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The effects of surface type on the removal of *Bacillus cereus* and *Pseudomonas fluorescens* single and dual species biofilms

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Abstract

The aim of this work was to assess the effectiveness of the biocide benzyldimethyldodecyl ammonium chloride (BDMDAC) on the removal of single and dual species biofilms of *Bacillus cereus* and *Pseudomonas fluorescens* formed in a rotating cylinder reactor (RCR), using AISI316 stainless steel (SS) and polymethyl methacrylate (PMMA) as adhesion surfaces. Additional tests were performed to understand the adhesion of *B. cereus* and *P. fluorescens* to the selected surfaces.

Predictions of the adhesion potential according to the thermodynamic theory showed more favourable adhesion on SS than on PMMA, for both species. Thermodynamically, adhesion was more favourable for *B. cereus*. After BDMDAC treatment, thermodynamic adhesion ability was favoured for *P. flurescens* and decreased for *B. cereus*, mainly on PMMA. Both bacteria had negative surface charge and the exposure to BDMDAC increased the charge to less negative values. *In vitro* adhesion results were, for most cases, contradictory to those predicted by the thermodynamic theory. Single and dual species biofilms were formed in the RCR for 7 days. Afterwards, the biofilms were exposed to the chemical (use of BDMDAC) and to hydrodynamic stresses (use of increasing Reynolds number of agitation), alone and combined. The applications of BDMDAC or hydrodynamic stress, when applied alone, were insufficient to remove the biofilms from the surfaces. The combined effects of BDMDAC with a series of increasing Reynolds number of agitation promoted additional biofilm removal. This effect was dependent on the surface used. For PMMA, the hydrodynamic stress was more effective on the removal of BDMDAC-treated dual species biofilms. For SS, the synergy of the chemical and hydrodynamic stresses removed more *B. cereus* and dual species biofilms. The overall results

demonstrate that the species association was not advantageous in biofilm resistance to removal when compared with the single species biofilms, particularly those of *P. fluorescens*. In general, removal by hydrodynamic stress, alone and preceded by the BDMDAC treatment, was higher for biofilms formed on SS. However, even the combined action of BDMDAC and the exposure to a series of increasing Reynolds number of agitation were not effective to obtain biofilm-free surfaces.

1. Introduction

Food industries face costly losses due to food spoilage and contamination with pathogenic microorganisms that often survive as biofilms (Brooks and Flint, 2008), which are highly resistant to antimicrobials (Bridier et al., 2011; Mah and O'Toole, 2001). Conventional strategies for disinfection leave residual microorganisms on the equipment surfaces, allowing the biofilm re-establishment (Fletcher, 1994; Verran, 2002). An effective biofilm control strategy must include the combination of disinfection with a mechanical removal step (Pechaud et al., 2012; Simões et al., 2005). Previous works using similar strategies have been done, though using only one material as adhesion surface and mostly one bacterium for biofilm formation (Simões et al., 2005, 2008b,c, 2009). However, the biofilm formation process can be affected by the type of adhesion surface (Tuson and Weibel, 2013), and further evidences are needed on the practical influence of this aspect on surface cleaning.

In this study, a rotating cylinder reactor (RCR) was used for biofilm formation on stainless steel (SS) and polymethyl methacrylate (PMMA) surfaces, providing hydrodynamic conditions similar to those found in industrial plants (Simões et al., 2009). Stainless steel is the most common material used in industrial processes (Zacheus et al., 2000). Polymethyl methacrylate (PMMA) is widely used in laboratory-scale biore- actors (Ferreira et al., 2010; Manuel et al., 2007; Matsuura et al., 2013; Reedy et al., 2011). To understand the microbial behaviour in the first steps of the biofilm formation process, the effects of the surface type and the exposure to BDMDAC was assessed on the bacterial physicochemical surface properties and on the initial adhesion to the selected surfaces.

2. Materials and methods

2.1. Bacteria and culture conditions

Biofilms were formed by *Pseudomonas fluorescens* ATCC 13525^{T} and a *Bacillus*

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 (PB) (KH_2PO_4 ; Na_2HPO_4) at pH 7. All the medium components were purchased from Merck (VWR, Portugal). For biofilm formation a sterile diluted nutrient medium (DNM) was used. The DNM was a 1:100 dilution of the CNM in 0.2 M PB.

2.2. Biocide

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

2.3. Surfaces

The materials tested were AISI316 stainless Steel (SS) (Neves & Neves, Portugal) and polymethyl methacrylate (PMMA) (Neves & Neves, Portugal). The substratum used were flat slides of either 3 cm^2 for contact angle measurements or 1 cm^2 for the adhesion assays. The surfaces were cleaned and sterilized according to the method described by (Simões et al., 2007a). In the RCR, biofilms were grown in cylinders of PMMA with a surface area of 44.0 cm^2 (diameter = 2.8 cm, length = 5.0 cm) or SS with a surface area of 34.6 cm^2 (diameter = 2.2 cm, length = 5.0 cm). The cylinders were prepared as described above and placed in the reactor. The RCR was sterilized with the cylinders mounted in place, by recirculating a solution of 15% (v/v) of sodium hypochlorite (Sigma–Aldrich, Portugal) during 10 h. After the sterilization, the system was rinsed twice with sterile distilled water to remove the residual sodium hypochlorite.

2.4. Measurement of the surface free energy

The bacterial cell surface free energy was determined by the sessile drop contact angle measurement on bacterial layers, according to Busscher et al.

(1984). Contact angles of the bacteria non-exposed and exposed to BDMDAC, for 30 min, and of PMMA and SS were determined automatically using an OCA 15 Plus (DATAPHYSICS, Germany) video-based optical measuring instrument, allowing image acquisition and data analysis, and ≥25 contact angle measurements (per liquid and sample) were carried out according to Simões et al. (2007b). The surface energy components of bacteria, PMMA and SS were obtained by measuring the contact angles with three pure liquids: water, formamide and α-bromonaphthalene (Sigma, Portugal). Reference values for these liquids surface tension components were obtained from the literature (Janczuk et al., 1993). The approach developed by van Oss et al. (1987, 1988, 1989) was used to determine the hydrophobicity after contact angle measurement. As stated by this approach, the degree of hydrophobicity of a given material (i) is expressed as the free energy of interaction between two entities of that material, when immersed in water (w) [ΔG_{iwi} (mJ m^{-2})]. If the interaction between the two entities is stronger than the interaction of each entity with water $\Delta G_{iwi} < 0$, the material is considered hydrophobic. Conversely, if $\Delta G_{iwi} > 0$ the material is hydrophilic. ΔG_{iwi} can be calculated through the surface tension components of the interacting entities, according to Eq. (1):

$$\Delta G_{iwi} = -2\left(\sqrt{\gamma_S^{LW}} - \sqrt{\gamma_L^{LW}}\right)^2 + 4\left(\sqrt{\gamma_S^+\gamma_W^-} + \sqrt{\gamma_S^-\gamma_W^+} - \sqrt{\gamma_S^+\gamma_S^-} - \sqrt{\gamma_W^+\gamma_W^-}\right) \quad (1)$$

where γ^{LW} is the Lifshitz-van der Waals component of the surface free energy, and γ^+ and γ^- are, respectively, the electron acceptor and electron donor parameters of the Lewis acid–base component γ^{AB}, where $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$.

The surface energy components of a solid material were obtained by measuring the contact angles of the three pure liquids with known surface tension components. Once the values were obtained, three equations (2) were used, one for each liquid:

$$(1 + \cos\theta)\gamma_1^{TOT} = 2\left(\sqrt{\gamma_S^{LW}\gamma_W^{LW}} + \sqrt{\gamma_S^+\gamma_W^-} + \sqrt{\gamma_S^-\gamma_W^+}\right) \quad (2)$$

where θ is the contact angle and $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$

2.5. *Free energy of adhesion*

The free energy of adhesion between the bacterial cells and PMMA and SS surfaces was assessed according to Simões et al. (2008a). When studying the interaction free energy of adhesion – ΔG^{TOT} between surface *i* (bacterium) and *l* (PMMA or SS) that are immersed or dissolved in water, the total interaction energy, ΔG_{iwl}^{Tot}, can be expressed as:

$$\Delta G_{iwI}^{TOT} = \gamma_{iI}^{LW} - \gamma_{iw}^{LW} - \gamma_{Iw}^{LW} + 2\left[\sqrt{\gamma_W^+}\left(\sqrt{\gamma_i^-} + \sqrt{\gamma_I^-} - \sqrt{\gamma_W^-}\right) + \sqrt{\gamma_W^-}\right] \times \left(\sqrt{\gamma_i^+} + \sqrt{\gamma_I^+} - \sqrt{\gamma_W^+}\right) - \sqrt{\gamma_i^+\gamma_I^-} - \sqrt{\gamma_i^-\gamma_I^+} \quad (3)$$

Thermodynamically, if $\Delta G_{iwl}^{Tot} < 0$ adhesion is favoured, whereas adhesion is not expected to occur if ΔG_{iwl}^{Tot}>0 mJ m^{-2}.

2.6. *Bacterial surface charge – zeta potential*

The zeta potential of cells suspension before and after contact with BDMDAC was determined using a Nano Zetasizer (Malvern Instruments, UK). Cell suspensions without biocide were used as controls.

2.7. *Adhesion assays*

In vitro initial adhesion was assessed on PMMA and SS sur- faces (1 cm^2 slide per well) inserted in 48-well microtiter plates (Nunc, Denmark). A method developed by Simões et al. (2010) was used to allow the adhesion of single and dual species of *B. cereus* and *P. fluorescens*, for 2 h, on the selected sur- faces. Total bacterial counts were obtained by direct staining with 4',6-diamidino-2-phenylindole (DAPI, Sigma, Portugal) as described previously (Saby et al., 1997). The slides were examined using an epifluorescence microscopy (LEICA DMLB2) as previously described (Lemos et al., 2013).

2.8. *Biofilm formation*

Biofilms were grown on cylinders of PMMA or SS, using a 5 L RCR, according to Simões et al. (2005). The reactor had three cylinders, immersed in a bacterial suspension, rotating at the same speed, during the biofilm formation period. The three cylinders were driven by an overhead stirrer *via* a synchronizing belt. The rotation speed of the cylinder provided a Reynolds number of agitation (*ReA*) of

2400. The agitation flow is laminar for *ReA* < 10 and turbulent for *ReA* > 10^4 (Geankoplis, 1993). Therefore, in this study, the biofilms were formed under transitional agitation flow.

A planktonic culture of *B. cereus* or *P. fluorescens* grew in a 0.5 L chemostat fed with the CNM previously described, at a flow rate of 10 mL/h. This chemostat was agitated by a magnetic stirrer and fed the RCR at a steady flow rate, set by gravity. Those reactors were used alone or simultaneously to provide single or dual species biofilms, respectively. The RCR was continuously fed with the DNM (described in Section 2.1) at a flow rate of approximately 0.8 L/h, while for dual species biofilms it was 1.6 L/h, in order to establish the same dilution rate as for the single species scenario. The period for biofilm formation was 7 days in order to obtain steady-state biofilms (Simões et al., 2005). Sterile aeration *via* a cellulose acetate syringe filter with pore size of 0.22 *μ*m (Whatman, VWR, Portugal) was provided to the RCR and to the chemostat.

2.9. *Biofilm chemical treatment*

The cylinders with biofilm were removed from the 5 L reactor, and then immersed in 250 mL glass beakers containing 200 mL of BDMDAC solution. The exposure to the biocide was carried out for 30 min, under the same *ReA* used for biofilm formation. After biofilm chemical exposure, a neutralization step was performed to dilute BDMDAC to residual levels, as described by Johnston et al. (2002). The wet weight of the cylinders plus biofilm attached was determined before and after the exposure. The wet mass of the biofilm that was removed from the surface area of each cylinder was expressed in terms of percentage of biofilm removal, as defined by Eq. (4).

$$\text{Biofilm removal}\,(\%) = \frac{X}{X_{biofilm}} \times 100 \qquad (4)$$

where $X_{biofilm}$ is the wet mass of the biofilm before BDMDAC exposure and X is the wet mass of the biofilm that was removed due to BDMDAC exposure.

2.10. *Biofilm removal by hydrodynamic stress*

The biofilm removal by hydrodynamic stress was assessed according to the method described by Simões et al. (2005). Biofilm layers were removed by submitting the biofilms submerged in PB to 30 s pulse exposure to increasing *ReA* (from 4000 to 16100). The wet weight of the cylinders plus biofilm attached

was determined before and after exposure to the hydrodynamic stress. Additionally, the residual biofilms, covering the cylinders, were entirely removed with a stainless steel scraper and then the weight of cylinders without biofilm was determined, to quantify the remaining biofilm after the hydrodynamic treatment. The same procedure was followed with the control assay, *i.e.* with the cylinder plus biofilm non- exposed to BDMDAC.

The amount of biofilm that remained adhered after expo- sure to the complete series of *ReA* was expressed as percentage of biofilm remaining, according to Eq. (5).

$$\text{Biofilm remaining}\,(\%) = \frac{X_{remaining}}{X_{biofilm}} \times 100 \qquad (5)$$

where: $X_{biofilm}$ is the wet mass of the biofilm non-exposed to the series of increasing *ReA*; $X_{remaining}$ is the wet mass of the biofilm remaining adhered to the cylinder surface after the exposure to the series of increasing *ReA*.

2.11. *Statistical analysis*

The data were analyzed using the statistical programme 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 performed for each condition tested. All data were analyzed by the application of the non-parametric Kruskal–Wallis test (confidence level ≥95%).

3. Results and discussion

Initial tests regarding the hydrophobicity of the cells and the selected surfaces were performed in order to estimate the effects of BDMDAC on bacteria–surface interaction, according to the thermodynamic theory of adhesion (van Oss et al., 1987, 1988, 1989). According to the results presented in Table 1, *B. cereus* and *P. fluorescens* cell surfaces are hydrophilic ($\Delta G_{iwi} > 0$ mJ m^{-2}), this characteristic being statistically more pronounced in the case of *B. cereus* ($P < 0.05$). The values obtained for *P. fluorescens* are in agreement with a previous report (Simões et al., 2007a). The *P. fluorescens* cell surfaces became even more hydrophilic after the exposure to BDMDAC, whereas in the case of *B. cereus* no significant effect

was observed. The treatment of *P. fluorescens* with BDMDAC increased significantly ($P < 0.05$) its apolar properties (indicated by the Lifshitz-van der Waals component) and decreased significantly ($P < 0.05$) its polar properties (indicated by the Lewis acid–base component). Again, no significant effects occurred in these parameters after exposure of *B. cereus* to BDMDAC ($P > 0.05$). The surfaces of both cells were predominantly electron donors. This ability increased significantly due to BDMDAC exposure ($P < 0.05$). The electron accepting capacity of *P. fluorescens* ($P < 0.05$) decreased after biocide exposure, but the same did not occur for *B. cereus* ($P > 0.05$).

PMMA and SS are hydrophobic surfaces. However, PMMA is more hydrophobic and its apolar properties (γ^{LW}) were higher than for SS. Nevertheless, the ability to donate electrons (γ^-) was higher for SS ($P < 0.05$). The results obtained for the SS hydrophobicity are similar to previous observations (Simões et al., 2007a). The water contact angles of PMMA (data not shown) are also in agreement with earlier studies (Bruinsma et al., 2001; Gottenbos et al., 2000).

The results on the free energy of adhesion (Table 2) between the bacteria and the selected surfaces showed that adhesion was thermodynamically more favourable on PMMA than on SS, for both species. Before the chemical treatment, *P. fluorescens* adhesion seems to be less favourable than for *B. cereus*. The bacterial exposure to BDMDAC disfavoured the adhesion of *B. cereus*, particularly on PMMA. An opposite effect was observed for *P. fluorescens*.

The cell surface charge was determined in order to assess the influence of BDMDAC on this cell property (Table 3). Under physiological conditions *B. cereus* and *P. fluorescens* had negative surface charges. The treatment with BDMDAC increased the bacterial cell charge to less negative values. This effect is probably a result of the cationic nature of BDMDAC (Ferreira et al., 2011).

The mechanism of initial bacterial adhesion to surfaces has been studied by many researchers and was proposed to depend on both long-range forces like electrostatic and thermodynamic forces and short-range forces like van der Waals attraction (Bos et al., 1999; Geoghegan et al., 2008). In addition to thermodynamics, the cell surface charge was found to influence bacterial adhesion to surfaces through attractive or repulsive electrostatic forces (van Loosdrecht et al., 1987; Eboigbodin et al., 2007). Bellon-Fontaine et al. (1990) stated that accurate predictions of bacterial adhesion to surfaces was found to be made when electrostatic forces were considered in addition to thermodynamic properties of the cell surface. However, the *in vitro* adhesion results (Fig. 1) do not provide clear evidences on the role of thermodynamic aspects and surface charge on bacterial adhesion. Even if *B. cereus* was thermodynamically more prone to adhere to the surfaces, higher numbers of *P. fluorescens* cells adhered

to the surfaces, being the number of adhered cells similar on PMMA and SS ($P > 0.05$). The number of adhered cells for the combination of *B. cereus* and *P. fluorescens* is apparently an average of the numbers obtained with the single species. *In vitro* tests also indicated that BDMDAC decreased the numbers of adhered cells, being this effect stronger for *B. cereus* ($P < 0.05$) than for *P. fluorescens* ($P > 0.05$). The higher number of *P. fluorescens* remaining adhered, after BDMDAC exposure, might be due to the fact that Gram negative bacteria have a cell envelope/outer membrane which regulates the passage of substances to the periplasmic space and the cytoplasm. This fact may contribute to a higher intrinsic resistance of *P. fluorescens* to biocides than the Gram positive *B. cereus* (Cloete, 2003).

The disagreement between the thermodynamic study and *in vitro* adhesion results is arguably due to the limitations of the thermodynamic theory. This theory of adhesion may predict some outcomes of the bacterial adhesion. However, accurate values for bacterial surface free energies are very difficult to obtain as cells are living organisms, with complex chemistry and hydration processes. Moreover, this theory implies an equilibrium model and a closed system, where there are no energy exchanges with the outside (Hermansson, 1999). Bacterial cells are metabolically active, and energy consuming physiological mechanisms like the synthesis of EPS, may be influencing the adhesion events (Katsikogianni and Missirlis, 2004). In fact, Simões et al. (2010) had also found similar results studying the initial adhesion of drinking water-isolated bacteria. These findings underscore that despite the physicochemical surface properties of both the bacterium and substratum being crucial for the adhesion phenomena, other biological aspects such as phenotypic and genetic switching, and the production of EPS, may play a significant role on biofilm formation and differentiation. Motility processes ruled by extracellular appendages (pili, flagella, fimbriae), the presence of outer membrane proteins and quorum sensing mechanisms also play an important role in the attachment to surfaces (Bullitt and Makowski, 1995; Nadell et al., 2009; Thomas et al., 2004).

The 7 days-aged biofilms (shown in Fig. 2 prior to the treatments) were exposed to BDMDAC under the same hydro- dynamic conditions used for its formation. The percentage of the remaining biofilm on PMMA and SS cylinders was determined for the following scenarios (Fig. 3): after the action of BDMDAC alone, for 30 min; after the exposure to increasing *ReA*; and after applying the two treatments combined (BDMDAC exposure followed by hydrodynamic stress).

The percentage of biofilm removed after exposure to BDMDAC was significantly higher for *B. cereus* and dual species biofilms than for the *P. fluorescens* single species adhered on PMMA. Conversely, when the biofilms

were formed on SS, *P. fluorescens* biofilms were more susceptible to this treatment, followed by the dual species and the *B. cereus* single species biofilms. The latter results contradict those found by Simões et al. (2009), who observed that *P. fluorescens* biofilms formed on SS surfaces were the most resistant to the chemical treatment with the biocides glutaraldehyde and cetyl trimethyl ammonium bromide (CTAB). These differences are likely due to distinct chemicals used, proposing that no general conclusion on a biofilm control strategy can be withdrawn from experiments based on a single antimicrobial agent, even if they share the same chemical nature as occurs for BDMADC and CTAB (both are quaternary ammonium compounds).

After the chemical treatments, the biofilms were subjected to a series of increasing *ReA* (Fig. 3). Control tests were performed subjecting untreated biofilms to the same hydro- dynamic stress. The results shown that the aim of obtaining biofilm-free surfaces was not achieved, for all conditions tested. In fact, after the hydrodynamic stress tests, significant amounts of *B. cereus*, *P. fluorescens* and dual species biofilms remained on the PMMA (37%, 44% and 31%, respectively) and the SS cylinders (14%, 35% and 21%, respectively). However, the removal values were higher than those achieved with only mechanical action, with the exception of *P. fluorescens* biofilms formed on SS, where no significant difference was observed ($P > 0.05$). *P. fluorescens* biofilms were the most resistant to the combined treatment, especially when formed on PMMA. BDMDAC is a quaternary ammonium compound (QAC) and therefore acts by destabilizing the cell membranes, causing rapid cell lysis (Chapman, 2003). Its action against bacteria was described by Ferreira et al. (2011), including its efficacy in the inactivation of biofilms (Ferreira et al., 2010). No reports are available on the mode of action and/or resistance of BDMDAC on biofilms. A study performed with two quaternary ammonium compounds (benzalkonium chloride and cetyltrimethyl ammonium bromide) demonstrated the reduction of their antimicrobial effects against *B. cereus* and *P. fluorescens* due to interaction with biofilm components (Araújo et al., 2013). Campanac et al. (2002) found that resistance of the Gram negative bacterium *Pseudomonas aeruginosa* biofilms to diverse QAC's was due to the involvement of the negatively charged EPS produced by the bacterium and the reduce diffusion of antimicrobials through the biofilm. In the case of the Gram positive bacterium *Staphylococcus aureus*, the role of the three-dimensional structure was limited and drastic physio- logical changes in the biofilm cells were the most important aspect implicated in resistance. It is recognized that the EPS matrix can cause mass transfer limitations, and that inter- actions between their molecules and the biocide may occur, reducing its availability to interact with the biofilm cells

(Melo, 2005; Peyton, 1996; Zhang and Bishop, 1994). A previous report (Simões et al., 2007a), described that the *B. cereus* strain used in the present study is a low biofilm EPS producer compared to the *P. fluorescens* biofilms. This high EPS content of *P. fluorescens* biofilms can help to explain their higher resistance to removal. In fact, one of the most important functions of EPS is supposed to be their role as fundamental structural elements of the biofilm matrix determining the mechanical stability of biofilms, mediated by non-covalent interactions either directly between the polymeric chains or indirectly *via* multivalent cation bridges (Flemming, 1996; Allison, 2003).

The species association was not advantageous in forming biofilm resistant to removal. The biofilm remaining on the surfaces after the chemical and hydrodynamic stresses, alone and/or combined, was, for most of the cases, lower for the dual species biofilms. This result contradicts previous studies, where species association increased biofilm resistance (Lindsay et al., 2002; Giaouris et al., 2013; Lee et al., 2014). However, in those studies, the biofilms were challenged with antimicrobial chemicals and the viability of the biofilm cells was analyzed. In the present study, the outcomes of the chemical and/or hydrodynamic stresses were only assessed in terms of biofilm removal. It is likely that the bacteria in the dual species biofilms had higher resistance to killing but a decreased resistance to removal, compared with the single species biofilms, particularly those formed by *P. fluorescens*. In fact, the killing and the removal of a biofilm are distinct processes (Araújo et al., 2014; Chen and Stewart, 2000). It is possible to kill a biofilm without promoting its removal from the surface (Simões et al., 2009).

Although total removal of the biofilm was not achieved, the overall results underscore that SS should be preferred to other materials as it facilitates the sanitization procedures, reinforcing previous findings (Allion et al., 2011; Lee et al., 2010).

4. Conclusions

Biofilm formation on SS and PMMA surfaces was monitored on a RCR. This bioreactor proved to be a versatile tool to ascertain the effectiveness of the biocide BDMDAC to be used in the removal of biofilms. The thermodynamic approach was not reliable in the prediction of bacterial initial adhesion to the selected surfaces. Laboratorial adhesion assays demonstrated that *P. fluorescens* had the highest ability to adhered to PMMA and SS surfaces, while *B. cereus* had the lowest ability. The number of adhered cells for the combination of *B. cereus* and *P. fluorescens* was apparently an average of the numbers obtained with the

single species. BDMDAC decreased moderately the numbers of adhered cells, being this effect stronger for *B. cereus* single cells than for *P. fluorescens* single cells and combined. Using the RCR, neither the application of BDM- DAC nor the increasing series of *ReA*, when applied alone or combined with the chemical, were able to totally remove the biofilms from the surfaces. Biofilm removal was dependent on the adhesion surface and on the microbial species. *P. fluorescens* biofilms were the most resistant to removal, while dual species biofilms had the lowest mechanical stability, for most of the cases. No advantage in biofilm resistance to removal was found for the dual species biofilms. SS was the surface for which biofilm removal by the hydrodynamic stress, alone and preceded by the BDMDAC treatment, was higher. However, even if additional removal was achieved by the combination of both treatments, total removal was not achieved, regardless the biofilm forming species and the surface material used.

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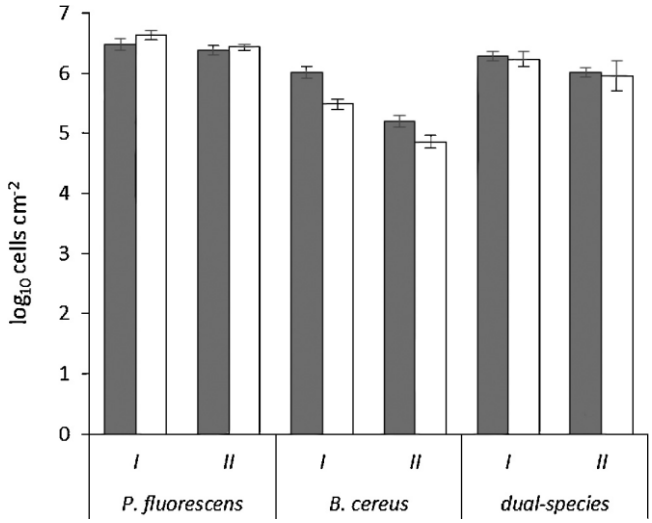

Fig. 1 – Numbers of *P. fluorescens* and *B. cereus* single and dual species cells previously adhered for 2 h on SS ( ) and PMMA (D) surfaces, before (I) and after a 30 min treatment with BDMDAC (II). Mean values standard deviation for at least three replicates are illustrated.

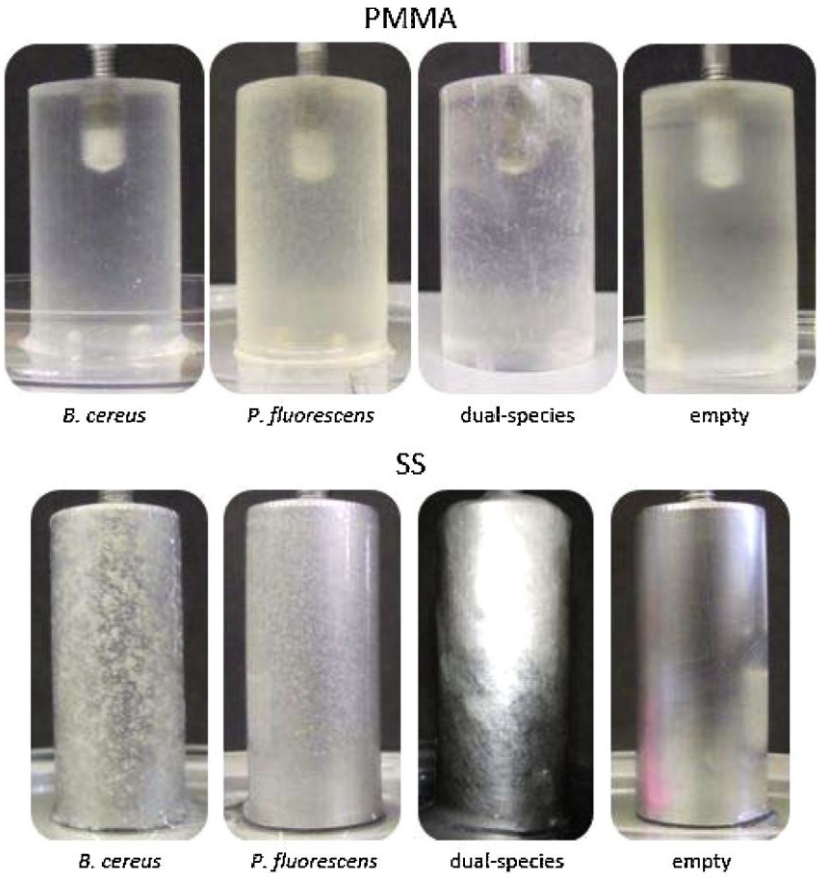

Fig. 2 – Photographs of the cylinders of PMMA and SS covered with *B. cereus*, *P. fluorescens* single and dual species biofilms, before any treatment. For both

materials, a photograph of the cylinder without biofilm (empty) is shown for comparison.

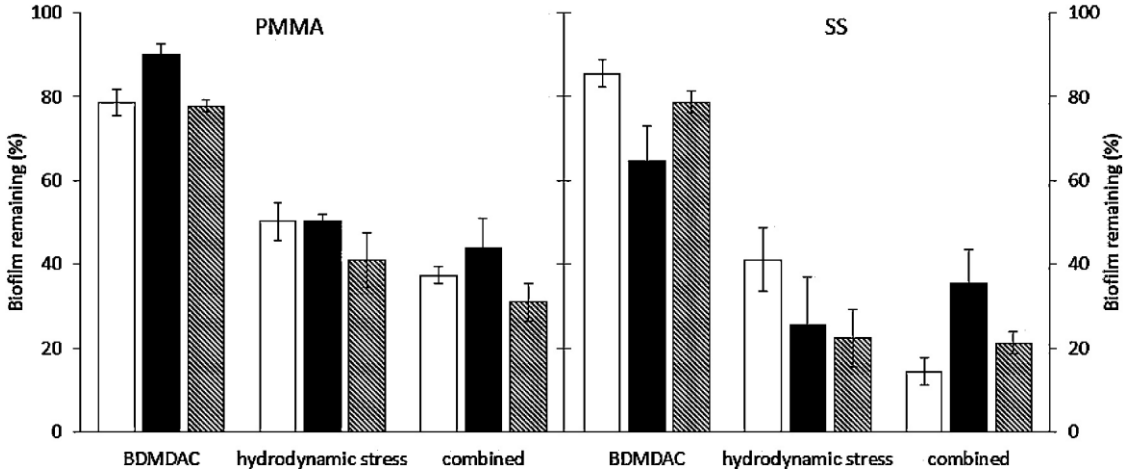

Fig. 3 – Biofilm remaining (%) after submitting the biofilms to BDMDAC treatment alone, to the complete series of hydrodynamic stresses and to combination of both treatments. B. cereus (D), P. fluorescens (•) single and dual species ( ) biofilms formed on PMMA (a) and on SS (b) cylinders using the RCR. The means ± SDs for at least three replicates are illustrated

Table 1 – Surface energy parameters (Lifshitz-van der Waals component – γ^{LW}; electron acceptor component – γ^+ and electron donor component – γ^-) and hydrophobicity ΔG_{iwi} for the untreated (control) and BDMDAC-treated cells, and for PMMA and SS surfaces. The means ± SD for at least three replicates are given.

	Surface energy parameters (mJ m^{-2})				ΔG_{iwi} (mJ m^{-2})
	γ^{LW}	γ^{AB}	γ^+	γ^-	
B. cereus					
Control	37.9 ± 2.5	15.9 ± 3.5	1.2 ± 0.4	47.4 ± 7.5	29.5 ± 6.0
BDMDAC	37.7 ± 1.2	14.3 ± 2.2	1.0 ± 0.2	57.7 ± 1.9	37.5 ± 3.2
P. fluorescens					
Control	18.0 ± 0.9	43.2 ± 1.7	9.6 ± 1.0	48.9 ± 2.3	14.8 ± 2.2
BDMDAC	39.5 ± 1.7	9.3 ± 2.5	0.3 ± 0.1	64.2 ± 3.9	49.3 ± 7.2
PMMA	40.9 ± 0.9	4.2 ± 1.0	1.5 ± 0.4	2.4 ± 0.5	−58.4 ± 7.3
SS	37.6 ± 1.1	4.0 ± 0.4	0.6 ± 0.1	5.9 ± 1.3	−44.8 ± 7.9

Table 2 – Free energy of adhesion (ΔG_{iwi}^{TOT} – mJ m^{-2}) between *B. cereus* or *P. fluorescens*, untreated (control) and BDMDAC-treated cells, and PMMA and SS surfaces. The means for at least three replicates are given.

	B. cereus		*P. fluorescens*	
	Control	BDMDAC	Control	BDMDAC
PMMA	−19.0	−14.2	2.6	−14.6
SS	−9.3	−3.7	7.7	−2.9

Table 3 – Zeta potential values (mV) of the untreated (control) and BDMDAC-treated *B. cereus* and *P. fluorescens*, PMMA and SS. The means ± SD for at least three replicates are given.

	Control	BDMDAC
Bacteria		
B. cereus	−18.7 ± 0.9	−15.6 ± 2.0
P. fluorescens	−9.8 ± 0.5	−4.1 ± 0.1
Materials		
PMMA	−1.7 ± 0.3	
SS	−2.1 ± 0.3	