Role of benzalkonium chloride surface preconditioning in the increased resistance of biofilms to removal and disinfection

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Abstract
The main goals of the present study was to ascertain the role of surface preconditioning in the biofilm sanitation (removal and disinfection) ability of the cationic surfactant benzalkonium chloride (BC) and to investigated whether BC preconditioning can be a factor of the increased resistance of the *Pseudomonas fluorescens* biofilms to the surfactant. Prior to biofilm formation, coupons of two distinct materials (stainless steel and silicone rubber) currently used on medical and industrial processing facilities were exposed to several sub-effective concentrations of BC for 30 min. Afterwards, these conditioned coupons were used as the surfaces to form biofilms in a chemostat for 6 days. The antimicrobial action of BC on the biofilms was assessed by means of respiratory activity, due to oxygen consumption, and biofilm mass. The results showed that BC preconditioning, by itself, did not prevent or impair biofilm formation. In general, the mass and respiratory activity of the biofilms developed on the conditioned coupons increased with the increase of the BC concentration used in the preconditioning. The data related with BC application to the bacterial biofilms formed on the conditioned metal and rubber coupons showed that biofilms became more difficult to inactivate, especially those that have been developed in the coupons preconditioned with the higher BC concentrations. Thus, it can be concluded that the antimicrobial ability of BC was considerably disturbed when the surfaces are preconditioned with the surfactant. Based merely on this data, it can be speculated that, in the initial adhesion stage, the contact of the *P. fluorescens* with the BC residues adsorbed on the coupons surface, due to preconditioning, induces bacteria resistance when they are entrapped in a developed biofilm and submitted to BC aggression.

Keywords: Biofilm resistance; Biofilm control; Benzalkonium chloride; Surfactant; Surface Preconditioning

Introduction
Microorganism's deposition on solid surfaces, and consequent biofilm formation, are phenomena that happen naturally but are also microorganism's strategies to protect themselves from external toxic factors. Biofilm microorganisms can cause serious problems in industry and medical area, since they often present reduced susceptibility to the action of antimicrobial agents than their liquid suspended counterparts.
In recent years, several studies have been carried out in order to develop suitable and efficient protocols to avoid biofilm accumulation on the most diverse surfaces. The control and prevention of undesirable biofilms is usually achieved with the increase of the frequency of the cleaning and disinfection (sanitation) programmes that often includes the application of chemical products with antimicrobial properties, such as

biocides and surfactants. Surfactants are added to increase the washing effect of the sanitation practices. If these practices are not effective, microrganisms and product residues can remain in the equipment surface at concentrations that may affect the quality and safety of the food product (Gibson et al. 1999). Also, those products residues and remaining microrganisms can contribute to the re-growth of biofilms with persistent characteristics to the sanitation products (Gilbert and McBain, 2003).
In recent years, there is growing concern about the increased number of reports noticing phenomena of bacterial adaptation and resistance to those biocides and surfactants. In order to overcome this drawback, it is crucial to comprehend all the parameters that can contribute to the prevalence of biocide and surfactant resistance and thus to biofilm recalcitrance. One of the factors that can contribute to that understanding it is to establish whether pre-contact of bacteria with a chemical can contribute to their reduced susceptibility to that product.
Therefore, the main objectives of this work was to ascertain whether BC residues adsorbed on the adhesion surfaces can alter the characteristics of *P. fluoresces* biofilms (in terms of biomass and respiratory activity) subsequently developed on those surfaces, and to investigate the impact of those residues in the sanitation ability (removal and inactivation) of BC when used in the treatment of well established biofilms and in the prevalence of biofilm resistance to chemical treatment.

Materials and Methods

Microorganism and Culture conditions

Pseudomonas fluorescens ATCC 13525^{T} was the microorganism used to produce biofilm. This bacterium was maintained in nutrient agar plates and, when required, fresh liquid cultures were prepared to implement the biofilm formation assays. The growth conditions were 27 ± 1 ºC, pH 7, and glucose as the carbon source. The bacterial planktonic culture was grown in a 0.5 L chemostat aerated, agitated, and continuously fed with a sterile concentrated nutrient solution (5 g/L glucose, 2.5 g/L peptone and 1.25 g/L yeast extract, in phosphate buffer at pH 7) at a flow rate of 10 mL/h (Simões *et al.* 2005a). The bacteria were let to grow in that fermenter as a batch for approximately one day (to reach the steady state) before the beginning of the continuous feeding process.

Biofilm formation

Biofilms were developed on metal and rubber coupons (2 cm x 2 cm and 1 mm thick) placed in a well stirred continuous 3 L glass reactor at 27 ± 1 ºC, suitable aerated and magnetically agitated. This reactor was continuously inoculated (10 mL/h) with bacteria in the exponential phase of growth supplied by the above referred 0.5 L chemostat and fed with 0.97 L/h of a sterile nutrient solution consisting of 40 mg/L glucose, 20 mg/L peptone and 10 mg/L yeast extract prepared in phosphate buffer pH 7. Twelve slides of ASI 316 stainless steel (SS) and twelve slides made of silicone rubber (SR) were placed within the reactor contained the bacterial suspension during 6 d for biofilm growth in order to obtain steady-state biofilms (Pereira *et al*. 2001). The slides were degreased, rinsed twice with water and sterilised before they were hung in the reactor through a device that was suitable fitted in the reactor and could be removed for biofilm sampling. Prior to each experiment all system components were sterilised by autoclaving at 120 ºC and 1 atm for 25 min.

Antimicrobial chemical

Benzalkonium chloride (BC), a cationic surfactant, obtained from Calbiochem (CMC of 5.00 mM; Cat. No. 198901) was the antimicrobial chemical used throughout this work

to preconditioning the coupons, before biofilm formation, and to treat the steady state biofilms. Before each experiment, BC solutions were prepared to the required concentration, with sterile distilled water.

Surfaces preconditioning
Biofilms were formed on stainless steel and silicone rubber coupons treated or not with the surfactant. The treatment, denominated preconditioning, consisted on the pre-contact of all the coupons with BC solutions of 0.0625 mM, 0.125 mM, 0.25 mM, and 0.5 mM mM, for 30 min.

Surfactant application
After 6 days of growth, all the coupons covered with biofilm were carefully removed from the reactor and immersed in flasks containing BC solutions of different concentration ($1.96x10^{-3}$, 0.00391, 0.00781 and 0.0156 mM) for 30 min. In each experiment, some SS and SR coupons with biofilm were immersed in a flask with pH 7 phosphate buffer (control).

Biofilm characterization
After BC exposure, the biofilm that covered the metal slides was entirely scraped off from the slides and resuspended into 20 ml of phosphate buffer pH 7. These biofilm suspensions were used to assess the cellular respiratory activity of the biofilm through oxygen uptake rates due to glucose oxidation and afterwards biofilm mass.

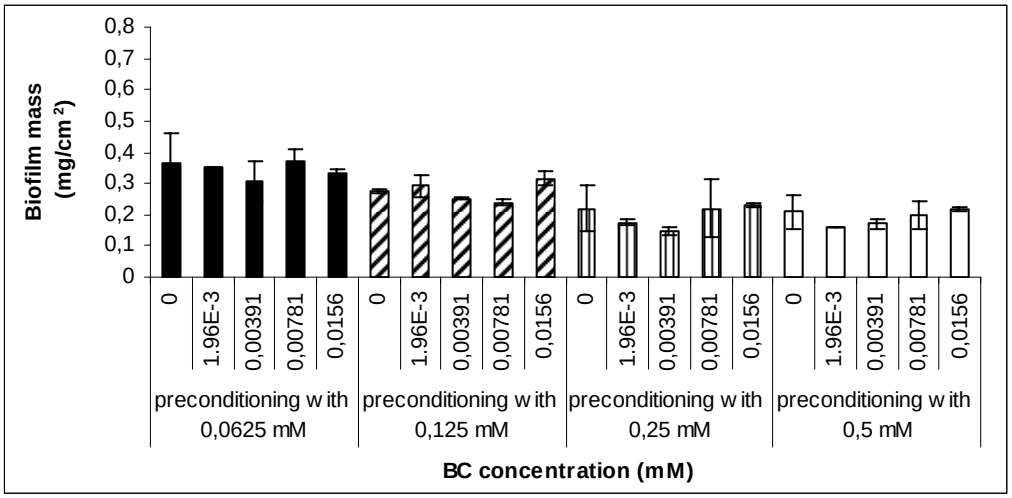
Respiratory activity assessment
The respiratory activity of the biofilm was evaluated by measuring oxygen uptake rates due to glucose consumption in a biological oxygen monitor (BOM) in short-term assays. The assays were performed in a Yellow Springs Instruments BOM (Model 53) and the procedure used was described elsewhere (Simões et al., 2005b). Briefly, the biofilm suspensions were placed in the temperature-controlled vessels of the BOM (T = 27 ºC ± 1ºC), each one contained a dissolved oxygen (DO) probe connected to a DO meter. Once inside the vessel, the samples were aerated for 30 min to ensure oxygen saturation. After that, vessels were closed and the decrease of the oxygen concentration was monitored over time. The initial linear decrease observed corresponded to the endogenous respiration rate. To determine the oxygen uptake due to substrate oxidation, a small volume (50 μl) of a glucose solution (100 mg/l) was injected within each vessel. The slope of the initial linear decrease in the DO concentration, after glucose injection, corresponded to the total respiration rate. The difference between the two respiration rates gives the oxygen uptake rate due to the glucose oxidation. This respiratory activity was expressed in mg of O_2 per g dry biofilm mass per minute.

Biofilm mass quantification
The dry biofilm mass was assessed by the determination of the total volatile solids (TVS) of the homogenised biofilm suspensions, according to the Standard Methods (1989), method number 2490 A-D. The dry biofilm mass accumulated on the metal and rubber surfaces was expressed in g of TVS *per* cm^2 of surface area of the coupons.

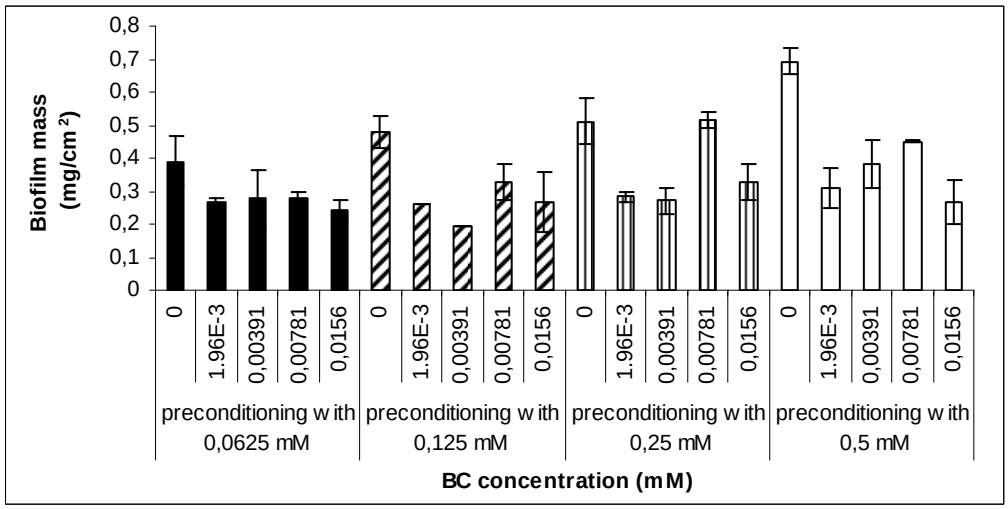
Statistical analysis
The mean and standard deviation within samples were calculated for all cases.

Results and Discussion

In order to investigate whether the presence of BC residues on the adhesion surfaces could have any impact in the posterior BC treatment of biofilms formed on those surfaces, several metal and silicone coupons were previously conditioned with several concentrations of the surfactant. The antimicrobial action of BC against the *P. fluorescens* biofilms was assessed by means of biofilm mass and respiratory activity. Fig. 1 shows the amount of biofilm mass accumulated on the SS (Fig. 1a) and SR (Fig. 1b) conditioned coupons after treatment with several BC concentrations.



a)



b)

Fig. 1 Mass of the biofilms formed on BC preconditioned SS (a) and SR (b) coupons, after treatment with several concentrations of BC (bars represent the standard deviation); "0 mM" means that these biofilms were not submitted to BC treatment (control).

This figure shows that the increase of the BC concentration used to pre-treat the adhesion surfaces caused different behaviours in terms of biofilm accumulation according to the type of the material of the coupon. In fact, the preconditioning of the SS coupons seemed not to significantly amend biomass accumulation since all the biofilm mass values observed on the treated coupons are similar (Fig. 1a). Concerning the rubber surfaces, their preconditioning appeared to cause the increase of the amount of biomass accumulated on coupons, since biofilm mass increased with the increase of the concentration used in the preconditioning. These results clearly showed that the existence of BC residues on the adhesion surfaces did not prevent or impair biofilm

formation. This event it is unexpected since BC it is a chemical with marked surfactant properties and previous works (Meylheuc et al. 2001) have demonstrated that the adsorption of a biosurfactant to a stainless steel surface reduced the adhesion of *L. monocytogenes* on that metal surface.

The data related with the posterior BC application to the bacterial biofilms formed on the metal and rubber coupons, preconditioned with the surfactant, showed, once more, that biofilm behaviour changes according to the material of the adhesion surface (Fig. 1). The application of BC to the *P. fluorescens* biofilms formed on the conditioned SS coupons did not cause any significant variation of the biomass accumulated. Concerning biofilms formed on the SR coupons, BC application caused the reduction of the biomass accumulated on the conditioned coupons. This reduction was more evident for the biofilms formed on the silicone coupons conditioned with the higher BC concentrations (0.25 mM and 0.5 mM) but almost independent of the concentration used in biofilm treatment. These results indicate that the presence and quantity of BC residues on the silicone coupons give rise to the formation of biofilms with more mass but more susceptible to the removal action of the surfactant.

The inactivation effect of BC when applied against *P. fluorescens* biofilms formed on the coupons previously conditioned with BC is depicted in Fig. 2.

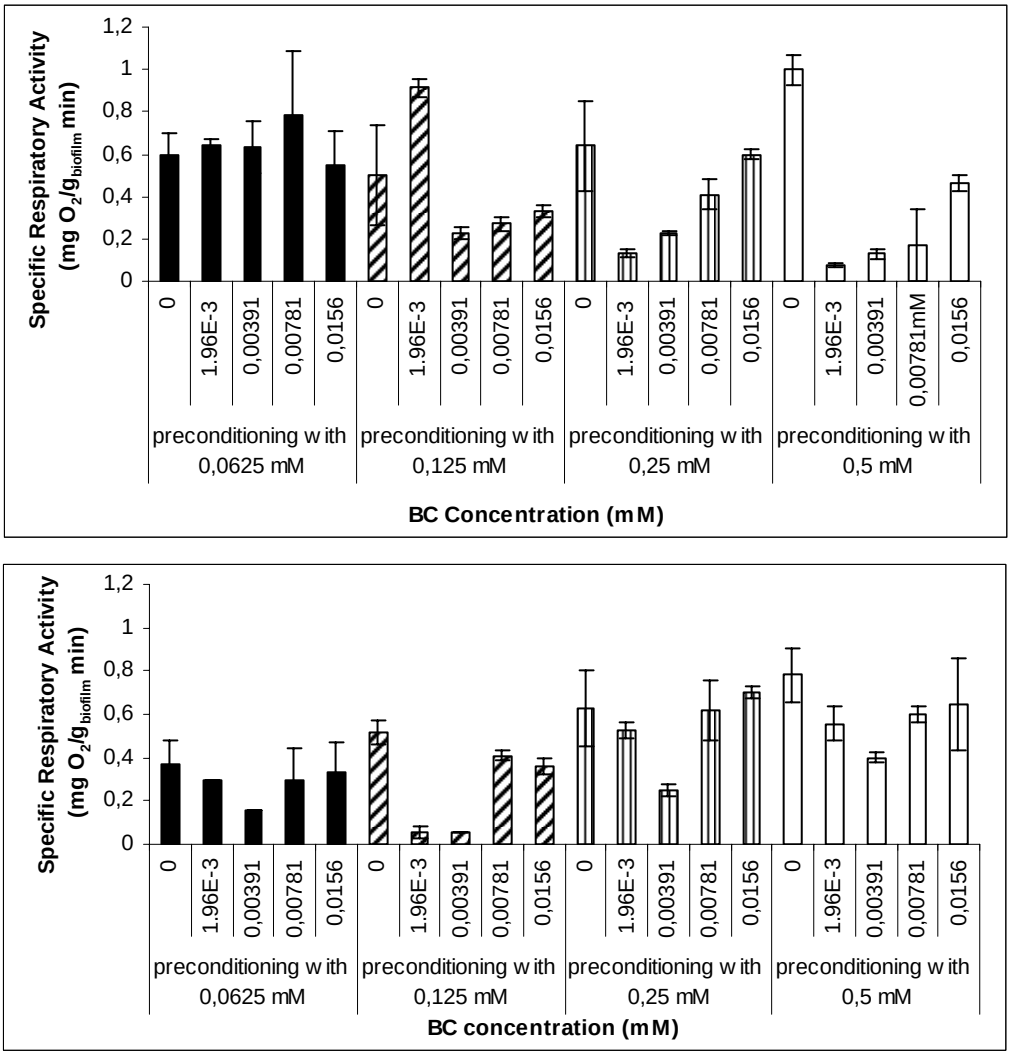


Fig. 2 Respiratory activity of the biofilms formed on BC preconditioned SS (a) and SR (b) coupons, after treatment with several concentrations of BC (bars represent the

standard deviation); “0 mM” means that these biofilms were not submitted to BC treatment (control).

Fig. 2 highlighted that, for both material types, the respiratory activity of the untreated *P. fluorescens* biofilms accumulated on the preconditioned surfaces increased gradually with the increase of BC concentration used in the preconditioning of the coupons. This data indicates that the presence and quantity of BC residues adsorbed on the adhesion surfaces have an important role in biofilm activity. Furthermore, Fig. 2 also showed that the respiratory activity of the untreated biofilms formed on the SS coupons is, in general, higher than the one monitored in the biofilms accumulated on the SR coupons. This fact assumes special interest because the untreated biofilms formed on the metal coupons presented noticeable less mass than the ones formed on the silicone (Fig. 1). This evidence emphasised the role of the type of material of the coupons in biofilm physiology, since silicone, contrary to what happens with stainless steel, seems to induce biofilms with more mass but less active.
Concerning biofilm respiratory activity after BC treatment, Fig. 2 showed that, in general, the application of the surfactant to *P. fluorescens* biofilms caused the reduction of their activity. Unexpectedly, this reduction was more pronounced for the lower BC concentrations used in biofilm treatment ($1.96x10^{-3}$ and 0.00391 mM). In fact, the reduction of the biofilm respiratory activity caused by BC application decreased with the increase of the surfactant concentration applied. This trend is more notorious for the biofilms formed on the coupons conditioned with the higher BC concentrations (0.25 and 0.5 mM). In general, the biofilms formed on those coupons appeared not suffer any negative impact with the application of 0.0156 mM of BC (the higher concentration used to treat biofilms), since they presented respiratory activities of the same magnitude of the control. Based on these results it can be speculate that surface preconditioning seem to induce biofilm insusceptibility to BC treatment, this insusceptibility being more significant when higher BC concentrations are used.
From the overall results, it was possible to detect that BC was more efficient in the inactivation of biofilms than in its removal, regardless the fact of total inactivation was not achieved. The results seem to suggest that the presence of BC residues adsorbed on the adhesion surfaces, no matter the type of the material, before the establishment of the *P. fluorescens* biofilms, gave rise to the formation of biofilms with recalcitrant properties, since the bacteria entrapped in those biofilms became more difficult to inactivate and to remove. So, it can be speculated that, in the initial adhesion stage, the contact of the *P. fluorescens* with the BC adsorbed on the coupons surface, due to preconditioning, induces bacteria resistance when they are entrapped in a developed biofilm and submitted to BC aggression. This biofilm resistance was more evident when higher BC concentrations were applied.

Conclusions

The experimental work gathered in this study permitted to conclude that the type of material of the adhesion surfaces, and the pre-treatment of the material, plays an important role in the characteristics of the biofilm formed on those surfaces, as well as, in the susceptibility of biofilms to the surfactant. Despite BC showed to have considerable antimicrobial capacity, more in terms of respiratory inactivation than in biomass removal, that ability was considerably disturbed when the surfaces have BC residues adsorbed. In fact, the existence of BC residues on the adhesion surfaces and their amount seemed to promote biofilm resistance to BC, since biofilms became more

difficult to eradicate, mainly when higher concentrations of BC are used in the preconditioning of the surfaces.

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