CONTROL OF FLOW-GENERATED BIOFILMS WITH SURFACTANTS - EVIDENCE OF RESISTANCE AND RECOVERY

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ABSTRACT

The action of the cetyltrimethyl ammonium bromide (CTAB) and sodium dodecyl sulfate (SDS), respectively a cationic and an anionic surfactant were investigated to control turbulent and laminar flow-generated biofilms formed by P. fluorescens. The disinfectant action of different concentrations of CTAB and SDS on biofilms was assessed by means of cellular respiratory activity and variation of biofilm mass, immediately, 3, 7 and 12 h after the application of the surfactants. The experiments along 12 h post-surfactant treatment were made in order to assess biofilm recovery. The results showed that, laminar flow-generated biofilms were more susceptible to the action of CTAB than those formed under turbulent flow. Total inactivation of the cells within the biofilms was not achieved for both types of flow-generated biofilms. Concerning SDS, higher concentrations applied promoted significant biofilm inactivation and turbulent and laminar flow had analogous susceptibility to SDS application. CTAB and SDS application did not promote the detachment of biofilms from the surfaces. Post-surfactant action results shown that biofilms recovered its respiratory activity, that in some cases reached higher values than the ones found without chemical treatment. CTAB application promoted similar recovery in the respiratory activity for both biofilms. Turbulent flow-generated biofilms showed a higher potential to recover their metabolic activity than laminar flow-generated biofilms when previously challenged with SDS. Conversely, concerning biofilm mass, any significant variation (increase or decrease) was detected after 12 h of surfactant treatment. This study highlights the need of care in choosing the correct procedure for biofilm control and the influence of hydrodynamic conditions on the persistent and recalcitrant properties of P. fluorescens biofilms.

Keywords: biofilm control, recovery, resistance, hydrodynamic conditions, surfactant

INTRODUCTION

The unwanted accumulation of biofilms in industrial equipment under aqueous environments, currently called biofouling, is a natural occurrence. Biocides still represent the more significant countermeasure to control biofilm formation (Chen and Stewart, 2000). However, these chemical substances may kill the attached microorganisms but must not be effective in removing the biofilm, remaining biomass in the surface. This fact may contribute to microbial recovery. Thus, in order to improve biofilm control procedures, industry has moved progressively towards the use of surface active compounds (surfactants) which present more biodegradable and less toxic properties. Surfactants are classified according to the ionic nature of their hydrophilic group, namely, as anionic, cationic, non-ionic and zwitterionic. The chemical nature of surfactants causes alteration of the surface properties of the submerged surfaces by decreasing their surface tension; preventing attachment of microorganism with

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potential to form biofilm and promoting the detachment of these microorganisms from the adhesion surface (MacDonald *et al*., 2000). Since bacteria within biofilms are protected from even the most aggressive of treatment regimens, it is expected that biofilms have a reactive behaviour when exposed to chemical treatment (biocide/surfactant), due to the possible alterations of their biofilm structure, composition and metabolic state.
The aim of this work was to assess the efficacy of surfactant treatment (CTAB/SDS) in the control of turbulent and laminar flow-generated biofilms and to characterize the biofilm behaviour post-surfactant treatment. Biofilms were formed by *Pseudomonas fluorescens*, an abundant bacteria in industrial biofilms, under turbulent and laminar flow in a simple flow cell reactor (Pereira *et al*., 2002) that allows biofilm sampling without disturbing the system.

MATERIAL AND METHODS

Microorganism and Culture Conditions

The microorganism used was *Pseudomonas fluorescens* (ATCC 13525^{T}), cultured as described elsewhere (Pereira *et al.*, 2002).

Flow Cell Reactor

A flow cell described by Pereira *et al.* (2002) was used to form biofilm under different flow conditions on ASI 316 stainless steel (SS) slides. Two flow cells were operating in parallel, to obtain one flow cell working under laminar flow (Reynolds number - Re=2000, u=0.204 m s^{-1}) and the other under turbulent flow (Re=5200, u=0.532 m s^{-1}). The system was continuously fed with sterile medium containing of 50 mg L^{-1} glucose, 25 mg L^{-1} peptone, 12.5 mg L^{-1} yeast extract in phosphate buffer (pH 7) and *P. fluorescens* in the exponential phase of growth in order to circulate a diluted bacterial suspension in the flow cells. The biofilm was allowed to grow for 7 days before beginning the surfactant treatment.

Surfactants

Cetyltrimethyl ammonium bromide (CTAB) with a critical micellar concentration (CMC) of 1.00 mM. The concentrations tested were 0.125, 0.250, 0.500 and 0.900 mM.
Sodium dodecyl sulfate (SDS) with a CMC of 8.30 mM. The concentrations tested were 0.500, 1, 3 and 7 mM.

Surfactant Treatment

The biofilms formed on the metal slides, of each parallel flow cell reactor, were exposed to different surfactant concentrations for 30 min. Each concentration was tested in an independent experiment and each experiment was performed three times. During the treatment period, the surfactant solution replaced the diluted bacterial suspension flowing in the flow cells. After the exposure time to the surfactant, the flow of the surfactant solution through the flow cells was stopped and the initial bacterial suspension was re-introduced in the system, in order to restore the conditions prior to surfactant application and to mimic real situations encountered in industrial processes. Immediately after the surfactant treatment (0 h), two metal slides of each flow cell were sampled. The biofilms that covered the SS slides were completely scraped, resuspended in 10 ml of a neutralization solution, which consisted of phosphate buffer containing (w.v^{-1}) 0.1 % peptone, 0.5 % Tween 80 and 0.07 % lecithin and left for 10 min. After that, the biofilm suspensions were vortexed during 30 s, washed two times with saline phosphate buffer (PBS), resuspended in phosphate buffer and used immediately to assess the bacterial respiratory activity and biofilm mass. In order to assess whether time plays a significant role on the action of SDS and CTAB, namely if it prevents a subsequent biofilm growth, the remaining slides were left in the flow cells with the operation

conditions restored and were only sampled 3, 7 and 12 h after surfactant application in order to assess the post-surfactant action. The control experiments were performed in the same operational conditions, but with the addition of PBS instead the surfactant solution.

Biofilm Mass

The dry mass of the biofilm accumulated on the slides was assessed by the determination of the total volatile solids (TVS) of the homogenised biofilm suspensions, according to the Standard Methods (APHA, AWWA, WPCF, 1989), method number 2540 A-D. The biofilm mass accumulated was expressed in mg of biofilm *per* cm^2 of surface area of the slide (mg $_{biofilm}$ cm^{-2}).

In each experiment, the percentage of the biofilm removal was determined through the following equation:

Biofilm removal (%) = $[(W-W_1)\ W^{-1}] \times 100$ (1)

where W is the biofilm mass without surfactant application (mg $_{biofilm}$ cm^{-2}) and W_1 is the biofilm mass after surfactant treatment (mg $_{biofilm}$ cm^{-2}).

Oxygen Uptake Rate

The respiratory activity of the several samples was evaluated by measuring the oxygen uptake rate needed to oxidise glucose 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.* 2005).

The decrease in the bacterial activity observed due to the application of the different concentrations of surfactant to both flow-generated biofilms was determined as the difference between the respiratory activity of the samples before (control) and immediately after the treatment period with surfactant, and expressed as the percentage of inactivation according to the following equation:

Inactivation (%) = $[(A_0-A_1)\ A_0^{-1}] \times 100$ (2)

where A_0 is the respiratory activity of the control assay, *i.e.*, without surfactant treatment (mg O_2 g $_{biofilm}$.min^{-1}), and A_1 is the respiratory activity immediately after the application of each surfactant concentration (mg O_2 g $_{biofilm}$.min^{-1}).

All the respirometric tests were carried out at least three times, for each condition tested.

Scanning Electron Microscopy Observations

Scanning electron microscopy (SEM) inspections were performed according to the procedure described by Simões *et al.* (2003) in order to assess potential structural alterations caused.

Statistical Analysis

The mean and standard deviation within samples were calculated for all cases. Statistical comparisons of biofilm inactivation, biofilm removal and recovery were analysed by *t* Student's test.

RESULTS AND ANALYSIS

Biofilm Inactivation and Removal due to CTAB and SDS Application

The effects of the application of CTAB and SDS for 30 min against biofilms formed on SS slides, under turbulent and laminar flow, were assessed either by determining the respiratory activity due to glucose oxidation and the variation of the mass of biofilm. Those results are presented in terms of percentage of biofilm inactivation and biofilm removal (Fig. 1), immediately after surfactant application.

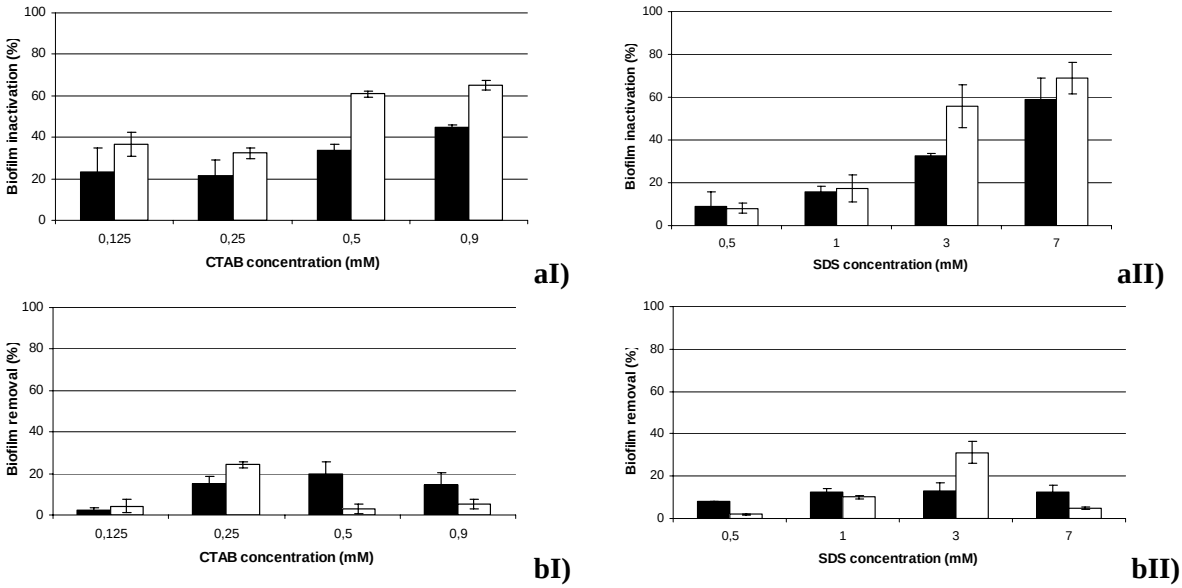

Fig. 1 - Biofilm inactivation (a) and removal (b) due to application of different concentrations of CTAB (I) and SDS (II). Each symbol indicates the means ± SD. ■ - Turbulent; □ - Laminar.

The application of CTAB to biofilms formed in the flow cell reactors resulted in the inactivation of the bacteria within the biofilms, which increased with the increase of the surfactant concentration (Fig. 1aI). Concerning the flow conditions under which biofilms were generated, the inactivation effect of CTAB was more pronounced for laminar flow-generated biofilms ($P < 0.05$). SDS also promoted biofilm inactivation, being this effect concentration dependent ($P < 0.05$). Comparing the inactivation data for turbulent and laminar flow-generated biofilms (Figs. aI and aII), a statistical analysis showed that both biofilms had similar susceptibility to the SDS action ($P > 0.1$). However, the overall data related with biofilm inactivation highlighted that both surfactants in the range of concentrations tested did not cause total inactivation (maximum of inactivation was around 60-70 %). Figs. 1bI and 1bII also demonstrate that both surfactants did not promoted biofilm detachment, since removal was always less than 30 %, independently on the concentration ($P > 0.05$ – for both surfactants). The higher amount of biofilm detached from the SS slides was observed for laminar flow-generated biofilms, after treatment with respectively, 0.250 mM of CTAB and 3 mM of SDS.

Structural Changes due to Surfactant Application

The evidence of bacterial biofilm in the metal slides before the treatment and the possible damage resulting from CTAB and SDS treatment was inspected by SEM, as displayed in Fig. 2.

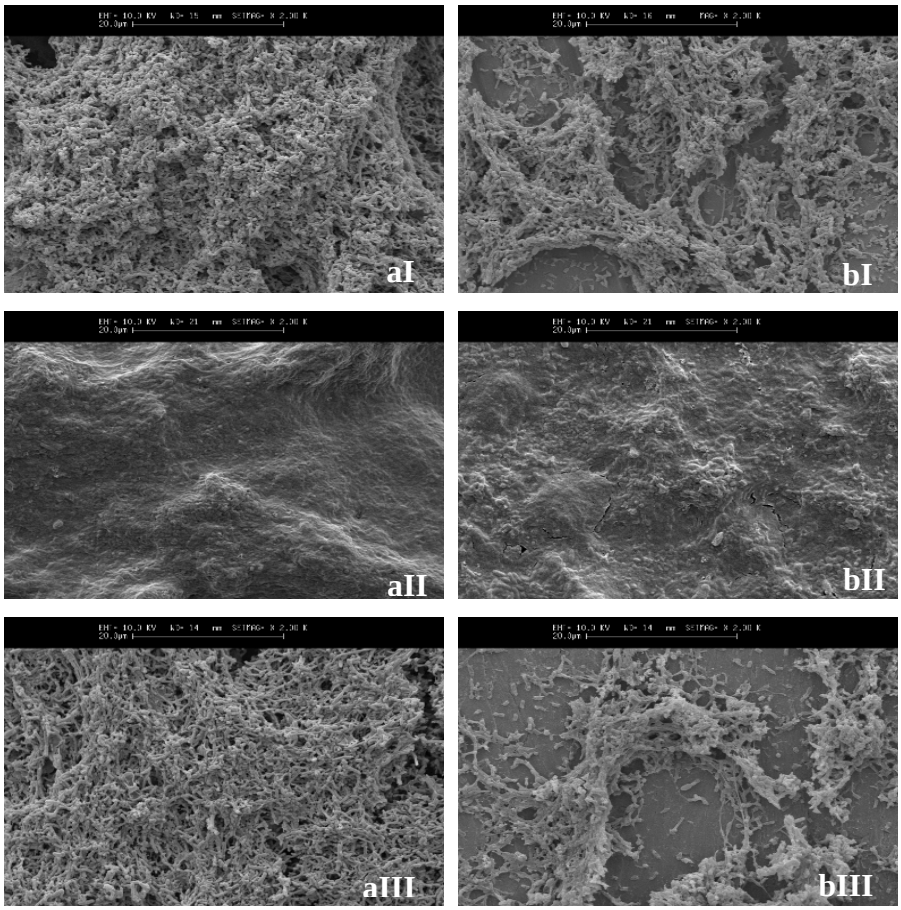

Fig. 2 - SEM microphotographs of a 7 d old *P. fluorescens* biofilms formed on stainless steel slides under turbulent (a) and laminar flow (b) without surfactant application (I), after treatment with 0.5 mM of CTAB (II) and after treatment with 3 mM of SDS (III) during 30 min. X2000 magnification, bar = 10 μm.

SEM observations (Fig. 2) show that biofilms formed under different flow regimes present significant morphological differences (Figs. 2aI and 2bI) and that the surfactants damaged the biofilm structure. The probable phenomenon behind this fact is related with the reaction with cationic and anionic reactive sites existent in the biofilm, respectively for SDS and CTAB. However, the structural changes are more evident for CTAB treated biofilms. In fact, after treatment with 0.5 mM of CTAB the structure of the biofilm was significantly altered. The reactivity of SDS with the biofilms is not as evident as for CTAB, probably due to electrostatic interactions. However, it can be observed that the treatment of laminar flow-generated biofilms with 3 mM of SDS clearly reduced the amount of biofilm. This SEM evidence is in accordance with the data related with biofilm removal, since is that SDS concentration that causes higher biofilm detachment (Fig. 1bII).

Biofilm Recovery after Treatment with CTAB and SDS

The results presented in Fig. 1 emphasize that after 30 min of contact with the surfactants, and for all the concentrations tested, biofilms still show respiratory activity. In order to know whether this fact could lead to biofilm recovery, the post-surfactant effect was evaluated along 12 h. Fig. 3 presents the biofilm behaviour, in terms of respiratory activity and mass, of turbulent and laminar flow-generated biofilms, after surfactant treatment. That post-surfactant response was evaluated after 3, 7 and 12 h later and compared with the results obtained immediately after the chemical treatment (0 h).

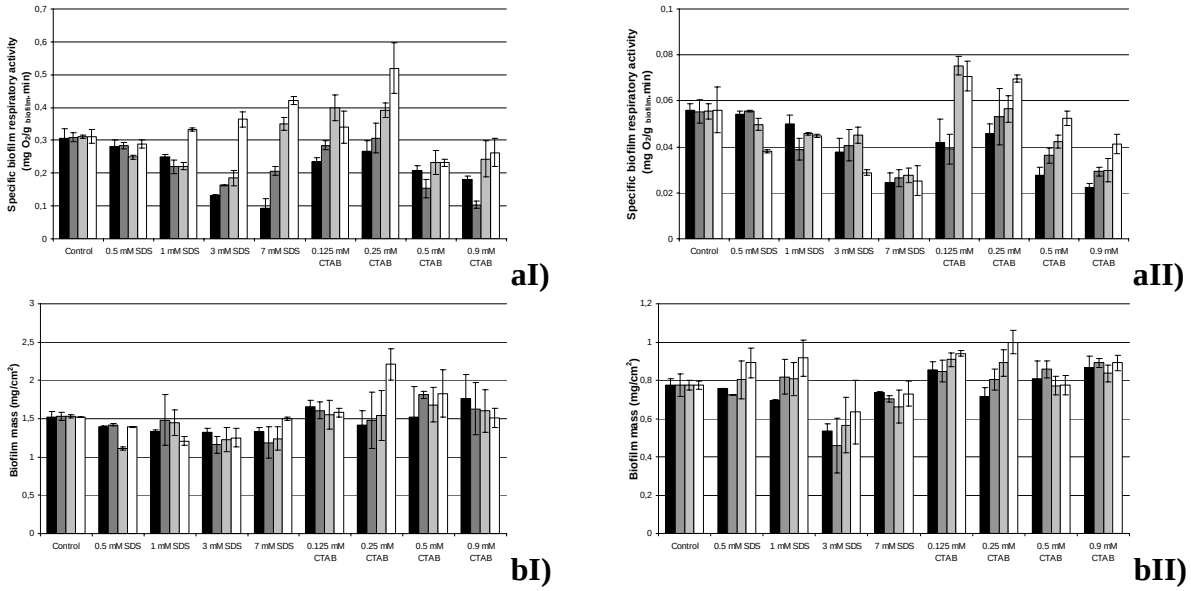

Fig. 3 - Biofilm respiratory activity (a) and mass (b) after chemical treatment (0 h) and 3, 7 and 12 h later for biofilms formed under turbulent (I) and laminar (II) flow. Control means without surfactant treatment. ■ 0 h; ▩ 3 h; ▩ 7 h; □ 12 h.

From the results obtained after treatment with SDS, the activity of biofilms increased with time (Fig. 3aI), particularly when 3 mM and 7 mM of SDS were applied to the turbulent flow-generated biofilms ($P < 0.05$), being the respiratory activity recovery more pronounced with the increase of the SDS concentration applied to the biofilms.

Concerning CTAB, in general the respiratory activity increased with the time between CTAB application and biofilm sampling, reaching values higher than the ones observed in the control experiment, *i.e.*, without surfactant application. Both turbulent and laminar flow-generated biofilms had similar recovery profiles when comparing statistically ($P > 0.05$).

The control experiments showed that the biofilm activity was almost independently of the time ($P > 0.05$) since the 7 d old biofilms exhibit the same respiratory activity during the time (12 h). Comparing the results of biofilm recovery after CTAB and SDS application, the recovery is more evident for biofilms treated with CTAB and less clear to laminar biofilms treated with SDS (Figs 3aI and 3bI).

In terms of total biofilm mass, only small variations were achieved with the surfactant treatment, being those variations more noticeable for laminar biofilms treated with SDS (Fig. 3bII). The application of CTAB to both turbulent and laminar biofilms did not give rise to the biomass decrease (Figs. 3aII and 3bII). On the contrary, it seems that the application of CTAB increased the amount of biofilm adhered to the SS slides. Therefore, it is clear that the application of SDS or CTAB and the time did not promoted any significant additional biofilm removal or growth, for any conditions tested and for any sampling time ($P > 0.05$ – for both surfactants and for every condition tested).

DISCUSSION AND CONCLUSIONS

The results depicted in Fig. 1, clearly show that the application of the surfactants decrease the respiratory activity and mass of both turbulent and laminar flow-generated biofilms. However, for the range of concentrations tested, total inactivation and removal was not achieved. Comparing hydrodynamic distinct biofilms, it is noticeable that the hydrodynamic conditions under which the biofilms were formed played a significant role on the resistance

to the chemical agents since laminar flow-generated biofilms were more susceptible to CTAB action than the turbulent ones. The higher inactivation effect on laminar flow-generated biofilms is probably related with the less amount of mass formed, compared with the turbulent ones (Simões, 2005) and consequently, to the less content of proteins which increased the surfactant available for reaction with the cells. This surfactant/exopolymeric matrix interaction is reinforced by previous tests carried out with planktonic cells, which showed that the inactivation effect of CTAB and SDS was significantly reduced in the presence of bovine serum albumin (Simões, 2005). In the present study, the low efficacy of the surfactants to control biofilms may be related with its chemical reaction with proteins of the exopolymeric matrix, as evidenced by SEM inspections (Fig. 2). The effect of the surfactants on the biofilm structure is more evident after CTAB application. Probably, the anionic properties of the biofilm matrix (Simões, 2005), quenched the effect of SDS on the biofilm structure due to electrostatic interactions, being the cells embedded in the biofilm the main target of SDS.

In both hydrodynamic situations and for both surfactants, problems associated with mass transfer limitations within the biofilms can, always, decrease the surfactant action. In fact, the understanding of the effect of operational parameters that affect the biofilm formation and subsequent disinfection plays a basic role on the establishment of a biofilm control program. Previous studies made by some authors (Pereira *et al.*, 2002; Vieira *et al.* 1993), concerning the characterisation of biofilms formed under turbulent and laminar flow, showed that biofilms formed under turbulent flow are more active and have a higher content of proteins than laminar biofilms and that their physical structure is different.

Biofilm removal results demonstrate that inactivation and removal are distinct processes (Fig. 1). The ability of CTAB and SDS to inactivate was higher than to remove biofilms from surfaces, leaving the biofilms on the surface not fully inactivated. The biofilms left in the flow cells after surfactant treatment recovered their respiratory activity in a period of time less than 12 h. This recover is more evident for biofilms treated with CTAB and less clear to laminar flow-generated biofilms treated with SDS. The overall results suggested that if the biofilms were left in the flow cell reactors more time, probably, the recovery of biofilm will be more evident and consistent. Furthermore, the ionic nature of the surfactant seems to be responsible for the alteration effects of the biofilm respiratory activity, playing a biofilm preventive action when the surfactant concentrations applied were near the CMC. In both cases, it was expected a more sustained antibacterial effect, since the biofilms which were not immediately sampled after surfactant application were not subjected to the neutralization step. Thus, the surfactant retained within the biofilm matrix had more chance to act on the bacteria. Forsyth and Hayes (1998) stated that surfaces treated with cationic surfactants could retain a bacteriostatic film, due to the adsorption of the chemical on the surface, this could prevent the subsequent growth of residual bacteria. Nevertheless, data presented in this study proved that the surfactants did not induced suppression of biofilm recovery for both biofilms. The biofilm recovery must be associated with the stress conferred by the surfactant application. Probably, the surfactant may have increased the availability of nutrients to the cells embedded in the biofilms (promoting bacterial recovery) since the surfactant have changed the structure of the biofilm matrix (Fig. 2), namely the porosity of the biofilm, and thus favouring nutrient diffusion inside the matrix. This effect occurred, probably, without killing the microorganisms.

The permanence of a remaining pellicle, as evidenced by the biofilm mass results and SEM inspections, that is still active, or in another metabolic state, may be a source of problems, such as biofilm recovery, development of resistant biofilms or a substrate for other microorganisms. According to Stewart (2003), an inefficient biofilm control could lead to the existence of persister bacteria, which may be recalcitrant to a subsequent disinfection process.

In conclusion, this study shows that a better understanding of biofilm response face to an external stress condition is essential for the emerge of new strategies for controlling biofilms. Biofilms formed under laminar flow were more susceptibly to the inactivation effect than turbulent flow-generated biofilms, but none of them were removed by the surfactants tested. The biofilm structure was markedly changed after surfactant treatment, mainly for CTAB treated biofilms. A post-surfactant effect was noticed for both biofilms since they recovered their metabolic activity along time, after surfactant treatment. Concerning biofilm mass, the surfactants did not promote a slow biofilm detachment or the increase in the biofilm mass, probably due to the limited time of experiment. This improvement in the understanding of the relationship between surfactant molecular properties and antibacterial properties and mechanisms of action could facilitate the design of chemical mixtures that more effectively control biofilms.

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