IChemE
www.icheme.org/fbp
doi: 10.1205/fbp06022

0960–3085/06/$30.00+0.00

Trans IChemE, Part C, December 2006
Food and Bioproducts Processing, 84(C4): 338–345

CONTROL OF FLOW-GENERATED BIOFILMS WITH SURFACTANTS
Evidence of Resistance and Recovery

M. SIMÕES, L. C. SIMÕES, I. MACHADO, M. O. PEREIRA and M. J. VIEIRA*

Centro de Engenharia Biológica—CEB, Universidade do Minho, Braga, Portugal

The action of cetyltrimethyl ammonium bromide (CTAB) and sodium dodecyl sulphate (SDS), a cationic and an anionic surfactant respectively, were investigated for their ability to control turbulent and laminar flow-generated biofilms formed by *P. fluorescens*. The disinfectant action of CTAB and SDS on biofilms was assessed by respiratory activity, variation of mass and structure, immediately, 3, 7 and 12 h after the application of the surfactants. 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 either type of flow-generated biofilms. For SDS, higher concentrations promoted significant biofilm inactivation, for both turbulent and laminar flow-generated biofilms. CTAB and SDS application did not promote the detachment of biofilms from the surfaces. Post-surfactant treatment, biofilms recovered respiratory activity, in some cases, reaching values higher than those found without chemical treatment. After CTAB treatment, the recovery of respiratory activity was not affected by the hydrodynamic conditions. Conversely, turbulent flow-generated biofilms showed a higher potential to recover their metabolic activity than laminar flow-generated biofilms, when previously challenged with SDS. Concerning biofilm mass, no significant variation (increase or decrease) was detected after 12 h of surfactant treatment. This study shows that care is needed when selecting the correct procedure and agent for biofilm control and demonstrates the influence of hydrodynamic conditions on the persistent and recalcitrant properties of *P. fluorescens* biofilms.

Keywords: biofilm control; hydrodynamic conditions; recovery; resistance; surfactant.

INTRODUCTION

The unwanted accumulation of biofilms in industrial equipment under aqueous environments, currently called biofouling, is a natural occurrence (Verran, 2002; Simões *et al.*, 2005a). Biocides still represent the more significant countermeasure to control biofilm formation (Chen and Stewart, 2000; Simões *et al.*, 2003a; 2005a). However, these chemical substances may kill the attached microorganisms but may not be effective in biofilm removal, leaving biomass on the surface that may contribute to microbial recovery and biofilm regrowth (Simões, 2005). 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 (Simões, 2005). 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 potential to form biofilm and promoting the detachment of these microorganisms from the adhesion surface (MacDonald *et al.*, 2000). As bacteria within biofilms are protected from even the most aggressive of treatment regimens, it is expected that they behave differently from the planktonic state, when exposed to chemical treatment (biocide/surfactant), due to possible alterations of their metabolic activity, biofilm structure and composition (Simões, 2005). Furthermore, biofilm development, behaviour, population characteristics and response to the action of antimicrobial agents are strongly influenced by many environmental factors, such as physical forces acting on the biofilm and by intrinsic biological properties (Purevdorj *et al.*, 2002; Simões *et al.*, 2003a, b; Stoodley *et al.*, 1999; Vieira *et al.*, 1993). One of the most important factors is the velocity field of the fluid in contact with the microbial layer (Pereira *et al.*, 2002a; Purevdorj *et al.*, 2002; Stoodley *et al.*, 1999; Vieira *et al.*, 1993).

The aim of this work was to assess the efficacy of surfactant treatment (CTAB and SDS) in the control of turbulent

**Correspondence to*: Professor M. João Vieira, Centro de Engenharia Biológica, Universidade do Minho, 4710-057 Braga, Portugal.
E-mail: mjv@deb. uminho.pt

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 environments (Pereira *et al.*, 2002a; Simões 2005; Simões *et al.*, 2005a; 2006; Wiedmann *et al.*, 2000), in a simple flow cell reactor (Pereira *et al.*, 2002b) that allows biofilm sampling without disturbing the system.

MATERIAL AND METHODS

Microorganism and Culture Conditions

Pseudomonas fluorescens ATCC 13525^{T} was obtained from the American Type Culture Collection and preserved in criovials (Nalgene) at $-80 \pm 2^{\circ}$C. The growth conditions were $27 \pm 1^{\circ}$C, pH 7.0 (0.02 M phosphate buffer), with 5 g L^{-1} glucose, 1.25 g L^{-1} yeast extract and 2.5 g L^{-1} peptone as nutrients. All the medium components were purchased from Merck (VWR, Portugal).

Biofilm Reactors

A continuous pure culture of *P. fluorescens* was grown in a 0.5 L glass chemostat, at 27°C, aerated (air flow rate = 0.425 min^{-1}) and agitated with a magnetic stirrer (Heidolph Mr 3001). The chemostat was continuously fed (peristaltic pump, Ismatec Reglo) with 10 mL h^{-1} of sterile medium containing 5 g L^{-1} glucose, 2.5 g L^{-1} peptone and 1.25 g L^{-1} yeast extract in 0.02 M phosphate buffer pH 7.0. The 0.5 L chemostat was used to continuously inoculate (10 mL h^{-1}) a 3.5 L reactor which was aerated (air flow rate = 0.243 min^{-1}) and agitated with a magnetic stirrer. This reactor was fed with a diluted nutrient medium consisting of 0.05 g L^{-1} glucose, 0.025 g L^{-1} peptone and 0.0125 g L^{-1} yeast extract in 0.02 M phosphate buffer (pH 7), at a flow rate of 1.7 L h^{-1}, which supported a bacterial cell density of approximately 6×10^{7} cells mL^{-1}. This diluted bacterial suspension obtained in the 3.5 L reactor was pumped back (centrifugal pumps, Eheim Typ 1060 and Eheim Typ 1048) through the flow cell reactors and to the 3.5 L reactor.

A flow cell reactor system described by Pereira *et al.* (2002b) was used as the device for biofilm formation, as depicted in Figure 1. This type of device offers a simple means to study and characterize biofilms in a well-controlled and reproducible manner. This device consists of a semicircular Perspex (polymethyl methacrylate) duct (45 cm length and 1.6 cm of hydraulic diameter) with 10 apertures on its flat wall, to suitably fit several removable rectangular pieces of Perspex. Stainless steel (ASI 316) slides (1.75 cm × 1.25 cm) were glued onto the Perspex faces. Biofilms were formed on those metal slides whose

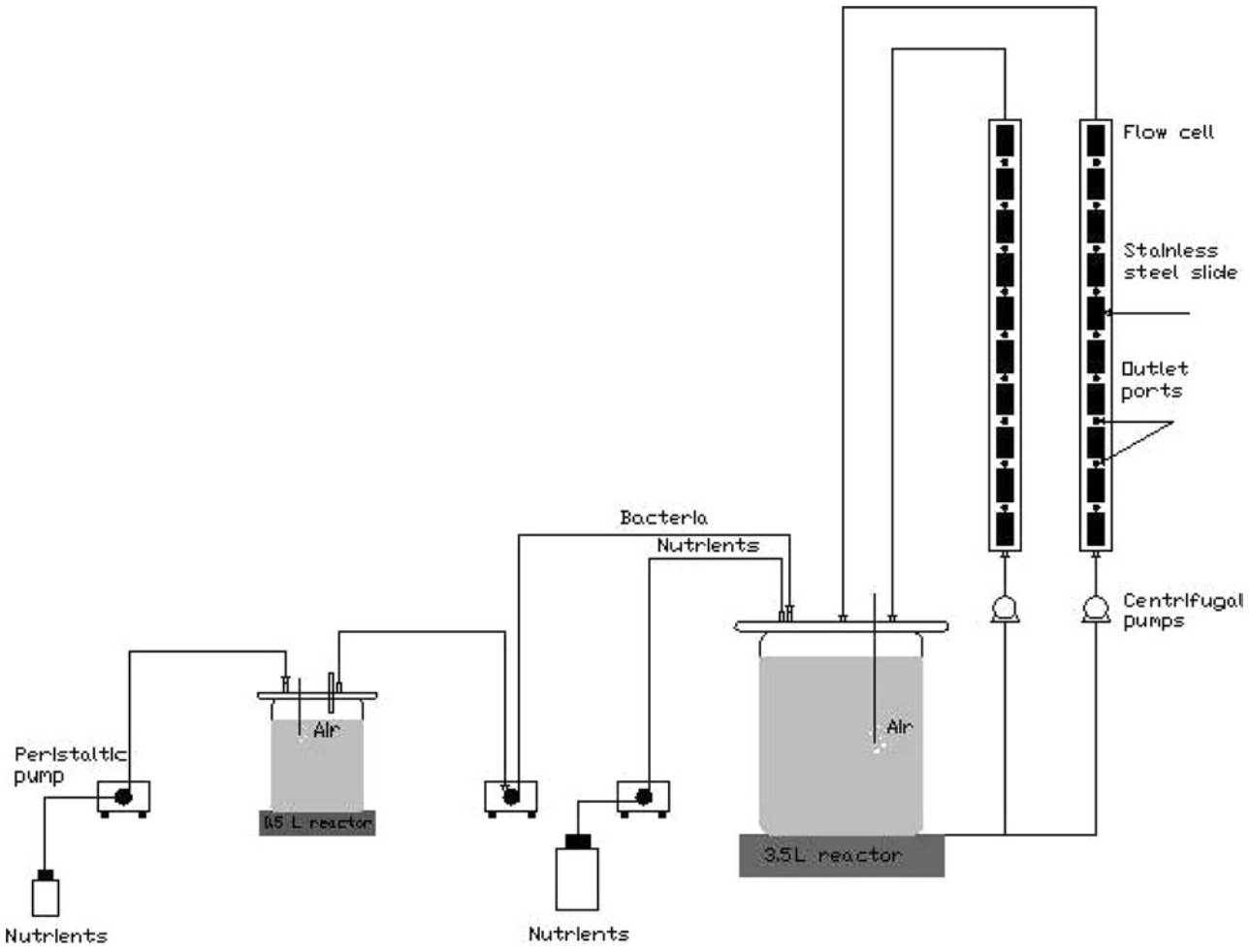

Figure 1. Schematic representation of the system used to perform biofilm formation on the flow cell reactors.

upper faces were in contact with the bacterial suspension passing through the flow cell reactor. Each of the rectangular pieces can be removed separately without disturbing the biofilm formed on other pieces and without stopping the flow. This is managed because outlet ports are placed on the round face of the flow cell between each two adjacent removable pieces of Perspex, thus allowing diversion of the circulating flow from the point where the reactor was opened. Two parallel similar flow cell reactors were used simultaneously in such a way that biofilms were formed under turbulent (Reynolds number—$Re = 5200$, $u = 0.532\ m\ s^{-1}$) and laminar ($Re = 2000$, $u = 0.204\ m\ s^{-1}$) conditions, respectively, in each flow cell, in order to mimic flow-dependent processes encountered in industrial environments (Pereira *et al.*, 2002a, b; Simões *et al.*, 2003a, b; Simões, 2005). The biofilms were allowed to grow for 7 days to ensure that steady-state biofilms were used in every experiment (Pereira *et al.*, 2002b).

Surfactants

Cetyltrimethyl ammonium bromide (CTAB), a cationic surfactant, purchased from Merck (Critical micellar concentration—CMC—1.00 mM; cat. no. 102342). The concentrations tested were 0.125, 0.250, 0.500 and 0.900 mM.

Sodium dodecyl sulfate (SDS), an anionic surfactant, purchased from Riedel-de-Haën (CMC—8.30 mM; cat. no. 62862). The concentrations tested were 0.5, 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 stainless steel slides were completely scraped, resuspended in 10 ml of a neutralization solution (not interfering with the bacterial respiratory activity), which consisted of phosphate buffer containing (w/v) 0.1% peptone, 0.5% Tween 80 and 0.07% lecithin and left for 10 min, according to the European Standard EN-1276 (1997). After that, the biofilm suspensions were vortexed (Heidolph, model Reax top) for 30 s with 100% power input, according to the methodology described by Simões *et al.* (2003a), washed twice with saline phosphate buffer, resuspended in 10 mL of 0.02 M phosphate buffer (pH 7) and used immediately to assess, sequentially, 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. Two metal slides were sampled for each sampling time. The control experiments were performed in the same operational conditions, but with the addition of phosphate buffer instead the surfactant solution.

Oxygen Uptake Rate—Respiratory Activity Assessment

The assays were performed in a model 53 Yellow Springs Instruments (Ohio, USA) Biological oxygen monitor (BOM) as described previously (Simões *et al.*, 2003a; 2005b). The homogenized biofilm suspensions were placed in the temperature-controlled vessel of the BOM ($T = 27^{\circ}C \pm 1^{\circ}C$). Each one contains a dissolved oxygen (DO) probe connected to a DO meter. Once inside the vessel, the samples were aerated for 30 min to ensure the oxygen saturation ($[O_2] = 9.2\ mg\ L^{-1} - 27^{\circ}C$, 1 atm). The vessel was closed and the decrease of the oxygen concentration was monitored over time. The initial linear decrease observed corresponds to the endogenous respiration rate. To determine the oxygen uptake due to substrate oxidation, 50 μL of a glucose solution ($100\ mg\ L^{-1}$) was introduced in each vessel. The slope of the initial linear decrease in the DO concentration, after glucose injection, corresponds to the total respiration rate. The difference between the two respiration rates gives the oxygen uptake rate due to the glucose oxidation.

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:

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

where A_0 is the respiratory activity of the control assay, i.e., without surfactant treatment ($mgO_2/g_{biofilm} \cdot min$), and A_1 is the respiratory activity immediately after the application of each surfactant concentration ($mgO_2/g_{biofilm} \cdot min$). Each A_1 value was standardized per respective biofilm mass remaining adhered after surfactant treatment.

Biofilm Mass

The dry mass of the biofilm accumulated on the slides, after the respiratory activity determination, was assessed by the determination of the total volatile solids (TVS) of the homogenized biofilm suspensions, according to the Standard Methods (APHA, AWWA, WPCF, 1989), method number 2540 A-D. According to this methodology the TVS assessed at $550 \pm 5^{\circ}C$ in a furnace (Lenton thermal designs) for 2 h is equivalent to the amount of biological mass. 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:

$$\text{Biofilm removal } (\%) = [(W - W_1)W^{-1}] \times 100 \qquad (2)$$

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

Scanning Electron Microscopy Observations

During the experiments, several stainless steel slides covered with biofilms were observed by scanning electron microscopy (SEM). The SEM inspections always comprised the observation of at least 15 fields of each biofilm-covered slide. Prior to SEM observations, biofilm samples were gradually dehydrated in ethanol (Merck) series to 100% (15 min each in 10, 25, 40, 50, 70, 80, 90 and 100% v/v), and dried in a desicator for 3 days. The samples were sputter-coated with gold and examined with a Leica S360 scanning electron microscope at 10–15 kV. The slides were not fixed because fixation procedures involves the use of chemicals that tend to react with some of the components at the biological matrix, as documented by Azeredo *et al.* (1999), hence modifying the real biofilm structure. SEM observations were documented through the acquisition of at least 20 representative microphotographs for each experiment.

Statistical Analysis

The data were analysed using the statistical program SPSS version 11.5 (Statistical Package for the Social Sciences). The mean and standard deviation within samples were calculated for all cases. Statistical comparisons of biofilm inactivation, biofilm removal and recovery were analysed by Student's t-test. Statistical calculations were based on confidence level equal or higher than 95% ($P < 0.05$ was considered statistically significant).

RESULTS AND ANALYSIS

Biofilm Inactivation and Removal due to CTAB and SDS Application

The effects of the application of CTAB and SDS during 30 min against biofilms formed on stainless steel slides, under turbulent and laminar flow, were assessed by the determination of 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 (Figure 2), immediately after surfactant application.

The application of CTAB to biofilms formed in the flow cell reactors resulted in the inactivation of the bacteria within the biofilms [Figure 2(aI)]. The concentrations of 0.125 and 0.25 mM had similar inactivation effects. Again, 0.5 and 0.9 mM produce similar biofilm inactivation. However, statistical analyses reveal that inactivation is concentration dependent ($P < 0.05$). 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, dependent on the SDS concentration ($P < 0.05$). Comparing the inactivation data of turbulent and laminar flow-generated biofilms [Figure 2(aI) and (aII)], a statistical analysis showed that both biofilms had similar susceptibility to SDS action ($P > 0.1$). However, the overall results related with biofilm inactivation highlighted that neither surfactants, in the range of concentrations tested, caused total inactivation

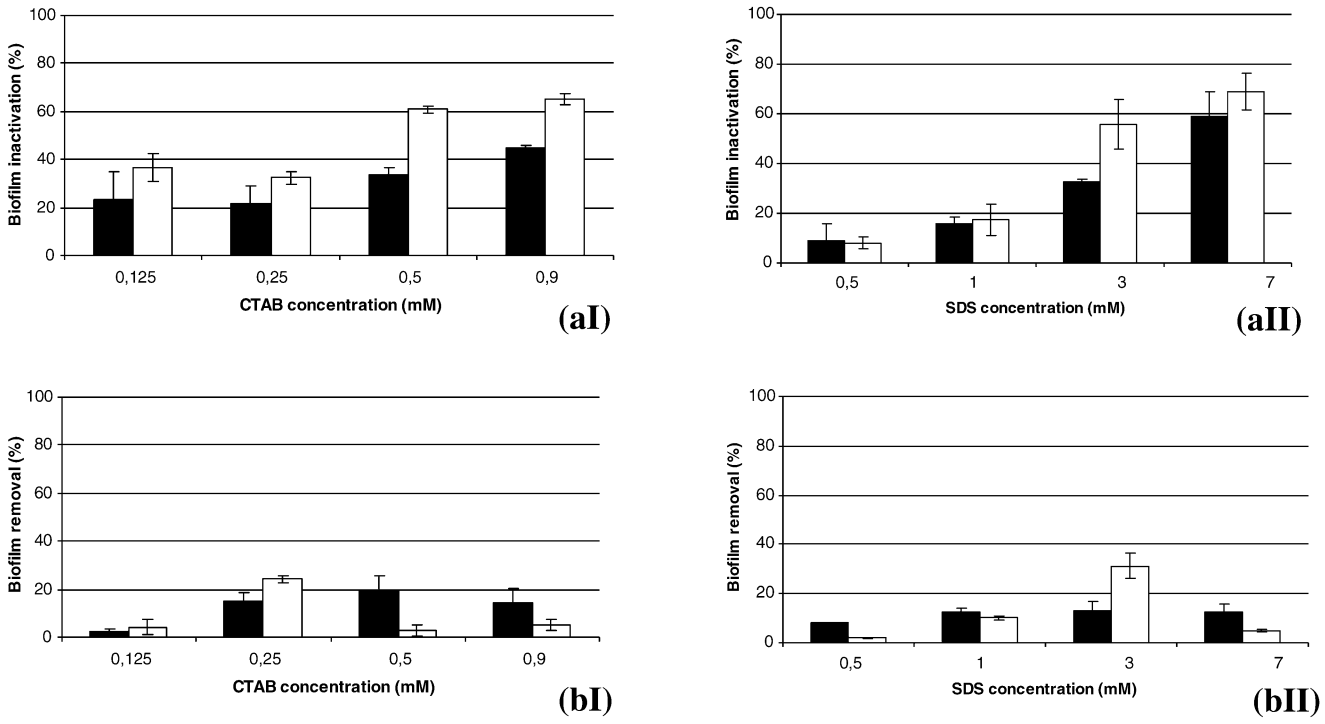

Figure 2. Biofilm inactivation (a) and removal (b) due exposure to different concentrations of CTAB (I) and SDS (II) for 30 min. Each symbol indicates the means ± SD. ■, Turbulent; □, laminar. The means ± SD for at least three replicates are illustrated.

(maximum of inactivation was around 60–70%). Figure 2(bI) and (bII) also demonstrate that neither surfactant promoted biofilm detachment, since removal was always less than 30%, independently on the concentration ($P > 0.05$ for both surfactants).

Structural Changes Due to Surfactant Application

Biofilm on the metal slides before surfactant treatment and the possible damage resulting from CTAB and SDS application, with representative concentrations (0.25 mM of CTAB and 3 mM of SDS), was inspected by SEM, as displayed in Figure 3.

SEM observations (Figure 3) show that biofilms formed under different flow regimes present significant morphological differences [Figure 3(aI) and (bI)] and that the surfactants altered the biofilm structure. This is probably due to CTAB and SDS reaction with cationic and anionic reactive sites respectively, within the biofilm. However, the biofilm structural changes are more evident for CTAB treated biofilms.

Biofilm Recovery after Treatment with CTAB and SDS

The results presented in Figure 2 emphasize that after 30 min of contact with the surfactants, and for all the concentrations tested, biofilms still show respiratory activity. In order to determine whether this property could lead to biofilm recovery, the post-surfactant effect was evaluated for up to 12 h. Figure 4 presents the biofilm behaviour, in terms of respiratory activity and mass, of turbulent and laminar flow-generated biofilms, after surfactant treatment.

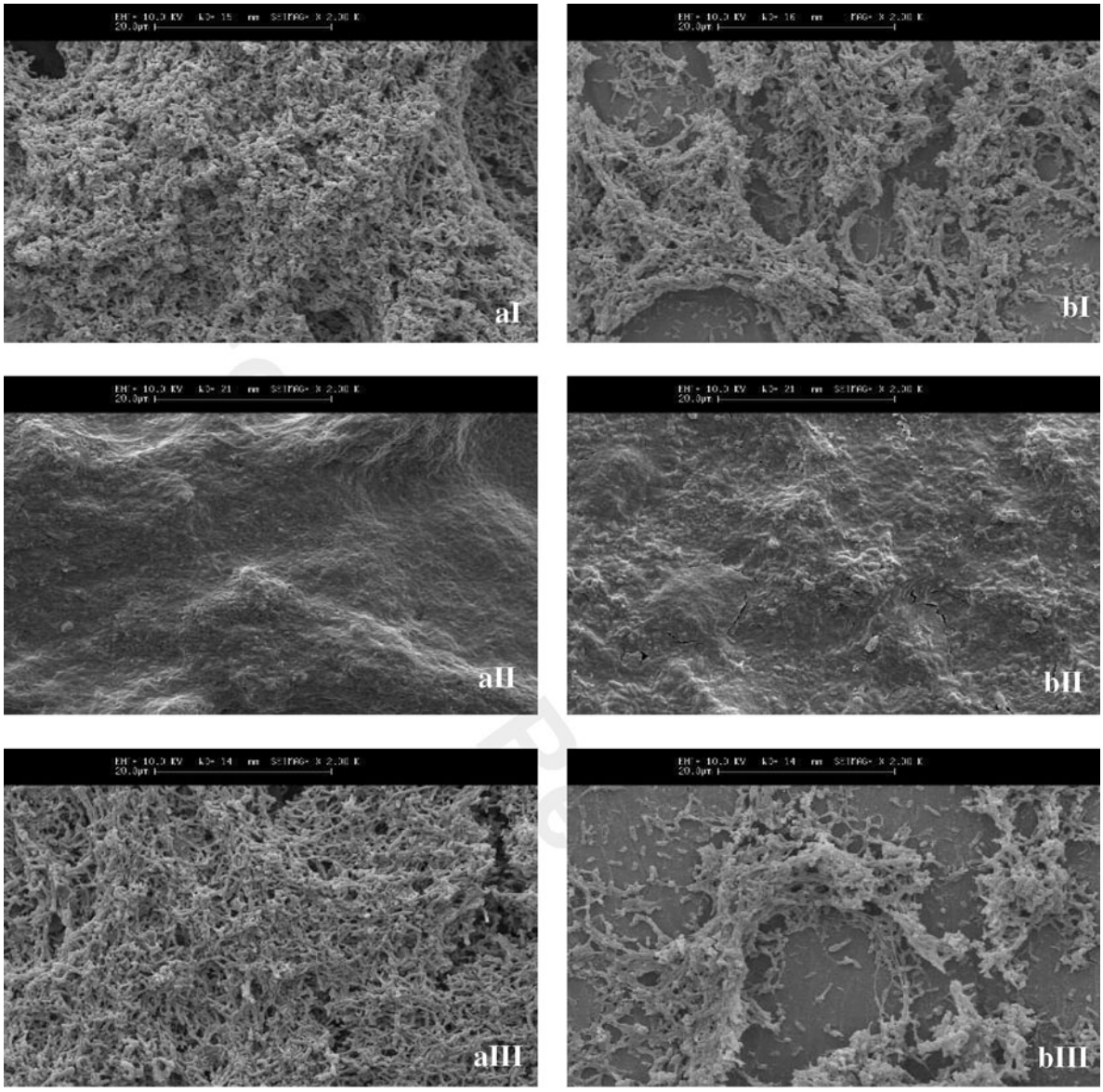

Figure 3. SEM microphotographs of a 7 day-old *P. fluorescens* biofilms formed on stainless steel slides under turbulent (a) and laminar flow (b) without surfactant application (I), after treatment with 0.5 mM of CTAB (II) and after treatment with 3 mM of SDS (III) during 30 min. ×2000 magnification, bar = 20 μm.

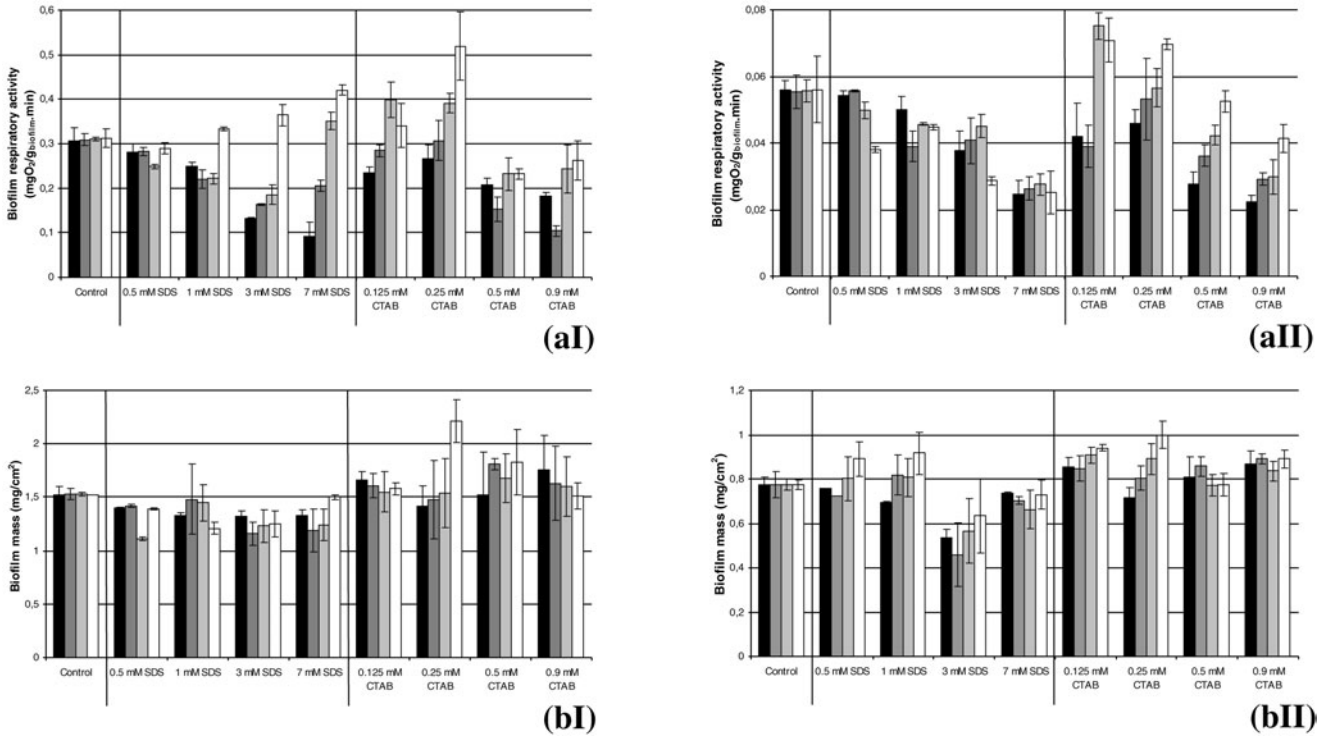

Figure 4. Biofilm respiratory activity (a) and mass (b) immediately after chemical treatment (0 h) and 3, 7 and 12 h later for biofilms formed under turbulent (I) and laminar (II) flow. Control means without surfactant treatment. ■, 0 h; ■, 3 h; ■, 7 h; □,12 h. The means ± SD for at least three replicates are illustrated.

The results obtained after SDS treatment showed that the activity of biofilms increased with time [Figure 4(aI)], particularly when 3 mM and 7 mM of SDS were applied to the turbulent flow-generated biofilms ($P < 0.05$). In fact, respiratory activity recovery was more pronounced with the increase of SDS concentration applied to the biofilms ($P < 0.05$). 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 ($P > 0.05$). 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 flow-generated biofilms treated with SDS [Figures 4(aI) and 3(bI)]. The control experiments showed that biofilm respiratory activity was almost independent of the time ($P > 0.1$) since the 7 day-old biofilms exhibit the same respiratory activity during the time of the experiments (12 h).

In terms of total biofilm mass, only small variations were achieved with the surfactant treatment, none being significant. Overall, the application of CTAB to both turbulent and laminar biofilms did not give rise to biomass decrease [Figure 4(aII) and (bII)]. On the contrary, it seems that the application of CTAB, particularly with 0.25 mM, increased the amount of biofilm adhered to the stainless steel slides, a phenomenon not statistically significant ($P > 0.05$). Therefore, it is clear that the application of SDS or CTAB and the recovery 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

Application of the surfactants increased inactivation and decreased the mass of both turbulent and laminar flow-generated biofilms. However, in the range of concentrations tested, total inactivation and removal was not achieved. The hydrodynamic conditions under which the biofilms were formed played a significant role in the resistance to the chemical agents, with laminar flow-generated biofilms being more susceptible to CTAB action. The higher inactivation effect on laminar flow-generated biofilms is probably related to the lower amount of mass formed, compared with the turbulent ones (Pereira *et al.*, 2002a; Simões *et al.*, 2003b, 2004; Simões, 2005), especially with respect to proteins. Proteins (one of the major biofilm exopolymeric matrix constituent—Pereira and Vieira, 2001; Simões *et al.*, 2003a, b, 2004) can react with surfactants, decreasing their availability for reaction with the cells (Pereira and Vieira, 2001; Simões *et al.*, 2003a). 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 *et al.*, 2006). In the present study, the limited efficacy of the surfactants to control biofilms may be related with its chemical reaction with proteins of the exopolymeric matrix, as proposed in previous studies regarding biofilm cohesive forces (Simões *et al.*, 2005a). The effect of the surfactants on the biofilm

structure was more evident after CTAB application (Figure 3). Probably, the anionic properties of the biofilm matrix (Simões, 2005), quenched the effect of SDS on the biofilm structure, as the cells embedded in the biofilm are the main target of SDS. In both hydrodynamic situations and for both surfactants, problems associated with mass transfer limitations within the biofilms decrease the surfactant action. In fact, the understanding of the effect of operational parameters that affect the biofilm formation and subsequent disinfection are fundamental to the development of a biofilm control program. Previous studies made by some authors (Pereira *et al.*, 2002a; Simões *et al.*, 2004; Simões, 2005; Vieira *et al.*, 1993) concerning the characterization of biofilms formed under turbulent and laminar flow, showed that turbulent flow-generated biofilms are more active and have a higher content of proteins than laminar flow-generated biofilms and that their physical structure is different.

Biofilm removal results demonstrate that inactivation and removal are distinct processes. The ability of CTAB and SDS to inactivate biofilms was higher than that required to remove them; furthermore, residual biofilms were not fully inactivated (Figure 4). The biofilms left in the flow cells after surfactant treatment and exposed to nutrients, recovered their respiratory activity in less than 12 h. This recovery was more evident for biofilms treated with CTAB. Respiratory activity results were corroborated by epifluorescence microscopy analysis using a viability stain (results not shown). The overall results suggested that if the biofilms were left in the flow cell reactors for a longer period, that biofilm recovery may be more evident and consistent (Figure 4).

The ionic nature of the surfactant seems to be responsible for the effects on the biofilm respiratory activity (Figure 4). In both cases, a more sustained antibacterial effect was expected, since the biofilms which were not immediately sampled after surfactant application were not subjected to a surfactant neutralization or washing step. Thus, the surfactant retained within the biofilm matrix had more chance to act on the bacteria. Forsythe 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, preventing the subsequent growth of residual bacteria. According to Chandy and Angles (2001) one of the key factors that determine bacterial recovery in drinking water distribution systems is the availability of nutrients, a phenomenon verified in this study. Perhaps, the surfactant may have increased the availability of nutrients to the cells embedded in the biofilms (promoting bacterial recovery) by altering the structure of the biofilm matrix, without killing the microorganisms. This presence of residual active biofilm may be a source of problems in terms of biofilm recovery, development of resistant biofilms, or as a substratum for colonisation by other microorganisms. According to Stewart (2003), inefficient biofilm control could lead to the existence of persistent bacteria, which may be recalcitrant to a subsequent disinfection process.

In conclusion, this study shows that a better understanding of biofilm response to external stress conditions is essential for the successful development of new strategies for controlling biofilms. Biofilms formed under laminar flow were more susceptibly to the inactivation effect than turbulent flow-generated biofilms, mainly when CTAB was applied, but none were removed by the surfactants tested. The biofilm structure was markedly changed after surfactant treatment, but metabolic activity was recovered over time, after treatment. The surfactants did not promote a slow biofilm detachment or an increase in the biofilm mass. An 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.

REFERENCES

APHA, AWWA, WPCF, 1989, *Standard Methods for the Examination of Water and Wastewater*, 17th edition, in Clesceri, L.S., Greenberg, A.E. and Trussel, R.R. (eds) (American Public Health Association, Washington DC, USA).

Azeredo, J.A., Lazarova, V. and Oliveira, R., 1999, Methods to extract the exopolymeric matrix from biofilms: a comparative study, *Wat Sci Tech*, 39: 243–250.

Chandy, J.P. and Angles, M.L., 2001, Determination of nutrients limiting biofilm formation and the subsequent impact disinfectant decay, *Water Res*, 35: 2677–2682.

Chen, X. and Stewart, P.S., 2000, Biofilm removal caused by chemical treatments, *Water Res*, 34: 4229–4233.

European Standard—EN 1276, 1997, Chemical disinfectants and antiseptics—quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic, and institutional areas—test method and requirements (phase 2, step 1).

Forsythe, S.J. and Hayes, P.R., 1998, *Food Hygiene, Microbiology and HACCP*, 3rd edition, 327–371 (Aspen Publishers, Inc., Gaithersburg, Maryland).

MacDonald, R., Santa, M. and Brözel, V.S., 2000, The response of a bacterial biofilm community in a simulated industrial cooling water system to treatment with an anionic dispersant, *J Appl Microbiol*, 89: 225–235.

Pereira, M.O. and Vieira, M.J., 2001, Effects of the interactions between glutaraldehyde and the polymeric matrix on the efficacy of the biocide against *Pseudomonas fluorescens* biofilms, *Biofouling*, 17: 93–101.

Pereira, M.O., Kuehn, M., Wuertz, S., Neu, T. and Melo, L., 2002a, Effect of flow regime on the architecture of a *Pseudomonas fluorescens* biofilm, *Biotechnol Bioeng*, 78: 164–171.

Pereira, M.O., Morin, P., Vieira, M.J. and Melo, L.F., 2002b, A versatile reactor for continuous monitoring of biofilm properties in laboratory and industrial conditions, *Lett Appl Microbiol*, 34: 22–26.

Purevdorj, B., Costerton, J.W. and Stoodley, P., 2002, Influence of hydrodynamics and cell signalling on the structure and behaviour of *Pseudomonas aeruginosa* biofilms, *Appl Environ Microbiol*, 68: 4457–4464.

Simões, M., Carvalho, H., Pereira, M.O. and Vieira, M.J., 2003a, Effect of different concentrations of ortho-phthalaldehyde on biofilms formed by *Pseudomonas fluorescens* under different flow conditions, *Biofouling*, 19: 287–295.

Simões, M., Pereira, M.O. and Vieira, M.J., 2003b, Monitoring the effects of biocide treatment of *Pseudomonas fluorescens* biofilms formed under different flow regimes, *Water Sci Technol*, 47: 217–223.

Simões, M., Pereira, M. O. and Vieira, M.J., 2004, Cellular changes due to biofilm formation—influence of flow regime. *Proceedings of the International Specialised Conference on Biofilms 2004—Biofilm Structure and Activity*, Las Vegas, NV, USA, 105–109.

Simões, M., Pereira, M.O. and Vieira, M.J., 2005a, Effect of mechanical stress on biofilms challenged by different chemicals, *Water Res*, 39: 5142–5152.

Simões, M., Pereira, M.O. and Vieira, M.J., 2005b, Validation of respirometry as short-term method to assess the toxic effect of a biocide, *Biofouling*, 21: 9–17.

Simões, M., 2005, Use of biocides and surfactants to control *Pseudomonas fluorescens* biofilms—role of hydrodynamic conditions, PhD thesis, University of Minho.

Simões, M., Pereira M.O., Machado, I., Simões, L.C. and Vieira, M.J., 2006, Comparative antibacterial potential of selected aldehyde-based

biocides and surfactants against planktonic *Pseudomonas fluorescens*, *J Ind Microbiol Biotechnol*, 33: 741–749.

Stewart, P.S., 2003, Multicellular nature of biofilm protection from antimicrobial agents, in Biofilm communities: Order from Chaos, McBain, A., Allison, D., Brading, M., Rickard, A. Verran, J., Walker, J. (eds). 181–190 (Bioline, Cardiff).

Stoodley, P., Lewandowski, Z., Boyle, J.D. and Lappin-Scott, H.M., 1999, Influence of hydrodynamic conditions and nutrients on biofilm structure, *J Appl Microbiol*, 85: 19S–28S.

Verran, J., 2002, Biofouling in food processing: biofilm or biotransfer potential, *Food Bioprod Process*, 80: 1744–3571.

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

Wiedmann, M., Weilmeier, D., Dineen, S.S., Ralyea, R. and Boor, K.J., 2000, Molecular and phenotypic characterization of *Pseudomonas* spp. isolated from milk, *Appl Environ Microbiol*, 66: 2085–2095.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support provided by the Portuguese Foundation for Science and Technology (Project CHEMBIO—POCI/BIO/61872/2004, PhD Grant—Lúcia C. Simões and Post-Doc Grant—Manuel Simões).

The manuscript was received 28 April 2006 and accepted for publication after revision 23 July 2006.