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Effect of Different Concentrations of *Ortho*-phthalaldehyde on Biofilms Formed by *Pseudomonas fluorescens* Under Different Flow Conditions

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The effectiveness of different concentrations of *ortho*-phthalaldehyde (OPA) in controlling biofilms of *Pseudomonas fluorescens* formed on stainless steel slides, using flow cell reactors under laminar and turbulent flow, was investigated by determining the variation in mass and respiratory activity. The physical stability of the biofilm with and without exposure to OPA was studied in a rotating device as variation in the mass of the biofilm on the surface after exposure to different rotation velocities. The activity of OPA against bacterial suspended cultures was evaluated in the presence and absence of bovine serum albumin (BSA) in order to evaluate the interference of proteins on the activity of the biocide. The results showed that biofilms formed under different flow conditions had different properties and reacted differently after biocide application. Biofilms formed under laminar flow were more easily inactivated than those formed under turbulent conditions. However, OPA did not promote the detachment of biofilms from the surface. The exposure of biofilms to different shear stress conditions after OPA treatment enhanced removal from the surface, indicating that OPA may weaken the biofilm matrix. The biocide was more effective on suspended cells than on cells grown in biofilms. This fact may be explained by the reaction of the biocide with proteins of the polymeric matrix of the biofilm as suggested by the significant reduction of biocide action on suspended cells in the presence of BSA.

Keywords: biofilm; *ortho*-phthalaldehyde; flow regime; biocidal resistance; biofilm stability

INTRODUCTION

Undesirable biofilm formation occurs in all industrial processes using water (Matilla *et al*., 2002). A better understanding of biofilm behaviour is particularly important because of the many problems associated with their presence. Biofilms can be described as microsystems adhered to solid surfaces in which microorganisms are embedded in a matrix of extracellular polymeric substances which are responsible for the morphology, structure, adherence and physico-chemical and biological properties (Flemming & Wingender, 1999).

Understanding the relationship between biofilm structure and function and the factors that account for differences is crucial in order to more effectively utilize and control biofilms in real systems. Hydrodynamics play a significant role in the structure of biofilms. Biofilms grown in turbulent flow are very different from those grown in laminar flow (Vieira *et al*., 1993; Stoodley *et al*., 1999; Pereira *et al*., 2002b), and this may affect the action of the agents used in their control. As a consequence, studies on biofilm control should be carried out using biofilms with a structure similar to that found in real systems.

Biocides, used as part of an accurate combined cleaning and disinfection programme, are an essential tool to control undesired biofilms. However, appropriate selection and application of these chemical compounds should be made in order to obtain successful biofilm control (Cloëte *et al*., 1998).

Ortho-phthalaldehyde (OPA) is a new product that is claimed to have excellent microbiocidal, mycobactericidal and sporicidal activity (McDonnell & Russell, 1999; Rutala & Weber, 2001). OPA received clearance by the FDA (Food & Drug Administration, USA) in October 1999 and it is currently under study

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as a possible alternative to glutaraldehyde (GTA) for high level disinfection. OPA is an aromatic compound with two aldehyde groups (McDonnell & Russell, 1999). The aromatic component might allow OPA to penetrate the outer layers of cells, thus helping to explain the very high activity of this biocide against Gram-negative vegetative organisms, even though the degree of cross-linking seems to be less than that seen with GTA (Walsh *et al*., 1999b). OPA has several potential advantages compared to GTA; it is odourless, stable and effective over a wide pH range of 3–9 (Rutala & Weber, 2001), non-irritating to the eyes and nasal passages and does not require activation before its use.

So far, studies carried out to test the efficiency and mechanism of action of OPA have been performed using suspended cells (Walsh *et al*., 1999a; 1999b; 2001) or using cells that had adhered to a surface for a short period of time (Alfa & Sitter, 1994). In all cases, biocide efficiency was evaluated through plate counting techniques, which may overestimate OPA action since cells may remain viable after biocide treatment, but because they are stressed they may not able to grow on solid growth media (Stewart *et al*., 1994; McFeters *et al*., 1995) or may aggregate leading to the underestimation of the culturable cells. Recently, Simões *et al*. (2003b) demonstrated that the disinfection action of OPA was substantially reduced for biofilms formed under low shear compared to suspended bacteria. The interaction of the biocide with the proteins of the polymeric matrix was pointed out as the main reason for this different performance.

The aim of the present study was to evaluate how the flow regime (laminar or turbulent) could affect the efficiency of OPA against *Pseudomonas fluorescens* biofilms developed on stainless steel slides using flow cell reactors. The experimental tests were performed using biofilms grown for 7 d and the application of a range of concentrations of OPA over 30 min. In order to assess how biofilms behaved in response to a change in hydrodynamic conditions before and after biocide treatment, physical stability was monitored using biofilms developed in a rotating device. To investigate the possible interference of proteins with the biocidal action of OPA, further respiratory activity tests with suspended cultures were performed in the presence and absence of bovine serum albumin (BSA).

MATERIAL AND METHODS

Microorganism and Cell Growth

The microorganism used to produce biofilm was the Gram-negative aerobic bacterium *Pseudomonas fluorescens* (ATCC 13525). This bacterium is a good biofilm producer, and the optimal growth conditions were $27 \pm 1^{\circ}C$ at pH 7, with glucose as the carbon source (Oliveira *et al*., 1994).

A continuous culture of *P. fluorescens* was produced as described elsewhere (Pereira *et al*., 1998). The bacterial culture was grown in a 0.5 l glass reactor suitably aerated and agitated, and continuously fed with a sterile concentrated nutrient solution consisting of 5 g glucose l^{-1}, 2.5 g peptone l^{-1} and 1.25 g yeast extract l^{-1}, in phosphate buffer at pH 7, at a flow rate of 10 ml h^{-1}.

Biocide

The biocide used in this study was o*rtho*-phthalaldehyde obtained as a powder from Sigma (P-1378). Before each experiment, biocide solutions were prepared to the required concentration with sterile distilled water.

Experiments with Biofilms

Biofilm Set-up

The bacterial culture referred to above was used to continuously inoculate (10 ml h^{-1}) a 3.5 l reactor, aerated and agitated and fed with a minimal nutrient medium consisting of 0.05 g glucose l^{-1}, 0.025 g peptone l^{-1} and 0.0125 g yeast extract l^{-1} in phosphate buffer pH 7, at a flow rate of 1.7 l h^{-1}, to obtain a bacterial suspension with 6×10^7 cells ml^{-1}. This diluted bacterial suspension was pumped up, passing through the flow cell reactors and back to the reactor.

A flow cell reactor described by Pereira *et al*. (2002a) was used as the device for biofilm formation. It consisted of a semicircular Perspex duct (45 cm long and with a hydraulic diameter of 1.6 cm) with 10 apertures in its flat wall for the attachment of several removable rectangular pieces of Perspex. In the present study, these pieces of Perspex had stainless steel (ASI 316) (SS) slides (1.75×1.25 cm) glued to one of their faces. Biofilms were formed on the metal slides whose upper faces were in contact with the bacterial suspension circulating in the flow cell reactor. Each of the rectangular pieces could be removed separately without disturbing the biofilm formed on the others and without stopping the flow. This was managed *via* outlet ports on the round face of the flow cell between each two adjacent removable pieces of Perspex that allowed deviation 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 (Re = 5200, u = 0.532 m s^{-1}) and laminar (Re = 2000, u = 0.204 m s^{-1}) conditions, respectively, in each flow cell. The biofilms were

allowed to grow for 7 d to ensure that steady-state biofilms were used in every experiment (Pereira *et al*., 2002a).

Biocide Treatment

The 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 mg l^{-1}). During the biocide treatment period, the OPA solution replaced the diluted bacterial suspension flowing in the flow cell reactors. Each OPA 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. After this exposure time, the flow of OPA solution was interrupted and the bacterial suspension was re-introduced in the system in order to restore the conditions prior to biocide application. In each experiment, and prior to the initiation of the biocide treatment, three metal slides from each flow cell were sampled and used as controls.

Biofilms were sampled immediately after the exposure period to OPA and after 3 h. For every condition tested and for all lengths of exposure, triplicate stainless steel slides were sampled.

Scraping and Disaggregation of the Biofilms

Immediately after biocide treatment and 3 h later, the biofilms that covered the SS slides were completely scraped from the metal slides, resuspended into 10 ml phosphate buffer, pH 7, and homogenized in a vortex for 30 s with 100% power input. These homogenized suspensions of biofilms were washed three times with phosphate-buffered saline solution and used immediately to assess the respiratory activity of the biofilm through oxygen uptake rate and later to determine biofilm mass. The control biofilm suspensions that were not treated with OPA were also characterised in terms of activity, mass and chemical composition.

Physical Stability of the Biofilms

The physical stability of the biofilms was assessed by exposure to increasing rotating speeds in a rotating device described elsewhere (Azeredo & Oliveira, 2000; Simões *et al*., 2003a) followed by determination of loss of biomass. Three ASI 316 SS cylinders (surface area = 34.6 cm^2) were inserted in a 3.5 l reactor, operating under the same growth conditions as the flow cells (same growth medium, dilution rate, pH and temperature). The cylinders rotated at 300 rpm. After 7 d operation, the cylinders covered with biofilm were carefully removed from the reactor. One of the cylinders was immersed in a flask with phosphate buffer (pH 7) while the others were immersed in flasks containing different OPA solutions for 30 min (volume of each flask was 170 ml). The biocide treatment was carried out with the cylinders rotating at 300 rpm. After exposure to the biocide, the cylinders were removed from the OPA solutions, accurately weighed, introduced into flasks with phosphate buffer and consecutively subjected to serial velocities of rotation, *i.e*. 500, 1000, 1500, and 2000 rpm for a period of 30 s each. The wet weight of the cylinders plus attached biofilm was determined before and after each rotation. Several concentrations of OPA were tested (20, 50, 100, 200, 300 mg l^{-1}). The experiments were repeated on three different occasions for each biocide concentration tested.

The quantification of the wet mass of the biofilm that remained attached to each cylinder after exposure to different rotation speeds was measured as the difference between the combined weight of the cylinder plus attached biofilm and the respective weight of the clean cylinder. For each experiment, the SS cylinders were identified and weighed before being introduced in the flask. The same procedure was followed for the control assay, *i.e*. with the cylinder plus biofilm immersed in the buffer solution.

The wet mass of the biofilm that remained adhered to the surface of each cylinder after subjection to different rotation speeds was expressed as the percentage of biofilm remaining according to the equation:

$$\text{Biofilm remaining } (\%) = [1 - (W_0 - W_1)/W_1] \times 100$$

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

The results are expressed as the percentage of biofilm mass remaining after exposure to the complete series of rotation velocities.

Analytical Methods

Chemical Analysis of the Biofilm

The total protein and polysaccharide was quantitatively determined for biofilms that were not treated with biocide (control). Protein was determined using the Lowry modified method (Sigma-protein assay kit No P5656) with BSA as the standard and the polysaccharide composition was determined by the phenol-sulphuric acid method of Dubois *et al*. (1956) with glucose as standard.

Biofilm Mass

The dry mass of biofilm accumulated on the slides was assessed by determination of the total volatile

solids (TVS) of the homogenized biofilm suspensions, according to APHA, AWWA, WPCF Standard Methods (1989), method number 2540 A-D. The biofilm mass accumulated on the metal slides was expressed in g of biofilm per cm^2 of surface area of slide ($g_{biofilm}\ cm^{-2}$).

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

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

where W is the biofilm mass without biocide application and W_B is the biofilm mass after biocide treatment.

Tests with Suspended Microorganisms

A suitable volume of culture was harvested from the reactor, and washed with saline phosphate buffer (0.01 M, pH 7.0) by three consecutive centrifugation steps (3777 × g, 10 min) and resuspended in phosphate buffer pH 7.0 in order to obtain a suspension with an absorbance of 0.4 (λ = 640 nm) with phosphate buffer as blank. The bacterial culture was then divided between several sterilized glass flasks and transferred to an orbital shaker (120 rpm, 27°C). The culture was exposed to different biocide concentrations (between 2.5 and 300 mg l^{-1}). After 30 min contact, the effect of the biocide on respiratory activity was measured by oxygen consumption as described by Pereira and Vieira (2001).

The mass of bacterial cells present in each glass flask was estimated as the total volatile solids (TVS) according to APHA, AWWA, WPCF Standard Methods (1989).

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

Respiratory Activity Assessment

The respiratory activity of the several samples was evaluated by measuring oxygen uptake rates in a biological oxygen monitor (BOM) in short-term assays. The assays were performed in a Yellow Springs Instruments BOM (Model 53) by the procedure described in Pereira *et al*. (2002a). Before each respirometric assay, the samples were carefully washed three times with phosphate-buffered saline solution (NaCl 0.85%), resuspended in 10 ml of phosphate buffer at pH 7 and placed in the temperature-controlled vessel of the BOM (T = 27°C ± 1°C). This washing practice was performed in order to guarantee the absence of carbon sources and other external energy sources for the bacterial cells, and to reduce the residual OPA concentration to a level that did not cause any further cell damage (Johnston *et al*., 2002).

The temperature-controlled vessel of the BOM 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 and consumption of any residual carbon source. The vessel was closed and the decrease in 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^{-1}) was injected into 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 gave the specific oxygen uptake rate due to glucose oxidation, herewith referred to as respiratory activity, and expressed in mg of O_2 consumed per g of biofilm per min (mg O_2 $(g_{biofilm}\ min)^{-1}$) in the case of the biofilm solutions, and in mg of O_2 consumed per g of bacterial cell per min (mg O_2 $(g_{bacterial\ cells}\ min)^{-1}$), in the case of the suspended bacterial cultures. All the respirometric tests were carried out at least three times.

The decrease in respiratory activity due to the application of the different concentrations of OPA to both biofilms and suspended cultures of *P. fluorescens* was determined as the difference between the respiratory activities of the samples before (control) and after the treatment with OPA, and expressed as the percentage inactivation according to the following equation:

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

where A_0 is the respiratory activity of the control assay, *i.e*. without OPA treatment, and A_1 is the respiratory activity after the application of each OPA concentration.

The same approach was used when BSA (3 g l^{-1}) was added to the suspended bacterial cultures.

Statistical Analysis

The means and standard deviations within samples were calculated in all cases. The results were tested using the Student's *t* distribution to assess whether the differences between the experimental values obtained under the different process conditions were significant. Statistical significance was based on confidence levels equal to or > 95%. The data related to the physical stability of the biofilms were analysed using one-way ANOVA (statistics software package SPSS). The Dunnett method (Daniel, 1987) was used to calculate minimum significant differences (*p* values). Dunnet's method was usually followed

when the objective of an experiment was to compare a control against each of the several treatments.

RESULTS AND DISCUSSION

The action of OPA at different concentrations applied for 30 min to biofilms formed on SS slides under different flow conditions (turbulent and laminar) was assessed by determining the variation in biofilm mass and its respiratory activity. The physical stability of the biofilm before and after OPA treatment was also monitored using biofilms developed in a rotating device and exposing them to increasing rotating velocities. Appropriate control experiments were carried out for each condition tested.

The action of OPA on the respiratory activity of the biofilm was evaluated by determining the oxygen uptake rate due to glucose oxidation, which according to Stewart *et al*. (1994) and McFeters *et al*. (1995) may be more accurate than the traditional method of colony formation on agar medium. This latter method has received much criticism especially when used to evaluate the effect of the antimicrobial agents (MacDonald *et al*., 2000). The method may underestimate the actual viable population since bacteria in the biofilm can remain viable after biocide application but may not grow on solid media.

Table I presents the characteristics of *P. fluorescens* biofilms developed under turbulent and laminar flow. The results are the mean of all control assays carried out for each biocide application, each condition being tested on three separate occasions. The results show that the biofilms formed under turbulent flow were more active, and a higher mass of these accumulated on the metal surfaces and a higher amount of protein was formed per g of biofilm. No significant differences were found in polysaccharide content between the flow conditions tested. These data are in agreement with results of previous studies related to the macroscopic composition of *P. fluorescens* biofilms (Simões *et al*., 2003a), which emphasized that the hydrodynamic conditions under which the biofilms are formed play a significant role in their composition. Furthermore, Simões *et al*. (2003a) reported that for the same system used in the present study, *P. fluorescens* biofilm structure also depends on the flow conditions under which they are formed. Biofilms formed under turbulent flow appeared homogeneous and slimy, while those formed under laminar flow were not uniform, often appearing to be scattered on the surface and having protuberances. Other authors (Vieira *et al*., 1993; Stoodley *et al*., 1999; Pereira *et al*., 2002b) have shown that the flow regime is one of the most important factors affecting biofilm structure and activity since biofilm properties change in response to environmental conditions. Biofilms formed under turbulent flow are denser and have a stronger exopolysaccharide matrix as a consequence of the higher fluid velocities.

TABLE I Characteristics of *P. fluorescens* biofilms grown under turbulent and laminar flow

Biofilm characteristics	Turbulent flow	Laminar flow
Biofilm mass (mg cm^{-2})	1.43 ($\pm$ 0.22)	0.72 ($\pm$ 0.14)
Respiratory activity (mg O_2 ($g^{-1}_{biofilm}$ min^{-1}))	0.280 ($\pm$ 0.099)	0.056 ($\pm$ 0.020)
Total protein (mg $g^{-1}_{biofilm}$)	258 ($\pm$ 28)	125 ($\pm$ 19)
Total polysaccharide (mg $g^{-1}_{biofilm}$)	166 ($\pm$ 28)	170 ($\pm$ 23)

Mean values $\pm$ 95% confidence interval

Figure 1a and b shows the respiratory activity of the *P. fluorescens* biofilm formed under turbulent and laminar flow after application of different concentrations of OPA for 30 min. Figure 1a displays the respiratory activity of the biofilms immediately after OPA application whilst Figure 1b shows the respiratory activity of the biofilms 3 h later. Biofilm respiratory activity after OPA application shows that increasing the concentration of OPA enhanced biocidal activity both in laminar and turbulent flow. This trend, however, was only consistently evident for OPA concentrations > 50 mg l^{-1}. Therefore, it can be concluded from these results that the bacterial cells entrapped in the biofilm became progressively more susceptible to inactivation as the concentration of the antimicrobial agent increased.

To directly compare the results presented in Figure 1, the percentages of biofilm inactivation obtained after the application of different concentrations of OPA were calculated, and these are shown in Figure 2. The effect of the biocide was dependent on the flow regime under which the biofilm was formed, as might be expected from the different biofilm structures found for each regime. These differences were especially marked when higher concentrations of OPA were used. The biofilms grown under laminar and turbulent flow seemed to exhibit a very low susceptibility to low OPA concentrations, although the former biofilms showed slight inactivation. Conversely, for OPA concentrations equal to or > 100 mg^{-1} both turbulent and laminar biofilms began to be inactivated by the biocide, the effect being more pronounced for biofilms formed under laminar conditions. The effect of the flow regime on the organic constituents of the biofilm, and consequently on OPA activity, was found to be statistically significant ($p < 0.05$). These data proved the hypothesis that the flow regime under which the biofilms were formed had a strong impact on biocide activity, as previously shown by Simões *et al*. (2003a). OPA may more easily penetrate biofilms formed under laminar flow due to their structure leading to higher susceptibility to biocides.

Figure 2 also shows that inactivation achieved 3 h after OPA exposure was always less than that attained immediately after biocide treatment, suggesting that the bacterial biofilms recovered respiratory activity to some extent. This fact is better observed in Figure 1c and 1d where for each flow regime, respiratory activity at different OPA concentrations is plotted as a function of biofilm sampling time. Figure 1c and 1d clearly show that the respiratory activity observed 3 h after exposure to OPA was higher than that immediately after biocide treatment, regardless of the flow regime. This phenomenon was consistently observed for all concentrations of OPA and for both turbulent (Figure 1c) and laminar (Figure 1d) biofilms. The statistical comparison between the two sampling times (*i.e.* immediately after OPA treatment and 3 h later) showed significant differences ($p < 0.05$ for turbulent flow and $p < 0.02$ for laminar flow). The feature illustrated in Figure 1 acquired further importance since the same did not occur in the controls for either flow regime. Indeed, Figure 1 shows that the control biofilms exhibited approximately the same respiratory activity during the course of the experiment (the smallest differences observed are within the SD interval). This is not surprising since, in this study, all the experiments were performed with steady-state biofilms. It must be remembered that after exposure

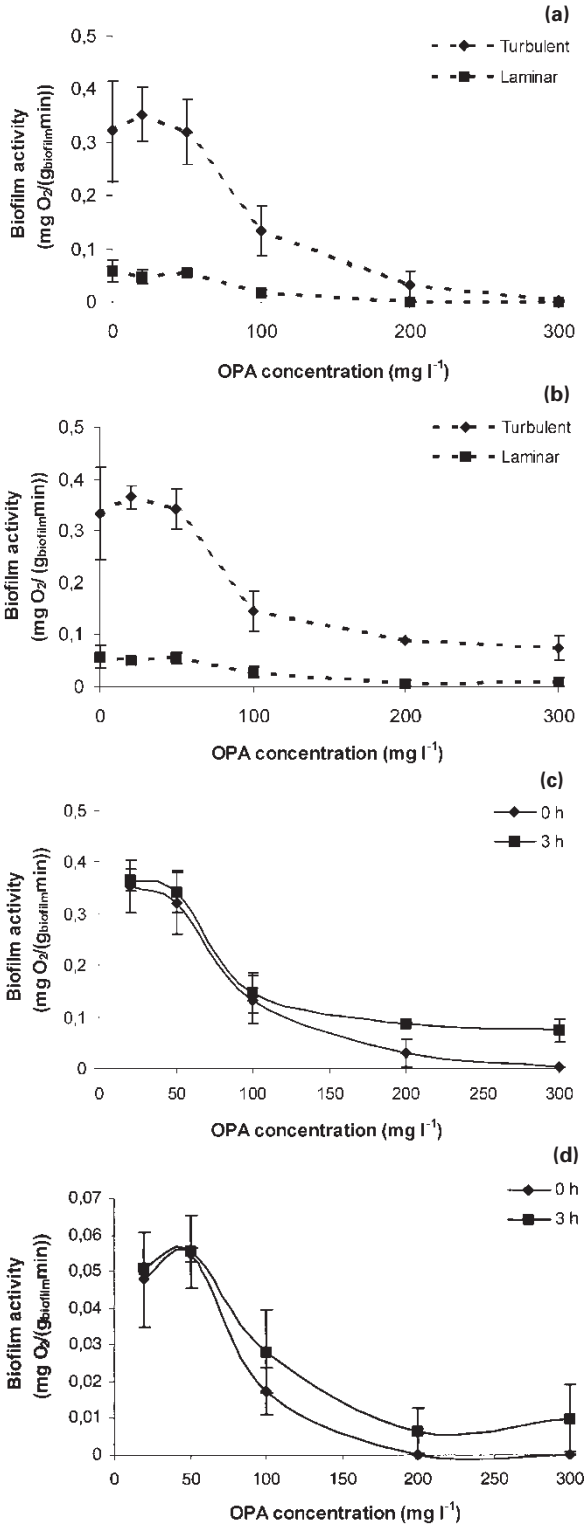

FIGURE 1 Biofilm respiratory activity immediately after OPA application a) and 3 h later b) for laminar and turbulent conditions and respiratory activity of biofilms formed under turbulent c) and laminar d) conditions, immediately after (0 h) application of different concentrations of OPA and 3 h later. Bars = the SD from the mean.

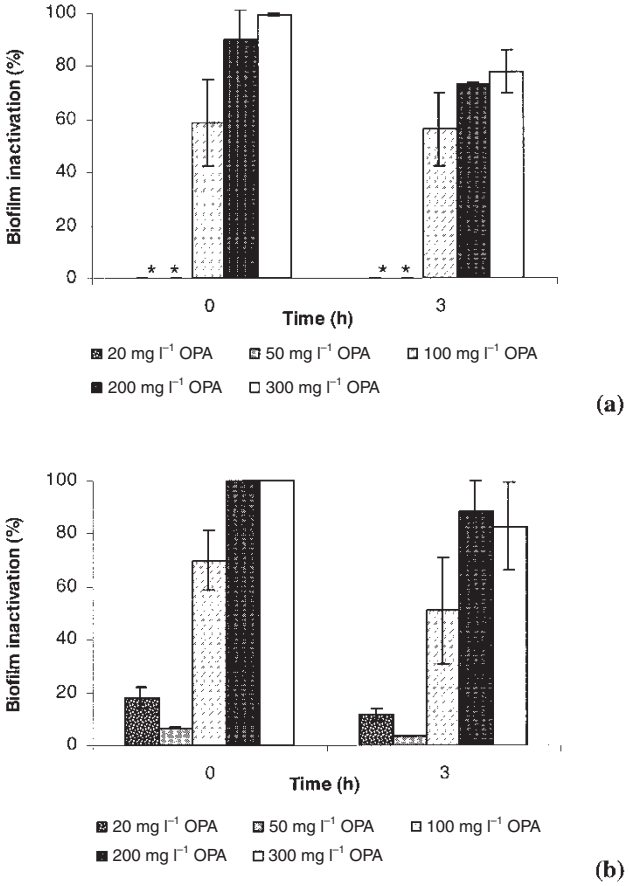

FIGURE 2 Biofilm inactivation after OPA application as a function of time. a) = turbulent flow; b) = laminar flow. Bars = SD. 0 h was immediately after biocide exposure; * = biofilm not inactivated.

to OPA, biofilms were again in contact with the bacterial suspension. Thus, the optimal conditions for biofilm development were restored. Consequently, based on these data it is possible to speculate that some biofilm recovery occurred after 3 h exposure to OPA. Nevertheless, further work needs to be carried out in order to determine how the bacterial biofilms recover.

The biocide had no effect on biofilm removal over the entire range of conditions studied since the mass of the different biofilms formed under laminar and turbulent flow did not show any significant variation due to OPA treatment (over 95% of the biofilm remained attached to the SS slides after OPA contact). The amount of biofilm mass that remained on the surface after each biocide treatment was within the same range as measured in the controls. Moreover, biofilm removal did not seem to be dependent on biocide concentration, since the large number of conditions tested revealed a standard deviation of zero, each concentration being tested three times. These results suggest that following OPA application, although biofilms can be partially or totally inactivated they stay attached to the surfaces, which is not desirable in industrial systems where biofilm accumulation is a problem. In these situations removal from the surfaces is essential and in order to promote biofilm removal, dispersants and surfactants need to be employed in combination with biocides (Paulus, 1993).

The results presented show that OPA is more effective in inactivating biofilms rather than in promoting their removal from surfaces. Based on these results, it can be speculated that OPA does not weaken the polymeric matrix and may even fix the biofilms to the surface as with other aldehydes (Workman & Day, 1984; Flemming & Schaule, 1996). However, the cohesive strength of the biofilm matrix may have been changed, thus the biofilm behaviour in response to different hydrodynamic conditions was investigated. Thus, in order to obtain a deeper knowledge of the effect of OPA on the remaining biofilm, a different set of experiments was carried out with the aim of characterizing the physical stability of the biofilms. The results expressed in terms of percentage of biofilm remaining on the surfaces of SS cylinders are shown in Figure 3. OPA appears to have a negative effect on the physical stability of the biofilm since the amount remaining adhered to the metal surface of the cylinders after OPA treatment and after submission to the rotation velocity series was always lower than that observed in the control assay, although similar values were obtained for all the OPA concentrations tested ($p > 0.05$). The differences observed for each OPA concentration are not significant when the SDs are taken into account. In the light of these results, it can be concluded that OPA promoted disturbance in the EPS matrix of the biofilms adhered to the cylinders, since some biofilm loss occurred as the rotation velocity increased. The negative effect of OPA on the physical stability of the biofilms is in contrast with the results obtained with GTA (Simões *et al.*, 2003a), where the physical stability of *P. fluorescens* biofilms, formed under the same conditions, was enhanced significantly with increasing concentrations of GTA applied to the system. For comparative purposes, Table II records the percentage of biofilm remaining adhered to the cylinders after treatment for 30 min with 200 mg l^{-1} of GTA and OPA, and after submission to the complete rotation velocity series.

As shown in Table II, the hydrodynamic changes caused by increasing rotation speeds did not result in the complete removal of biofilm from the cylinder surfaces. In the control assays, about 23–24% of the biofilm mass remained adhered to the cylinders. This was more pronounced when biofilms were previously treated with GTA, since the amount of biofilm mass remaining adhered to the cylinders after submission to the rotation velocities was considerably higher than that observed in the control assays. Conversely, in the case of OPA application, the amount of biofilm that remained adhered to the cylinders was lower than observed in the control. These results emphasise that GTA and

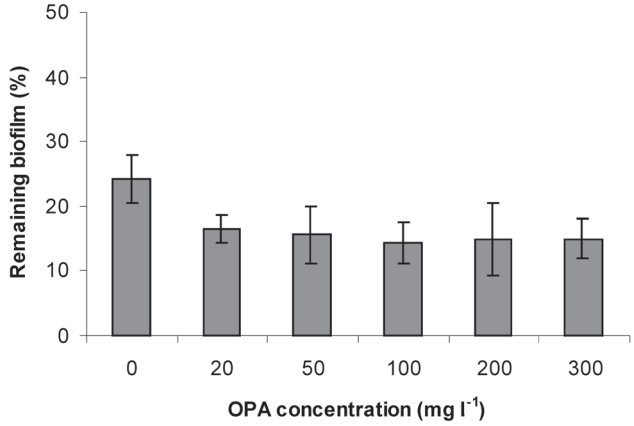

FIGURE 3 Percentage of biofilm mass remaining on the surface after submission to the complete series of rotation speeds. Bars = SD.

TABLE II Comparison of the percentage biofilm remaining on the surface after submission to different rotation speeds

	Remaining biofilm (%)	
	Control	Application of 200 mg l^{-1} of biocide
GTA		
Exposure time: 30 min	22.9 ± 3.7	35.4 ± 9.9
OPA		
Exposure time: 30 min	24.3 ± 4.0	14.8 ± 5.6

OPA, which both belong to the aldehyde family, interact in a different way with the matrix of the biofilms. GTA appears to promote strengthening of the structure of the biofilm, whereas OPA seems to cause weakening of the biofilm developed under the same conditions.

The bactericidal effect of OPA is due to its lipophilic aromatic nature that makes it easy to penetrate the outer layers of Gram-negative bacteria (Simons *et al*., 2000). Despite the fact that OPA is a chemical with two aldehyde groups, it is a less potent cross-linking agent than other aldehydes like GTA (Walsh *et al*, 1999a; 1999b; Simons *et al*., 2000). The results obtained from the data on the physical stability of the biofilm also revealed that a successive increase in the rotation velocity did not promote total removal from the surface. This result shows that after OPA treatment, even with the increase in biocide concentration there was no removal from the surface due to hydrodynamic forces.

In order to understand the decreased biofilm susceptibility to OPA, some additional tests were carried out where the action of OPA was assessed in cells in suspension, both in the presence and absence of protein (BSA). The interference of BSA with the efficacy of OPA against the suspended bacterial cultures is shown in Figure 4. In these tests the kinetics of inactivation were determined as a function of biocide concentration in the presence and absence of 3 g l^{-1} of BSA. The antimicrobial activity of OPA was significantly reduced when BSA was introduced into the bacterial suspended cultures, being particularly noticeable for OPA concentrations higher than 15 mg l^{-1}. Figure 4 also shows that in the absence of protein, there was a linear relationship between bacterial inactivation and OPA concentration up to 25 mg l^{-1}. For higher OPA concentrations total inactivation was almost achieved. This behaviour was no longer observed when BSA was added to the bacterial suspensions; the linear relationship was only observed for OPA concentrations < 15 mg l^{-1} and the total inactivation of the bacterial suspension was not observed even when high concentrations were used (the maximum inactivation achieved was around 90%).

Statistical analysis of the results obtained in the presence and absence of BSA showed that they were significantly different ($p < 0.01$). In the presence of BSA, even for an OPA concentration of 300 mg l^{-1}, total cellular inactivation was not observed. BSA is a protein that contains primary amine residues capable of reacting with free aldehyde groups. As a consequence, it reduces the availability of the groups necessary for biocidal activity and may also decrease the accessibility of the biocide to its target sites by forming a coating around the microbial cell (Fraud *et al*., 2001). BSA contains lysine, hystidine and glycine and glycine can be used to neutralize the OPA and make it safe for disposal (Rutala & Weber, 2001).

In the present work it was also shown that the bacterial cells entrapped in biofilms were more resistant than suspended cells, since the biocide concentration needed to achieve complete inactivation was higher for the biofilm (Figures 1 and 2) than for their suspended counterparts (Figure 4). There may be several reasons for this behaviour, such as an altered physiology of cells in the biofilm mode of growth, a different structure of the biofilm as a consequence of the hydrodynamic conditions under which it was formed, or by reaction of the biocide with a component of the matrix (Johansen *et al*., 1997; Pereira & Vieira, 2001).

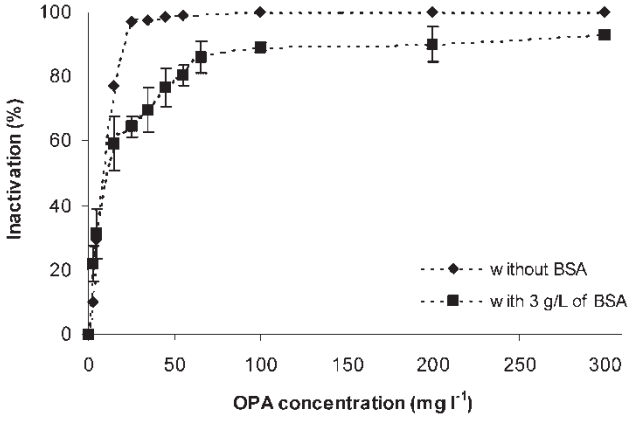

FIGURE 4 Inactivation of the respiratory activity of the suspended bacterial cells after treatment with different concentrations of OPA, with and without (control) BSA addition. Bars = SD.

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