethyldodecyl ammonium chloride (BDMDAC) (Sigma-Aldrich, Portugal), at a concentration of 300 μg mL^{-1}. This concentration was selected based on previous experiments with chemically related products (Simões et al., 2005).

2.3. *Biofilm formation*

Biofilms were grown on cylinders of AISI316 stainless steel (SS) with a surface area of 34.6 cm^2 (diameter = 2.2 cm, length = 5.0 cm), using a 5 L rotating cylinder reactor (RCR) (Fig. 1). The main reactor contained three cylinders immersed in a bacterial suspension. The three cylinders were driven at the same rotation speed by an overhead stirring engine via a synchronizing belt.

A planktonic culture of *B. cereus* grew in a 0.5 L chemostat fed with the CNM described above, at a flow rate of 10 mL h^{-1}. This chemostat was agitated by a magnetic stirrer and fed the RCR at a steady flow rate, set by gravity, at approximately 0.8 L h^{-1}. The period for biofilm formation and growth was 7 days, in order to obtain steady-state biofilms (Simões et al., 2008). Sterile aeration by filtration was provided to the RCR and to the chemostat.

The RCR was assumed to be an agitated vessel and there- fore the Reynolds number of agitation (Re_A) was calculated according to (Geankoplis, 1993):

$$Re_A = \frac{D_a^2 N \rho}{\mu} \quad (1)$$

where D_a is the diameter of the cylinder; N is the rotation speed, ρ is the fluid density and μ is the fluid viscosity.

The Fanning friction factor establishes the relation between the r_w and the velocity head $\rho V^2/2$, and is defined by (Perry and Green, 1999):

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

For the RCR, the relationship between the f and the Re_A for a rotating electrode under turbulent flow conditions (the critical Re_A is 200) by Gabe and Walsh (1983) was used:

$$f = 0.158 Re_A^{-0.3} \quad (3)$$

Table 1 presents the Re_A and the r_w values estimated at each rotation speeds used in this study.

2.4. *Biofilm sampling for characterization*

The cylinders were removed from the reactor and the biofilm wet biomass and thickness were measured. The biofilm (chemically untreated) was then removed using a stainless steel scraper, resuspended in 10 mL of PB, and homogenized by vortexing (Heidolph, model Reax top) for 30 s at 100% power input.

After homogenization, the biofilm suspensions were characterized in terms of total and matrix content (proteins and polysaccharides), biomass amount and cell (vegetative cells and spores) density. Three independent biofilm formation experiments were performed for each case studied.

2.4.1. *Thickness*

Biofilm thickness was determined using a digital micrometer (VS-30H, Mitsubishi Kasei Corporation) according to Teodósio et al. (2011).

2.4.2. *Biomass quantification*

For the determination of the wet mass, the cylinders were removed from the RCR and the biofilm accumulated on the top and bottom surfaces was discarded. The cylinders were then weighed and the wet mass obtained by subtracting the mass of the clean cylinders (without biofilm).

The dry mass of the biofilms was assessed by the determination of the total volatile solids (TVS) of the homogenized biofilm suspensions according to standard methods (American Public Health Association [APHA], American Water Works Association [AWWA], Water Pollution Control Federation [WPCF], 1989).

The water content was estimated as the difference between the wet mass and the dry mass. The biofilm density was calculated using biofilm dry mass and the volume of the biofilm, estimated from its thickness and the adhesion surface area (Melo and Bott, 1997).

2.4.3. *Cellular density*

For the enumeration of the total cell density, an aliquot of the homogenized biofilm suspension was microfiltered through a 0.22 μm Nucleopore® (Whatman, Middlesex, UK) black polycarbonate membrane. The membrane was stained with 4',6-diamidino-2-phenylindole (DAPI, VWR, Portugal). After 10 min of incubation in the dark, the membranes were mounted with non-fluorescent immersion oil on glass micro- scope slides, and the total cell counts were assessed using epifluorescence microscopy (LEICA DMLB2) as described by Lemos et al. (2013). *B. cereus* spore numbers were assessed by surface plating

(300 mL sample) after biofilm suspension heat treatment (80 °C, 5 min). The plates of solid CNM (13 g L^{-1} agar) were incubated at 27 °C for 72 h.

2.4.4. *Protein and polysaccharide quantification* Biofilm EPS (proteins and polysaccharides) extraction was pre- formed according to a previously described method (Frølund et al., 1996). The procedure for total (before EPS extraction) and extracellular biofilm protein quantification used was the Lowry et al. (1951) method as modified by Peterson (1979), using the Total Protein Kit, Micro Lowry, Peterson's Mod- ification (Sigma, Portugal), with bovine serum albumin as standard. The total and extracellular polysaccharides were quantified through the phenol–sulphuric acid method of Dubois et al. (1956), with glucose as standard.

2.5. *Biofilm chemical treatment*

The cylinders with biofilm were removed from the reactor and immersed in 250 mL glass beakers containing 200 mL of BDMDAC solution. Exposure to chemical treatment was carried out for 30 min, under constant r_w, equal to that used for biofilm growth. After exposure, a neutralization step (expo- sure to 0.2 M PB solution for 10 min, to dilute any BDMDAC present to residual levels) was performed to quench the residual antimicrobial activity of BDMDAC, according to Johnston et al. (2002). The wet weight of the cylinders plus biofilm attached was determined before and after the exposure. The amount of biofilm removed due to BDMDAC exposure was expressed in terms of mg cm^{-2}.

2.6. *Biofilm removal by hydrodynamic stress*

The biofilm removal by mechanical action was tested using the method described by Simões et al. (2005). Biofilm layers were removed by exposing the biofilms at rotational flow with increasing r_w during 30 s (r_w going from 0.07; 0.17; 0.57; 1.13 to 1.84 Pa, with 30 s exposure to each r_w). The wet weight of the cylinders plus biofilm attached was determined before the mechanical treatment and after exposure to r_w at 1.84 Pa. The same procedure was followed with the control assay, i.e. with the cylinder plus biofilm immersed in the PB solution (i.e. not exposed to BDMDAC). The residual biofilms, covering the cylinders, were removed completely with a stainless steel scraper and then the weight of cylinders without biofilm was measured, to assess the biofilm remaining after the mechanical treatment. The amount of biofilm remaining on the cylinders

surface was expressed in terms of mg cm^{-2}.

2.7. *Statistical analysis*

Data was analysed using the statistical program SPSS version 21.0 (Statistical Package for the Social Sciences). The mean and standard deviation within samples were calculated for all cases. At least three independent experiments were per- formed for each condition tested. All data were analysed by the application of the non-parametric Kruskal–Wallis test (confidence level ≥95%).

3. Results and discussion

The biofilm structure is determined essentially by the nature of the microorganisms and by the environmental conditions under which they are formed (Melo and Bott, 1997; van Loosdrecht et al., 1995). In this study, *B. cereus* biofilms were formed under different r_w (0.02, 0.12 and 0.17 Pa), over 7 days, using the RCR with constant nutrient loading rate, temperature and pH. The biofilms formed presented different characteristics (Table 2). Only the water content was statistically similar for the three biofilms ($P > 0.05$). These biofilms were mostly composed of water (>93% of the total mass). Higher r_w applied during formation resulted in lower biofilm thickness ($P < 0.05$). The thickness of a biofilm grown under 0.02 Pa was about three times higher than those formed under 0.17 Pa. The biofilms formed under 0.12 Pa were twice as thick as those formed under 0.17 Pa. On the other hand, increasing r_w caused an increase ($P < 0.05$) in the biofilm mass, volumetric density and cell density ($P < 0.05$). Previous works showed similar trends regarding the effects of hydrodynamic conditions on biofilm thickness, volumetric density and cell density (Coufort et al., 2007; Melo and Bott, 1997; Paul et al., 2012; Simões et al., 2007b). It is known that the flow velocity affects the transport of substrate to the surface of the biofilm, influencing microbial metabolism and growth (Simões et al., 2007b; Tsai, 2005). The main reasons for the distinct structure, physiological composition and metabolic characteristics of biofilms formed under distinct hydrodynamic conditions are attributed to the different transport rates of oxygen, nutrients and cells from the fluid to the biofilm, the effect of flow conditions on the structural plasticity of biofilms (mass transfer limitations) and the cellular induced reactions, acting as single or concomitant factors (Hall-Stoodley and Stoodley, 2002; Liu and Tay, 2001; Vieira et al., 1993). The influence of hydro- dynamic conditions on biofilm formation and characteristics might help to explain the increase in the dry mass and cell density and decrease in the biofilm thickness,

with increasing of r_w The differences in biofilm characteristics are more significant when comparing the biofilms formed under 0.02 Pa with those formed under higher r_w. The biofilm mass and cell density was not significantly different when the biofilms were formed under 0.12 and 0.17 Pa ($P > 0.05$). A previous study (Simões et al., 2007c) with different strains of *Pseudomonas fluorescens* demonstrated the presence of higher cell counts for turbulent flow-generated biofilms than for those formed under laminar flow. However, those biofilms were formed in a flow cell reactor, where the hydrodynamic regimes were laminar flow (*Re* = 2000, corresponding to a linear velocity of v = 0.20 m s^{-1}) and transition/turbulent flow (*Re* = 4200, v = 0.53 m s^{-1}), whereas in this work the *Re*A was essentially turbulent (above 100) and the maximum linear velocity used (for the highest *Re*A) was 0.51 m s^{-1}.

The numbers of spores were negligible (always lower than 2.2 $\log_{10}$ cells cm^{-2}) comparatively to the numbers of vegetative cells, for the three biofilms. This result corroborates previous findings where *B. cereus* biofilms had residual numbers of spores comparatively to the numbers of vegetative cells (Ryu and Beuchat, 2005; Simões et al., 2008). However, Faille et al. (2014) found that sporulation occurred in biofilms formed by *Bacillus* strains and suggested that biofilms would be a significant source of food contamination with spores. It is possible that the presence of spores in the biofilm may depend on the microbial strain and on the process conditions used, including the type and mode of operation of the bioreactor used for biofilm formation.

The biofilms presented different amounts of matrix proteins and polysaccharides (Table 2). The percentage of matrix polysaccharides was higher for the biofilms formed under 0.12 and 0.17 Pa. It has been proposed (Lazarova et al., 1994; Liu and Tay, 2002; Tay et al., 2001) that high detachment forces can induce the biofilms to secrete more EPS. However, there was no relationship apparent between the percentage of matrix proteins and polysaccharides r_w increased. In fact, the biofilms formed at 0.12 Pa presented the highest percentage of both proteins and polysaccharides.

A combined strategy of chemical and mechanical stress was applied to the *B. cereus* biofilms formed in the RCR in order to promote biofilm removal. This was performed by exposing the biofilms to BDMDAC at the same r_w used for its formation. This process was followed by exposing the biofilm to a series of increasing r_w values. The amount of biofilm mass removed with the chemical treatment is presented in Fig. 2. The highest amount of biomass removed was observed for the biofilms formed under 0.17 Pa while those formed at 0.02 Pa where the least affected by BDMDAC. In terms of percentage of the initial biofilm

that was removed the highest percentage was observed for biofilms formed at 0.17 Pa and the lowest percentage was obtained for biofilms formed at 0.12 Pa.

Quaternary ammonium compounds have demonstrated their potential to remove biofilms (Amalaradjou and Venkitanarayanan, 2014; Peng et al., 2002; Trueba et al., 2013). To our knowledge, this is the first study on the effects of BDMDAC on biofilm removal. It was found that BDMDAC only induced modest biofilm removal, being the amount of biomass removed lower for the biofilms formed under lower r_W. In fact, the use of high r_W during biofilm formation cause the compression of the matrix and facilitate the mass transfer of the biocides, allowing the complete penetration of the microbial layers (van Loosdrecht et al., 1995). Therefore, it is not surprising that the amount of biomass removed due to BDMDAC was higher for those biofilms formed under 0.17 Pa, being followed by those formed under 0.12 Pa.

The amount of biofilm remaining on the cylinders surface after the mechanical treatment and the synergic chemical and mechanical treatments is presented in Fig. 3. The application of increasing r_W was not sufficient to remove all the biofilm from the surfaces, neither was its synergy with BDMDAC. The biofilms formed under 0.02 Pa were the least resistant to both mechanical and combined chemical and mechanical treatments ($P < 0.05$). The amount of biomass remaining on the cylinders was higher for the biofilms formed at 0.12 and 0.17 Pa, after biofilm exposure to the mechanical treatment alone and combined with BDMDAC. The amount of biofilm remaining was statistically similar for both biofilms ($P > 0.05$).

For the three conditions, the synergy between the chemical and mechanical treatments removed an additional fraction of biofilm ($P < 0.05$), when compared with the mechanical treatment alone. This effect was more significant for those biofilms formed under higher shear stresses ($P < 0.05$).

Comparing the results from Figs. 2 and 3 it can be seen that biofilm formation under the lowest r_W increased biofilm removal due to chemical treatment (on an absolute mass basis). However, the biofilms formed under higher r_W were more resistant to removal due to the mechanical treatment alone and the combined with BDMDAC. A higher amount of biomass remained adhered to the surface. The results also show that the susceptibility to the mechanical and to the combined chemical and mechanical treatments was similar for the biofilms formed under 0.12 and 0.17 Pa ($P > 0.05$), even if the highest amounts of biofilm remaining were found for those biofilms formed under 0.12 Pa.

The resistance of biofilms was correlated with the percentage of matrix polysaccharides, when mechanical stress was used. The biofilms formed at 0.12 Pa demonstrated the highest resilience to mechanical stress (alone and combined

with BDMDAC). These biofilms had the highest percentages of matrix polysaccharides and proteins. EPS is known to strengthen the cohesive forces within the biofilm, thereby contributing to an enhanced inherent biofilm mechanical stability (Körstgens et al., 2001).

For all conditions tested, a layer of residual biofilm was found on the cylinder surface. It is interesting to note that after the combined treatment with both mechanical and mechanical actions there was always a layer of about 25% of the total biofilm mass that was still attached to the cylinders sur- face, for the three biofilms tested. Coufort et al. (2007) stated that whatever the environmental conditions are used during formation, biofilms will present a stratified structure where layers present different cohesion from the top to the bottom (close to the substratum). Later, Paul et al. (2012) also proposed the existence of a stratified structure of mature biofilms, with a strongly cohesive and dense basal layer. The results obtained in this study also indicate that the *B. cereus* biofilms had a basal layer strongly resistant to removal by chemical and mechanical action, regardless of the r_w value under which the biofilms were formed. Even if the biofilms were treated with a biocide with recognized antimicrobial efficacy (Ferreira et al., 2010, 2011), BDMDAC only promoted modest biofilm removal. In real process conditions, this result means that the sanitation strategy was not effective, and that this basal layer will help reseeding a new biofilm possibly with higher resistant and resilient properties than its predecessor.

4. Conclusions

The RCR allowed the formation of *B. cereus* biofilms under different hydrodynamic conditions. Increasing the r_w experienced during biofilm formation resulted in biofilms with lower thickness, higher dry mass, and higher volumetric and cell densities.

The biofilms formed under low r_w were more resistant to removal due to BDMDAC action alone since the low amounts of biomass were removed from the surface. However, the biofilms formed under higher r_w were more resistant to the mechanical and the combined chemical and mechanical treatments. The amount of biofilm remaining after mechanical or combined chemical and mechanical treatments was similar for both biofilms formed under 0.12 and 0.17 Pa.

The combined action of BDMDAC and mechanical treatment provided additional biofilm removal when compared with the single chemical or mechanical treatments. However, total biofilm removal was not achieved, neither by the use of mechanical treatment alone, neither with its combination

with BDMDAC. All the biofilms had a basal layer (about 25% of the initial biofilm mass) strongly resistant to removal by chemical and mechanical actions, which may promote the reseed of the biofilm after sanitation procedures.

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References

Amalaradjou, M., Venkitanarayanan, K., 2014. Antibiofilm effect of octenidine hydrochloride on *Staphylococcus aureus*, MRSA and VRSA. Pathogens 3 (2), 404–416.

APHA, AWWA and WPCF, 1989. Standard methods for the examination of water and wastewater. American Public Health Association, Washington DC, USA.

Blel, W., Legentilhomme, P., Legrand, J., Bénézech, T., Gentil-Lelièvre, C.L., 2008. Hygienic design: effect of hydrodynamics on the cleanability of a food processing line. AlChE J. 54 (10), 2553–2566.

Brooks, J.D., Flint, S.H., 2008. Biofilms in the food industry: problems and potential solutions. Int. J. Food Sci. Technol. 43 (12), 2163–2176.

Bryers, J., Characklis, W., 1981. Early fouling biofilm formation in a turbulent flow system: overall kinetics. Water Res. 15 (4), 483–491.

Cao, Y.S., Alaerts, G.J., 1995. Influence of reactor type and shear stress on aerobic biofilm morphology, population and kinetics. Water Res. 29 (1), 107–118.

Cloete, T.E., Jacobs, L., Brözel, V.S., 1998. The chemical control of biofouling in industrial water systems. Biodegradation 9 (1), 23–37.

Cloete, T.E., Westaard, D., Van Vuuren, S.J., 2003. Dynamic response of biofilm to pipe surface and fluid velocity. Water Sci. Technol. 47 (5), 57–59.

Costerton, J.W., Stewart, P.S., Greenberg, E.P., 1999. Bacterial biofilms: a common cause of persistent infections. Science 284 (5418), 1318–1322.

Coufort, C., Derlon, N., Ochoa-Chaves, J., Liné, A., Paul, E., 2007. Cohesion and detachment in biofilm systems for different electron acceptor and donors. Water Sci. Technol. 55 (8-9), 421–428.

Derlon, N., Massé, A., Escudié, R., Bernet, N., Paul, E., 2008. Stratification in the cohesion of biofilms grown under various environmental conditions. Water Res. 42 (8–9), 2102–2110.

Douterelo, I., Sharpe, R.L., Boxall, J.B., 2013. Influence of hydraulic regimes on bacterial community structure and composition in an experimental drinking water distribution system. Water Res. 47 (2), 503–516.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28 (3), 350–356.

Faille, C., Bénézech, T., Blel, W., Ronse, A., Ronse, G., Clarisse, M., Slomianny, C., 2013. Role of mechanical vs. chemical action in the removal of adherent *Bacillus* spores during CIP procedures. Food Microbiol. 33 (2), 149–157.

Faille, C., Bénézech, T., Midelet-Bourdin, G., Lequette, Y., Clarisse, M., Ronse, G., Ronse, A., Slomianny, C., 2014. Sporulation of *Bacillus* spp. within biofilms: a potential source of contamination in food processing environments. Food Microbiol. 40 (0), 64–74.

Ferreira, C., Rosmaninho, R., Simões, M., Pereira, M.C., Bastos, M.M.S.M., Nunes, O.C., Coelho, M., Melo, L.F., 2010. Biofouling control using microparticles carrying a biocide. Biofouling 26 (2), 205–212.

Ferreira, C., Pereira, A.M., Pereira, M.C., Melo, L.F., Simões, M., 2011. Physiological changes induced by the quaternary ammonium compound benzyldimethyldodecylammonium chloride on *Pseudomonas fluorescens*. J. Antimicrob. Chemother. 66 (5), 1036–1043.

Flemming, H.-C., 2011. Microbial biofouling: unsolved problems, insufficient approaches, and possible solutions. In: Flemming, H.-C., Wingender, J., Szewzyk, U. (Eds.), Biofilm Highlights. Springer, Berlin Heidelberg, pp. 81–109.

Frølund, B., Palmgren, R., Keiding, K., Nielsen, P.H., 1996. Extraction of extracellular polymers from activated sludge using a cation exchange resin. Water Res. 30 (8), 1749–1758.

Gabe, D.R., Walsh, F.C., 1983. The rotating cylinder electrode: a review of development. J. Appl. Electrochem. 13 (1), 3–21.

Geankoplis, C.J. (Ed.), 1993. Transport Processes and Unit Operations. Prentice-Hall International, Inc, New Jersey.

Gomes, I.B., Simões, M., Simões, L.C., 2014. An overview on the reactors to study drinking water biofilms. Water Res. 62 (0), 63–87.

Hall-Stoodley, L., Stoodley, P., 2002. Developmental regulation of microbial

biofilms. Curr. Opin. Biotechnol. 13 (3), 228–233.

Jensen, B.B.B., Friis, A., 2005. Predicting the cleanability of mix-proof valves by use of wall shear stress. J. Food Process Eng. 28 (2), 89–106.

Johnston, M.D., Lambert, R.J.W., Hanlon, G.W., Denyer, S.P., 2002. A rapid method for assessing the suitability of quenching agents for individual biocides as well as combinations. J. Appl. Microbiol. 92 (4), 784–789.

Körstgens, V., Flemming, H.C., Wingender, J., Borchard, W., 2001. Uniaxial compression measurement device for investigation of the mechanical stability of biofilms. J. Microbiol. Methods 46 (1), 9–17.

Lazarova, V., Pierzo, V., Fontvielle, D., Manem, J., 1994. Integrated approach for biofilm characterisation and biomass activity control. Water Sci. Technol. 29 (7), 345–354.

Lee, M.J., Ha, J.H., Kim, Y.S., Ryu, J.H., Ha, S.D., 2010. Reduction of *Bacillus cereus* contamination in biofilms on stainless steel surfaces by application of sanitizers and commercial detergent. J. Korean Soc. Appl. Biol. Chem. 53 (1), 89–93.

Lelièvre, C., Legentilhomme, P., Gaucher, C., Legrand, J., Faille, C., Bénézech, T., 2002. Cleaning in place: effect of local wall shear stress variation on bacterial removal from stainless steel equipment. Chem. Eng. Sci. 57 (8), 1287–1297.

Lemos, M., Borges, A., Teodósio, J., Araújo, P., Mergulhão, F., Melo, L., Simões, M., 2013. The effects of ferulic and salicylic acids on *Bacillus cereus* and *Pseudomonas fluorescens* single- and dual-species biofilms. Int. Biodeterior. Biodegrad. 86 (Part A), 42L 51.

Liu, Y., Tay, J.-H., 2001. Detachment forces and their influence on the structure and metabolic behaviour of biofilms. World J. Microbiol. Biotechnol. 17 (2), 111–117.

Liu, Y., Tay, J.-H., 2002. The essential role of hydrodynamic shear force in the formation of biofilm and granular sludge. Water Res. 36 (7), 1653–1665.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193 (1), 265–275.

Ludensky, M., 2003. Control and monitoring of biofilms in industrial applications. Int. Biodeter. Biodegrad. 51 (4), 255–263.

Manuel, C.M., Nunes, O.C., Melo, L.F., 2010. Unsteady state flow and stagnation in distribution systems affect the biological stability of drinking water. Biofouling 26 (2), 129–139.

Melo, L.F., Bott, T.R., 1997. Biofouling in water systems. Exp. Therm. Fluid Sci. 14 (4), 375–381.

Nam, H., Seo, H.-S., Bang, J., Kim, H., Beuchat, L.R., Ryu, J.-H., 2014. Efficacy of gaseous chlorine dioxide in inactivating *Bacillus cereus* spores attached to and

in a biofilm on stainless steel. Int. J. Food Microbiol. 188 (0), 122–127.

Paul, E., Ochoa, J.C., Pechaud, Y., Liu, Y., Liné, A., 2012. Effect of shear stress and growth conditions on detachment and physical properties of biofilms. Water Res. 46 (17), 5499–5508.

Peng, J.S., Tsai, W.C., Chou, C.C., 2002. Inactivation and removal of *Bacillus cereus* by sanitizer and detergent. Int. J. Food Microbiol. 77 (1-2), 11L 18.

Perry, R.H., Green, D.W., 1999. Perry's Chemical Engineers' Handbook. McGraw-Hill Professional Publishing, New York.

Peterson, G.L., 1979. Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Anal. Biochem. 100 (2), 201–220.

Peyton, B.M., 1996. Effects of shear stress and substrate loading rate on *Pseudomonas aeruginosa* biofilm thickness and density. Water Res. 30 (1), 29–36.

Rochex, A., Godon, J.-J., Bernet, N., Escudié, R., 2008. Role of shear stress on composition, diversity and dynamics of biofilm bacterial communities. Water Res. 42 (20), 4915–4922.

Ryu, J.H., Beuchat, L.R., 2005. Biofilm formation and sporulation by *Bacillus cereus* on a stainless steel surface and subsequent resistance of vegetative cells and spores to chlorine, chlorine dioxide, and a peroxyacetic acid-based sanitizer. J. Food Prot. 68 (12), 2614–2622.

Simões, L.C., Simões, M., Oliveira, R., Vieira, M.J., 2007 a. Potential of the adhesion of bacteria isolated from drinking water to materials. J. Basic Microbiol. 47 (2), 174–183.

Simões, M., Pereira, M.O., Vieira, M.J., 2005. Effect of mechanical stress on biofilms challenged by different chemicals. Water Res 39 (20), 5142–5152.

Simões, M., Pereira, M.O., Sillankorva, S., Azeredo, J., Vieira, M.J., 2007 b. The effect of hydrodynamic conditions on the phenotype of *Pseudomonas fluorescens* biofilms. Biofouling 23 (4), 249–258.

Simões, M., Pereira, M.O., Vieira, M.J., 2007 c. The role of hydrodynamic stress on the phenotypic characteristics of single and binary biofilms of *Pseudomonas fluorescens*? Water Sci. Technol. 55 (8–9), 437–445.

Simões, M., Simoes, L.C., Pereira, M.O., Vieira, M.J., 2008. Antagonism between *Bacillus cereus* and *Pseudomonas fluorescens* in planktonic systems and in biofilms. Biofouling 24 (5), 339–349.

Srey, S., Jahid, I.K., Ha, S.-D., 2013. Biofilm formation in food industries: a food safety concern. Food Control 31 (2), 572–585.

Stoodley, P., Dodds, I., Boyle, J.D., Lappin-Scott, H.M., 1999. Influence of hydrodynamics and nutrients on biofilm structure. J. Appl. Microbiol. 85 (Suppl. 1), S19–S28.

Tay, J.H., Liu, Q.S., Liu, Y., 2001. The effects of shear force on the formation,

structure and metabolism of aerobic granules. Appl. Microbiol. Biotechnol. 57 (1-2), 227L 233.

Teodósio, J.S., Simões, M., Melo, L.F., Mergulhão, F.J., 2011. Flow cell hydrodynamics and their effects on *E. coli* biofilm formation under different nutrient conditions and turbulent flow. Biofouling 27 (1), 1–11.

Trueba, A., Otero, F.M., González, J.A., Vega, L.M., García, S., 2013. Study of the activity of quaternary ammonium compounds in the mitigation of biofouling in heat exchangers–condensers cooled by seawater. Biofouling 29 (9), 1139–1151.

Tsai, Y.-P., 2005. Impact of flow velocity on the dynamic behaviour of biofilm bacteria. Biofouling 21 (5–6), 267L 277.

van Loosdrecht, M.C.M., Eikelboom, D., Gjaltema, A., Mulder, A., Tijhuis, L., Heijnen, J.J., 1995. Biofilm structures. Water Sci. Technol. 32 (8), 35–43.

Vieira, M.J., Melo, L.F., Pinheiro, M.M., 1993. Biofilm formation: hydrodynamic effects on internal diffusion and structure. Biofouling 7 (1), 67–80.

Vieira, M.J., Melo, L.F., 1999. Intrinsic kinetics of biofilms formed under turbulent flow and low substrate concentrations.

Bioprocess Eng. 20 (4), 369–375.

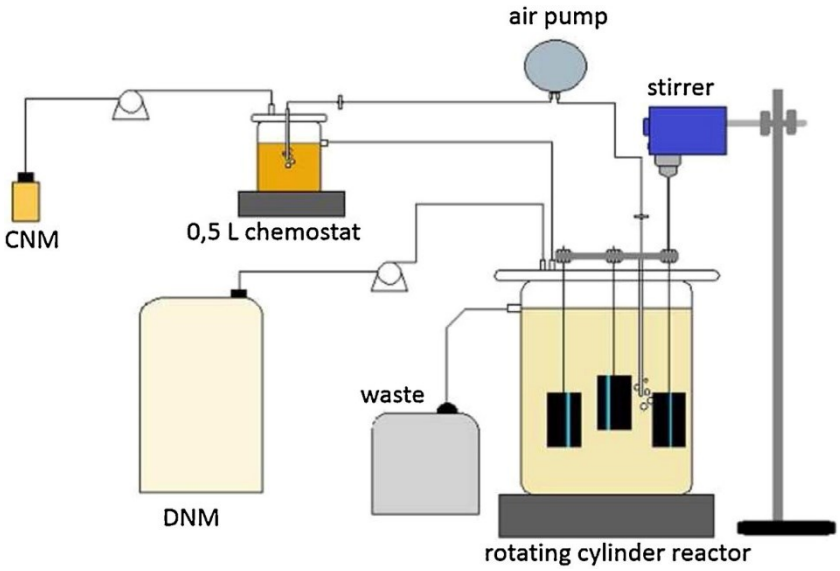

Fig. 1 – Schematic representation of the RCR system.

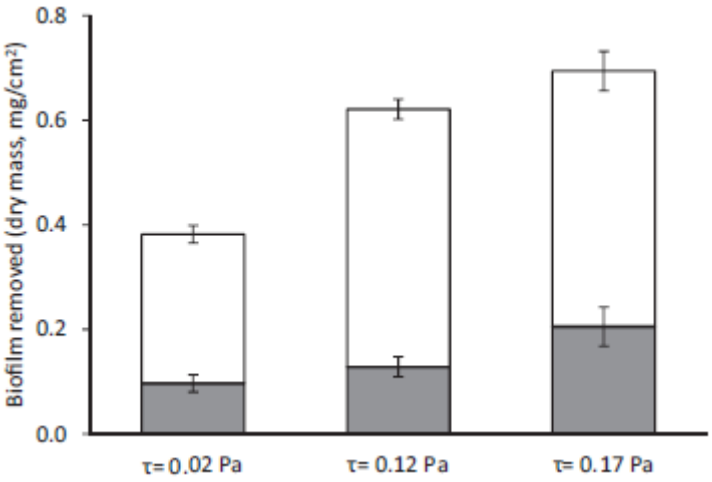

Fig. 2 – Biofilm removed (·) after submitting the biofilms to BDMDAC treatment for 30 min. The white bar represents the amount of biofilm remaining after the treatment and the complete bar represents the amount of biofilm formed, over 7 days. The means ± SDs for at least three replicates are plotted.

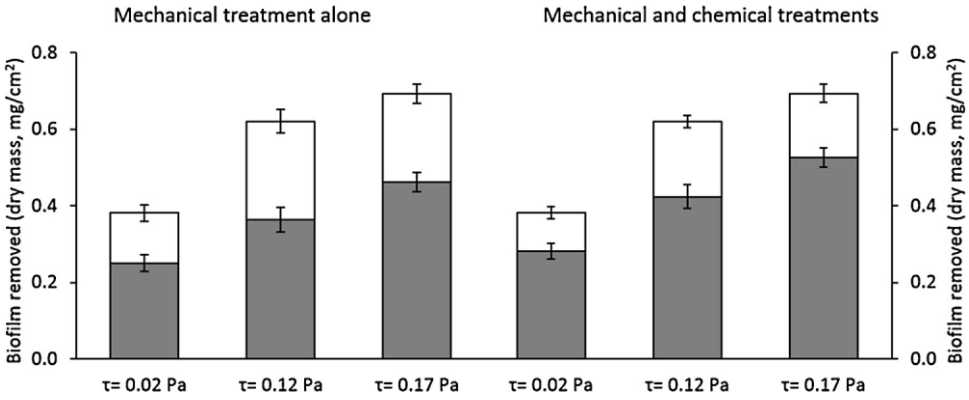

Fig. 3 – Biofilm removed (·) after submitting the biofilms to the mechanical treatment alone and to the combination of mechanical and chemical treatments. The white bar represents the amount of biofilm remaining after the treatment and the complete bar represents the amount of biofilm formed, over 7 days. The means ± SDs for at least three replicates are plotted.

Table 1 – Estimated values for τ_w and Re_A at the rotation speeds used in this study.

N (s^{-1})	Re_A	τ_w (Pa)
1.8	1000	0.02
5.9	3200	0.12
7.4	4000	0.17

Table 2 – Characteristics of *B. cereus* biofilms formed under different τ_w. These are the characteristics of the biofilms before any chemical or mechanical treatment. Values reported are the mean ± SD for at least three replicates.

τ_w (Pa)	0.02	0.12	0.17
Thickness (μm)	298 ± 17	220 ± 10	108 ± 20
Dry mass (mg cm^{-2})	0.382 ± 0.03	0.621 ± 0.02	0.694 ± 0.05
Volumetric density (mg cm^{-3})	12.9 ± 0.7	28.4 ± 1.3	66.8 ± 12.1
Cellular density (log_{10} cells cm^{-2})	7.60 ± 0.58	8.13 ± 0.09	8.37 ± 0.52
Water content (% of total biofilm mass)	97.0 ± 0.03	93.9 ± 0.37	95.1 ± 0.35
Extracellular polysaccharides (% of total biofilm polysaccharides)	31.5 ± 6.7	50.5 ± 0.7	40.4 ± 10.7
Extracellular proteins (% of total biofilm proteins)	29.5 ± 4.6	41.9 ± 1.6	22.7 ± 0.4