HOW THE HYDRODYNAMIC CONDITIONS UNDER WHICH BIOFILMS ARE FORMED AFFECT THE EFFECTIVENESS OF ALDEHYDE-BASED BIOCIDES

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The effectiveness of glutaraldehyde (GTA) and ortho-phthalaldehyde (OPA) to control biofilms formed by ***Pseudomonas fluorescens*** *on stainless steel slides under laminar and turbulent, was compared using a flow cell reactor. The action of the biocides was evaluated in terms of the metabolic activity of the biofilm and the mass of the biofilm that remained on the surface after the treatment. The physical stability of the biofilm was also assessed using a rotating device. The activity of the biocides against bacterial suspended cultures was evaluated in order to compare the action of both biocides in suspended and sessile bacteria. The interference of proteins on the action of the biocides was also assessed, as these components of the biofilm matrix may react with the biocides, decreasing their antimicrobial action.* ***P. fluorescens*** *biofilms grown under turbulent conditions appear to be more difficult to inactivate than those developed under laminar flow, OPA was more effective than GTA. Concerning biofilm removal, both biocides appeared to have little effect, independent of the flow regime under which biofilms are formed. As expected, the toxic action of the biocides was more pronounced in suspended bacteria, than in biofilms. This toxic action was significantly reduced when bovine serum albumin was added to the suspended cultures, demonstrating that these aldehyde-based biocides react strongly with proteins. The physical stability of the biofilm was altered by the biocide application: OPA increased biofilm removal regardless of the concentration tested; whereas, GTA promoted biofilm adhesion, since the amount of biomass that remained attached to the surface, after submission to the different shear stresses, increased with GTA application. The experimental methodologies used in this work were valuable tools in these biofilm/biocide-related studies.*

Introduction

Biocides and disinfectants have been the main weapons used to control unwanted biofilms, acting either by stopping growth or allowing natural detachment from the surface (Chen & Stewart 2000). Glutaraldehyde (GTA) and ortho-phthalaldehyde (OPA) are two aldehydes that are known to have good antimicrobial properties. GTA is a dialdehyde that has a broad spectrum of activity against bacteria and their spores, fungi and viruses. The mechanism of action involves a strong association with outer layers of bacterial cells, specifically with unprotonated amines on the cell surface (McDonnell & Russell 1999). OPA is an aromatic compound with two aldehyde groups and the action

of OPA is believed to be due to the interactions with the primary amino groups of the outer envelope or cell wall. However, the level of cross-linking associated with the outer membrane does not appear to be as extensive as that of GTA (Walsh *et al.* 1999a).

The aim of this work was to evaluate how the flow regime could affect the efficiency of OPA and GTA against *Pseudomonas fluorescens* biofilms formed under turbulent and laminar flow on stainless steel slides, as it has been known that the structure of the biofilms are different under the different flow rates (Vieira *et al.* 1993, Stoodley *et al.* 1999, Pereira *et al.* 2002b). The experimental tests with biofilms were performed using a range of concentrations of OPA for an exposure period of 30 min. Experimental tests with GTA were performed using a constant concentration and a range of exposure times.

Materials and methods

Microorganism and Cell Growth

P. fluorescens (ATCC 13525) was used to produce biofilm. The culture was grown in a 0.5 l glass reactor, aerated and agitated, continuously fed with 5 gl^{-1} glucose, 2.5 gl^{-1} peptone and 1.25 gl^{-1} yeast extract, in phosphate buffer at pH 7, at a flow rate of 10 ml/h.

Biocide

Two biocides were tested, ortho-phthalaldehyde (Sigma P-1378) and glutaraldehyde (Riedel-de-Ha'n 62621). Biocide solutions were diluted to the required concentration with sterile water.

Experiments with Biofilms

Biofilm set-up

The bacterial culture was used to continuously inoculate (10 mlh^{-1}) a 3.5 l reactor, aerated and agitated and fed with a solution of 0.05 gl^{-1} glucose, 0.025 gl^{-1} peptone and 0.0125 gl^{-1} yeast extract in phosphate buffer pH 7, at a flow rate of 1.7 lh^{-1}. This diluted bacterial suspension was pumped up, passing through the flow cell reactors and back to the reactor.

Two parallel similar flow cell reactors, described by Pereira, *et al.* (2002a), were used as the devices for biofilm formation, operating simultaneously in such a way that biofilms were formed under turbulent (Re=5200, u=0.532 m/s) and laminar (Re=2000, u=0.204 m/s) flow conditions, respectively, in each flow cell. The biofilms were allowed to grow for 7 days to ensure that steady-state biofilms were used in every experiment (Pereira *et al.* 2002a).

Biocide Treatment

Biofilms, formed on the metal slides of each parallel flow cell reactor, were exposed to OPA solutions of different concentrations (20, 50, 100, 200 and 300 mgl^{-1}) and to 200 mgl^{-1} of GTA. During the biocide treatment period, the

biocide solution replaced the diluted bacterial suspension flowing in the flow cell reactors. Each biocide concentration was tested in an independent experiment and each experiment was performed on three separate occasions. The continuous exposure period to OPA was 30 min. For GTA the system was exposed to the biocide for 30 min, 1 h, 2 h, 2 x 30 min and 2 x1 h. In the case of multiple applications they were separated by 2 h. After the biocide exposure time, the flow of the biocide solution was interrupted and the bacterial suspension was re-introduced in the system. In each experiment, and prior to the initiation of the biocide treatment, three metal slides of each flow cell were sampled and used as a control. Biofilms were sampled immediately after the exposure period to the biocide and after 3 h. For every condition tested and for all times of exposure, triplicate stainless steel slides were sampled.

Determination of the Inactivation and Removal of Biofilms due to Biocide Treatment

Immediately after treatment with the biocide and three hours later, the biofilms that covered the stainless steel slides were completely scraped from the metal slides, resuspended into 10 ml phosphate buffer, pH 7, and homogenized in a vortex for 30 s at 100% power input. These homogenized suspensions of biofilms were washed three times with phosphate-buffered saline solution and immediately used to assess the bacterial activity of the biofilm through oxygen uptake rate (Simoes *et al.* 2003b) and afterwards used to determine the biofilm mass. The biofilm suspensions that were not treated with OPA or GTA were also characterized in terms of biofilm activity, biofilm mass and chemical composition and used as controls (Simoes *et al.* 2003a). The inactivation of the biofilms due to the application of biocides was determined as the difference between the respiratory activities of the samples before (control) and after biocide treatment, and expressed as the percentage of inactivation according to the following equation

$$Inactivation\ (\%) = [(A_0 - A_1)/A_1] \times 100$$

Where A_0 is the respiratory activity of the control assay, i.e., without biocide treatment, and A_1 is the respiratory activity after the biocide application. The biofilm removal was determined through the following equation

$$Biofilm\ removal\ (\%) = [(W - W_B)/W] \times 100$$

Where W is the biofilm mass without biocide application and W_B is the biofilm mass after biocide treatment, both determined as the total volatile solids (TVS) of the homogenised biofilm suspensions, according to the Standard Methods (1989) and expressed in g of biofilm per cm^2 of surface area of the slide ($g_{biofilm}/cm^2$).

Physical Stability of the Biofilm

The physical stability of the biofilms was assessed by means of exposing

biofilms to increasing rotating speeds in a rotating device (Azeredo *et al.* 2000, Simoes *et al.* 2003a) and determining the biomass loss. Three ASI 316 stainless steel cylinders were inserted in a 3.5 l reactor, operating under the same growth conditions as the flow cells. The cylinders were rotating at 300 rpm. After 7 days of operation, the cylinders covered with biofilm were carefully removed from the reactor. One of the cylinders was immersed in a reactor with phosphate buffer (pH 7) while the others were immersed in reactors containing the biocide solution (170 ml). Concentrations of OPA (20, 50, 100, 200, 300 mgl^{-1}) and GTA (100, 200, 500 and 1000 mgl^{-1}) were tested at contact time of 30 min. The biocide treatment was carried out with the cylinders rotating at 300 rpm. After the exposure to the biocide, the cylinders were removed, accurately weighed, introduced in to reactors with phosphate buffer and consecutively subjected to serial velocities of rotation, i.e., 500, 1000, 1500, and 2000 rpm, for 30 s. The wet weight of the cylinders plus biofilm attached was determined before and after each rotation. The experiments were repeated on three different occasions for every biocide concentration tested. The quantification of the wet mass of the biofilm removed after submission to the rotation speeds was measured as the difference between the combined weight of the cylinder plus biofilm attached and the biofilm remaining adhered to the cylinders. For each experiment, the stainless steel cylinders were identified and weighed before being introduced in the reactor. The same procedure was followed with the control assay, i.e., with the cylinder plus biofilm immersed in the buffer solution.

The wet mass of the biofilm that was removed after each rotation speed, was expressed in percentage of biofilm removal according to the equation

$$Biofilm\ removal\ (\%) = [(W_0 - W_1)/W_1] \times 100$$

Where W_0 is the wet biofilm mass adhered on the surface of the cylinders after immersion in the biocide solutions and W_1 is the wet biofilm mass that remains adhered to the cylinders after submission to serial rotation speeds.

Tests with Suspended Microorganisms

A suitable volume of culture was harvested from the reactor, and washed with saline phosphate buffer by three consecutive steps of centrifugation (3777 g, 10 min) and ressuspended in phosphate buffer pH 7 in order to obtain a suspension with an optical density of 0.4 (λ = 640 nm). The bacterial culture was then divided into several sterilised glass flasks and put in an orbital shaker (120 rpm, 27 °C). The culture was exposed to different biocide concentrations of OPA (between 2.5 and 300 mgl^{-1}). After 30 min of contact with the biocide, the effect was assessed by determining the bacterial respiratory activity through oxygen consumption (Pereira & Vieira 2001). The same procedure was followed with GTA, however, with different concentrations (between 200 and 13000 mgl^{-1}). The mass of bacteria present in each flask was estimated by determining the total volatile solids (TVS) of the bacterial cultures (Standard Methods 1989). To

Table 1 Characteristics of *Pseudomonas fluorescens* biofilms grown under turbulent and laminar flow. Mean values ± standard deviation.

Flow	Biofilm activity ($mg\ O_2/(g_{biofilm}.min)$)	Biofilm mass (mg/cm^2)	Total polysaccharide ($mg/g_{biofilm}$)	Total protein ($mg.g_{biofilm}$)
Turbulent	0.27(±0.03)	1.56 (±0.17)	161(±21)	255(±23)
Laminar	0.06(±0.01)	0.74 (±0.14)	187(±24)	126(±27)

investigate the influence of the proteins on the biocide efficacy, the procedure described above was followed but with the addition of 3 g l^{-1} (European Standard –EN 1276) of bovine serum albumin (BSA, Merck 12018) to the bacterial suspension.

Results

Table 1 presents selected characteristics of the *Pseudomonas fluorescens* biofilms that developed under turbulent and laminar flow, before biocide treatment. The characterisation of *P. fluorescens* biofilms shows that the biofilms formed under turbulent flow were more metabolically active, have a higher content of proteins *per g* of biofilm, have less polysaccharide and have a higher amount of biofilm mass accumulated on the surfaces (Simoes *et al.* 2003a).

Application of Different OPA Concentrations

Figure 1a presents the percentage of biofilm inactivation as a function of OPA concentration. An increase in the OPA concentration particularly above 50 mgl^{-1} enhanced the biocidal activity, both in laminar and turbulent flow. The effect of the biocide seems to be dependent on the flow regime under which the biofilm was formed, especially in the cases when higher concentrations of OPA were used. Figure 1b (turbulent flow) and 1c (laminar flow) present the respiratory activity immediately after OPA treatment and 3 h later. Immediately after OPA application, an increase in OPA concentration decreased biofilm activity, both in turbulent and laminar flow (as previously, the data is more consistent for OPA at concentrations greater than 50 mgl^{-1}). The results demonstrate total biofilm inactivation under laminar flow conditions and at biocide concentrations greater than 50 mg/L. Figure 1b and 1c indicate that 3 h after OPA application, it seems that there is an increase in the biofilm respiratory activity compared to time zero for both flow regimes. The respiratory activity is always higher than that immediately after biocide treatment, suggesting that a recovery in the biofilm respiratory activity.

OPA does not promote the detachment of the biofilms from the surface, as shown by Figure 2a. Figure 2b and 2c present the biofilm mass after treatment at 0 and 3 h with different OPA concentrations. The biocide seems to have no effect on the variation of biofilm formed under turbulent and laminar flow, for both sampling times.

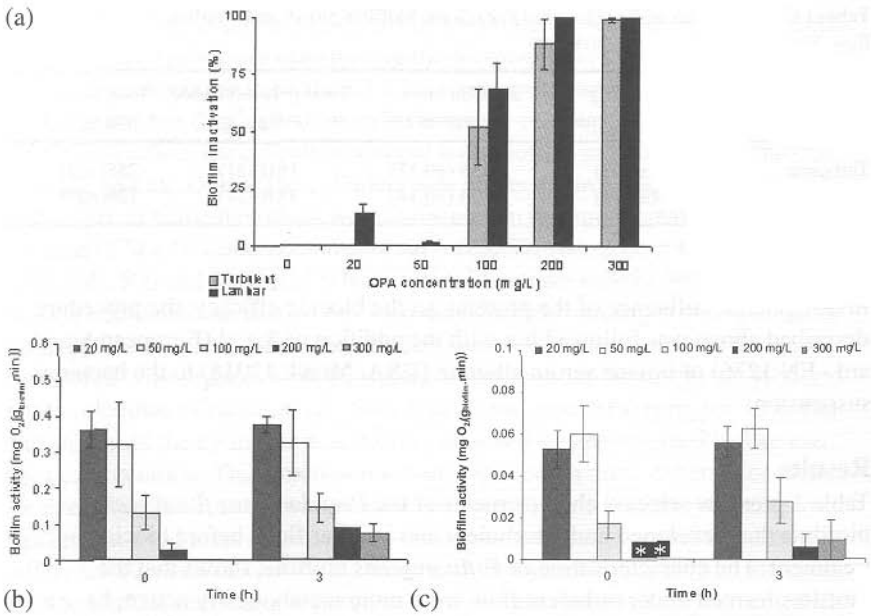

Figure 1 Biofilm inactivation (a) for both flow regime as a function of OPA concentration; biofilm activity immediately (0 h) after OPA treatment and 3 h later, for turbulent (b) and laminar (c) flow (bars represent the standard deviation). (*) – the asterisks indicate that the biofilm was completely inactivated.

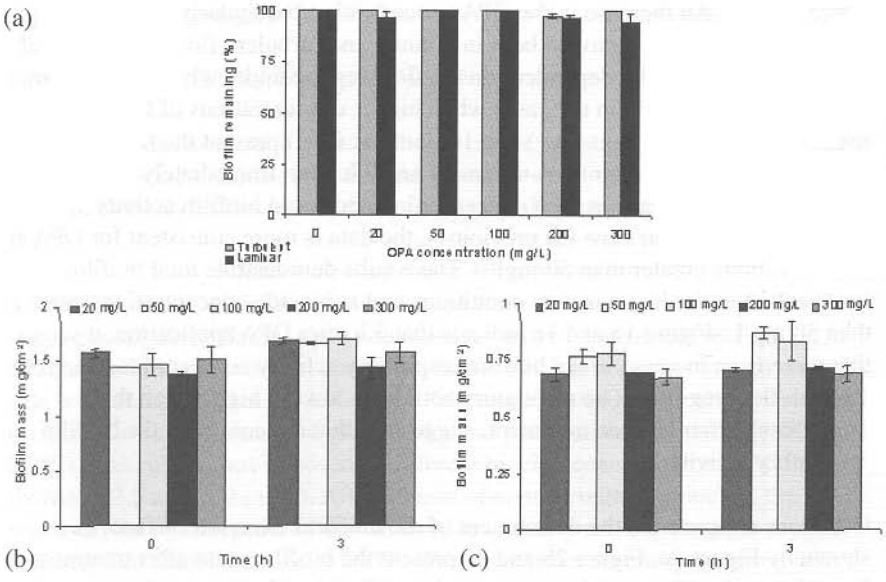

Figure 2 Biofilm removal (a) for both flow regime as a function of OPA concentration; biofilm mass after OPA treatment and 3 h later, for turbulent (b) and laminar (c) flow, after application of different concentrations of OPA (bars represent the standard deviation).

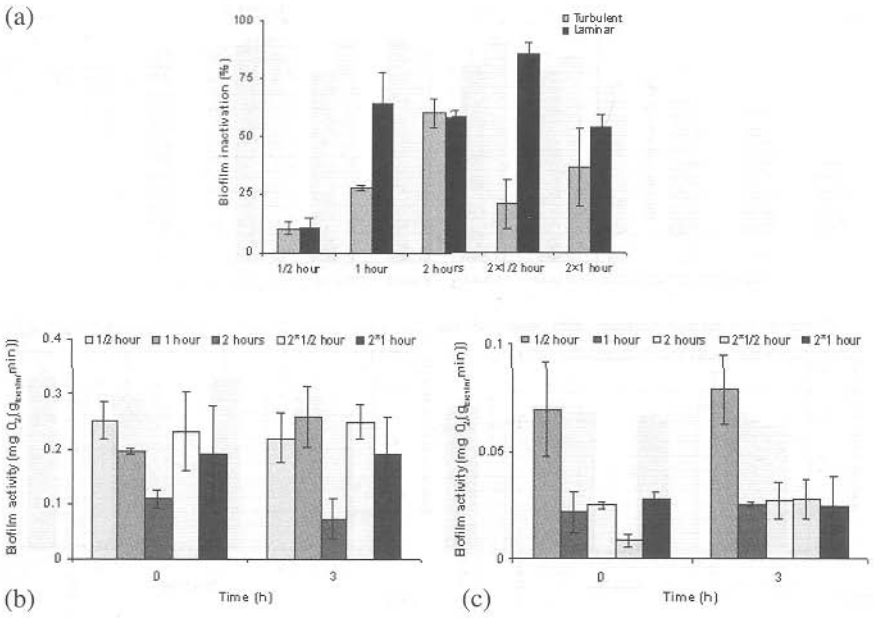

Figure 3 Biofilm inactivation (a) for both flow regime as a function of exposure time to GTA; biofilm activity after GTA treatment and 3 h later, for turbulent (a) and laminar (b) flow (bars represent the standard deviation).

Application of GTA During Different Intervals of Time

The action of GTA against biofilms formed under turbulent and laminar flow can be seen in Figures 3 and 4. Figure 3a presents the percentage of biofilm inactivation due to the application of 200 mgl^{-1} of GTA during different exposure periods for turbulent and laminar flow. GTA was more effective for longer exposure times. For the same total exposure time, the multiple application of biocide proved to be less efficient than the application in a single period of time. The flow regime under which the biofilm is formed appears to have a marked effect upon biocide effectiveness, since biofilms formed under laminar flow were more readily inactivated than the ones formed under turbulent flow. Figures 3b and 3c show, respectively, the respiratory activity for turbulent and laminar biofilms immediately after GTA treatment and 3 h later. After 3 h of GTA application, biofilm activity did not change for the exposure times studied.

Figure 4a presents the percentage of biofilm removal due to the application of 200 mgl^{-1} of GTA during different exposure periods, for turbulent and laminar flow. Biofilms were not significantly removed after the GTA treatment. Figures 4b and 4c respectively, show the biofilm mass for turbulent and laminar conditions, immediately after GTA treatment and 3 h later. After 3 h of GTA application the mass of the biofilm did not change for the exposure times studied.

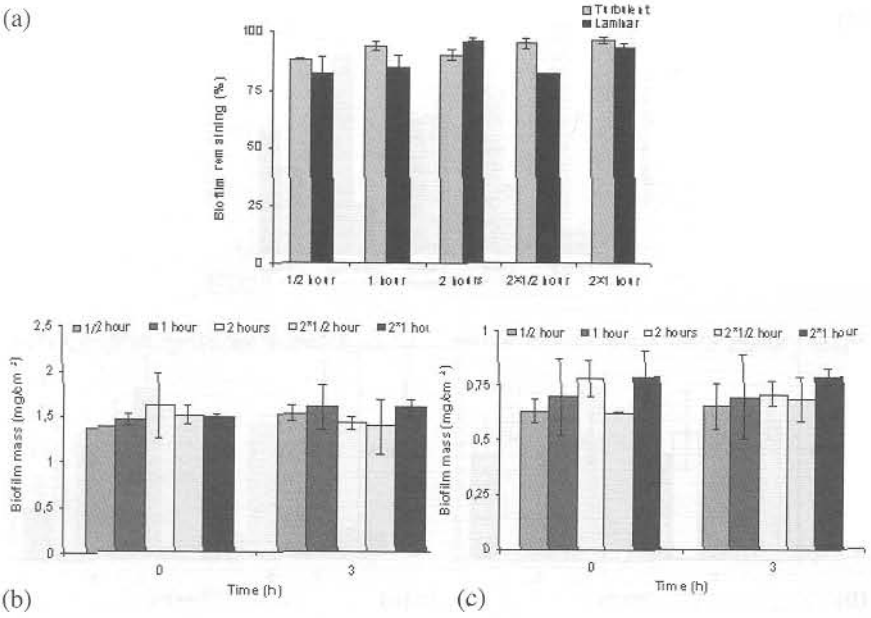

Figure 4 Biofilm removal (a) for both flow regime as a function of exposure time to GTA; biofilm mass after GTA treatment and 3 h later for turbulent (a) and laminar (b) flow (bars represent the standard deviation).

Physical Stability of the Biofilm

The results of the physical stability of the biofilm after biocide treatment, expressed in terms of percentage of biofilm that is removed due to sequential exposure of biofilm covered cylinders to increasing rotation speeds, are presented in Figure 5a and 5b. Smaller enhancement in the removal of the biofilm was achieved due to OPA application (near 8% more). However, the differences observed among the different OPA concentrations are not relevant especially when the standard deviations are taken into account. Conversely to the treatment with OPA, the application of GTA seems to have a different effect on the physical stability of the biofilm. In fact, the amount of biofilm that remains adhered to the surface after GTA treatment and exposure to the serial rotation speeds increases with GTA concentration. This evidence indicates that the physical stability of the biofilm increased for all the GTA concentrations applied.

Interference of BSA with the Activity of Suspended Bacteria

The interference of BSA with the efficacy of OPA and GTA against the suspended *P. fluorescens* cultures can be seen in Figure 6. From the results, it is clear that OPA is more effective in the inactivation of suspended cells than GTA. For an OPA concentration of 100 mgl^{-1}, the bacterial cells are totally inactive, whereas for a GTA concentration of 13000 mgl^{-1} there is no total inactivation.

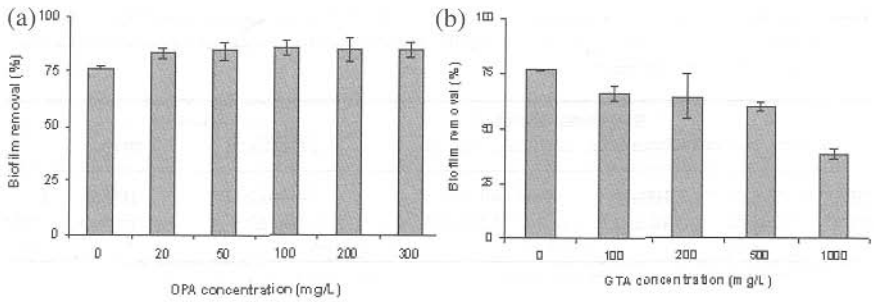

Figure 5 Percentage of biofilm removal after submitting the biofilm covered cylinders to different rotation speeds, after exposure to different OPA (a) and GTA (b) concentrations (bars represent the standard deviation).

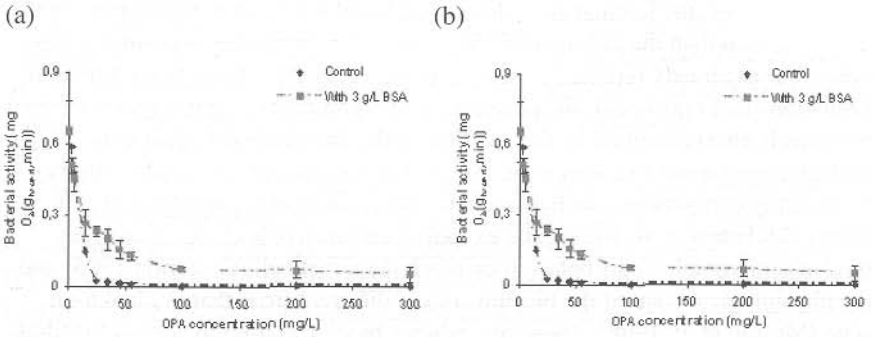

Figure 6 Respiratory activity of the suspended bacterial cultures after treatment with several concentrations of OPA (a) and GTA (b), with and without BSA addition (control).

The OPA and GTA efficacy was significantly reduced when BSA was introduced in the suspended bacterial cultures.

Discussion

As previously reported (Simoes *et al.* 2003a), the hydrodynamic conditions under which biofilms are formed play a significant role in the composition of the biofilms. The flow regime is an important factor that affects the biofilm structure and activity (Purevdorj *et al.* 2002, Vieira *et al.* 1993), since biofilm properties change in response to environmental conditions. Biofilm thickness and density have a marked influence on the microbial metabolism in the film, because they may influence diffusional limitations as well as affect the composition of the microenvironment surrounding the cells within the matrix (Pereira *et al.* 2002b, Purevdorj *et al.* 2002), and may also affect the signals diffusion (Purevdorj *et al.* 2002). Biofilms formed under turbulent flow were denser, has a stronger exopolymeric matrix as a response to the higher fluid velocities, were more active within the matrix and more protected from external aggressions

Table 2 Bacterial inactivation in the presence and absence of BSA and inactivation of biofilms formed under turbulent and laminar flow due to the application of 200 mgl^{-1} of OPA and GTA at 30 min. Values ± standard deviation.

	Suspended bacteria		Biofilm	
	Without BSA	With BSA	Turbulent	Laminar
OPA	100±0	89.3±1.3	88.6±10.8	100±0
GTA	48.8±2.9	5.82±0.3	10.3±2.8	10.8±3.5

(Vieira *et al.* 1993; Pereira *et al.* 2002b). Therefore, it is not surprising to find differences in biocide efficacy when applied to turbulent and laminar biofilms. In these studies, the laminar flow developed biofilm was more susceptible to the biocide action than the turbulent biofilm. Several reasons may account for this behaviour as already reported in several previous studies. Apart from diffusion limitations that may occur, the penetration of antimicrobial agents into microbial biofilms is also controlled by the reaction of the antimicrobial agent with biofilm components (Stewart *et al.* 2001). The biocide can also react with the community components, such as organic matter, inorganic particles and cell debris (McFeters *et al.* 1995). The exopolymeric matrix is charged, and may bind antimicrobial agents before they reach the target cell (Costerton 1985) and the physiological state of the biofilm cells is different from that in planktonic state (Morton *et al.* 1998). Therefore, when a biocide is used to control biofilms, the microbial response to the chemical agent will depend not only on the type of microorganisms and the type of chemical agent, but also on the complex interactions between the biocide and the biofilm matrix (Pereira & Vieira 2001). The higher content of proteins and biofilm mass present in biofilms formed under turbulent flow may have reacted with the biocides, lowering their concentrations and thus reducing their antimicrobial effect. In previous work, Pereira & Vieira (2001) suggested that GTA, when applied to biofilms, reacted with proteins of the polymeric matrix. As a consequence, the concentration of the biocide available for react with the bacteria is reduced. The results obtained in suspended tests in the presence of BSA reinforces this last statement (Figure 6).

Comparing the action OPA and GTA in the control of the *P. fluorescens* biofilms, OPA is more effective in inactivating bacteria and biofilms at lower concentrations than for GTA. The results presented in Table 2 give an overview of the inactivation by OPA and GTA (200 mgl^{-1}) at 30 min. In the case of suspended cultures, this efficiency was drastically reduced when BSA was present in the solution for both biocides. It is known that proteins acts as neutralizers of GTA and OPA (Rutala & Weber 2001, Walsh *et al.* 1999a, b), lowering the biocide concentration that is available to react with the cells. For higher OPA concentrations, total inactivation was achieved, but when BSA is added to the bacterial suspension the total inactivation is not observed even

Table 3 Biofilm formed under turbulent and laminar conditions, and under rotation, analysed in terms of removal after application of 200 mgl^{-1} of OPA and GTA during 1/2 h. Values ± standard deviation.

	Biofilm removal (%)		
	Turbulent	Laminar	Physical stability
Control	0±0	0±0	76.5±0.64
OPA	2.9±1.2	3.6±1.1	85.2±5.6
GTA	11.9±0.35	18.1±7.1	64.6±9.9

when OPA concentration is increased. As reported for OPA, BSA has a similar effect in the antimicrobial effect of GTA, however, GTA seems to be a less potent disinfectant than OPA. This latter evidence emphasizes OPA as a possible alternative to GTA for high level disinfection, as already suggested by Walsh *et al.* (1999a). With a concentration 130 fold higher than that used with OPA, GTA does not promote a total inactivation of the bacterial suspension.

The analysis of the results obtained for the biofilms 3 h after OPA treatment, shows that the respiratory activity, was always higher than the one achieved immediately after biocide treatment, regardless of the flow regime, while for GTA the activity of the biofilm seems to continue. This fact could be due to the higher level of cross-linking promoted by GTA (Simmons *et al.* 2000; Walsh *et al.* 1999a, 1999b) that could have a prolonged effect on post-biocide application and therefore in the biofilm development.

The effect of both biocides on the biofilm mass was similar, i.e., the application of OPA and GTA did not promote a significant biofilm removal. Hence, the biofilms can be inactive but stay attached to the surface, which is not convenient in industrial systems where biofilm accumulation is a problem and, as seen above, the remaining biofilm can restore its functions.

Table 3 shows the differences obtained in biofilm removal after a similar process of treatment, with OPA and GTA. The biofilm removal with OPA seems to be negligible for both turbulent and laminar biofilms. GTA promotes a small reduction in the biofilm mass, more significant in the laminar biofilm. Comparing the physical stability of the biofilm after OPA and GTA treatment with the control, the hydrodynamic changes caused by the implementation of increasing rotation speeds did not give rise to the complete removal of biofilm from the cylinder surface, despite the fact that OPA is a chemical with two aldehyde groups, as GTA, but with a less potent cross-linking ability (Simmons *et al.* 2001, Walsh *et al.* 1999a, 1999b). This fact is supported by the results obtained from the physical stability of the biofilm. In the case of OPA, the amount of biofilm that remains adhered to the cylinders was lower than the one observed in the control, suggesting that OPA causes the weakening of the biofilm, while

GTA appears to promote the strengthening, increasing the physical stability of the biofilm with the concentration (Figure 5b). Concerning OPA treatment, even with the increase of the biocide concentration there was no significant variation in the biofilm mass due to the hydrodynamic forces. The differences encountered can be explained by the strong ability of GTA to cross-link proteins, expressed as a fixative action of the biofilm to the surface, being GTA currently used as fixation agent in microscopy techniques.

Conclusions

The data presented in this work showed that OPA and GTA were more effective in the inactivation of biofilms that formed under laminar flow than those that formed under turbulent flow. Unlike OPA, the application of GTA did not cause total inactivation. Both biocides exhibited poor ability for the removal of laminar or turbulent biofilms from the surfaces. These results support the suggestion that the flow regime under which the biofilm are formed play an important role in the biocide action, especially when extremes conditions are tested (higher biocide concentrations or exposure times). Therefore, it can be concluded that biofilms are very stable structures that can be inactivated but stay attached to the surfaces. Both biocides proved to be more efficient against *P. fluorescens* suspended cultures than against the bacterial biofilms, emphasizing that bacteria entrapped in a biofilm are more resistant to antimicrobial agents than suspended cells. However, OPA was very effective in the inactivation of bacteria in suspension at concentrations far lower than GTA. The aldehyde based biocides, tested in this work, react strongly with proteins, hence their reduced efficacy in the presence of BSA.

GTA promotes an increase in the physical stability of the biofilm, conversely to what occurs with OPA. In fact, OPA has a small effect on the weakening of the physical stability of the biofilm that did not change with the OPA concentration. This different behaviour is probably related with the strong stabilizing influence of GTA that reflects the protein cross-linking effect, which is less extensive for OPA. This work showed again the inadequacy of planktonic testing methods for evaluating antimicrobial agents to be used against biofilms, and the need to use biofilm testing methods. From the results presented, it can be concluded that the experimental systems used (flow cell and rotating device) are suitable for the evaluation of biofilm growth, and represent very useful tools to monitor the behaviour of biofilms after biocide treatment.

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References

APHA. AWWA. WPCF (1989). Standard methods for the examination of water and wastewater, 17th edition. Edited by Clesceri, L. S., Greenberg, A. E. & Trussel, R. R. *American Public Health Association*, Washington DC, USA.

Azeredo, J. & Oliveira, R. (2000). The role of exopolymers produced by *Sphingomonas paucimobilis* in biofilm formation and composition, *Biofouling*, **16, 17-27.**

Chen, X. & Stewart, P.S. (2000). Biofilm removal caused by chemical treatments. *Water Research* **34**, 4229-4233.

Costerton, J.W. (1985). The role of bacterial exopolysaccarides in nature and disease. *Developments in Industrial Microbiology* **26**, 249-261.

McDonnell, G. & Russell, A.D. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clinical Microbiology Reviews*, **12**, 147-179.

McFeters, G.A., Yu, F.P., Pyle, B.H. & Stewart, P.S. (1995). Physiological methods to study biofilm disinfection. *Journal of Industrial Microbiology* **15**, 333-338.

Morton, L.H.G., Greenway, D.L.A., Gaylarde, C.C. & Surman, S.B. (1998). Consideration of some implications of the resistance of biofilms to biocides. *International Biodeterioration and Biodegradation* **41**, 247-259.

Pereira, M.O. & 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., Morin, P., Vieira, M.J. & Melo, L.F. (2002a). A versatile reactor for continuous monitoring of biofilm properties in laboratory and industrial conditions. *Letters in Applied Microbiology* **34**, 22-26.

Pereira, M.O., Kuehn, M., Wuertz, S., Neu, T. & Melo, L. (2002b). Effect of flow regime on the architecture of a *P. fluorescens* biofilm. *Biotechnology and Bioengineering* **78**, 164-171.

Purevdorj, B., Costerton, J.W. & Stoodley, P. (2002). Influence of hydrodynamics and cell signalling on the structure and behaviour of *Pseudomonas aeruginosa* biofilms. *Applied and Environmental Microbiology* **68**, 4457-4464.

Rutala, W.A. & Weber, D.J. (2001). New disinfection and sterilization methods. *Emerging Infections Diseases* **7**, 348-353.

Simmons, C., Walsh, S.E., Maillard, J.-Y. & Russell, A.D. (2000). A note: ortho-phthalaldehyde: proposed mechanism of action of a new antimicrobial agent. *Letters in Applied Microbiology* **31**, 299-302.

Simões, M., Pereira, M.O. & Vieira, M.J. (2003a). Monitoring the effects of biocide treatment of *Pseudomonas fluorescens* formed under different flow regimes. *Water Science and Technology* **47**, 217-223.

Simões, M., Carvalho, H., Pereira, M.O. & Vieira, M.J. (2003b). Studies on the behaviour of *Pseudomonas fluorescens* biofilms after ortho-phthalaldehyde treatment. *Biofouling* (In press).

Stewart, P.S., Rayner, J., Roe, F. & Rees, W.M. (2001). Biofilm penetration and disinfection efficacy of alkaline hypochlorite and chlorosulfamates. *Journal of Applied Microbiology* **91**, 525-532.

Stoodley, P., Boyle, J. D., DeBeer, D. & Lappin-Scott, H.M. (1999). Evolving perspectives of biofilm structure. *Biofouling* **14**, 75-90.

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

Walsh, S.E., Maillard, J.-Y. & Russell, A.D. (1999a). Ortho-phthalaldehyde: a possible alternative to glutaraldehyde for high level disinfection. *Journal of Applied Microbiology* **86**, 1039-1046.

Walsh, S.E., Maillard, J.-Y., Simmons, C. & Russell, A.D. (1999b). Studies on the mechanisms of the antibacterial action of ortho-phthalaldehyde. *Journal of Applied Microbiology* **87**, 702-710.