Monitoring the effects of biocide treatment of *Pseudomonas fluorescens* biofilms formed under different flow regimes

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Abstract The effectiveness of glutaraldehyde – a very common biocide – to control biofilms formed by *Pseudomonas fluorescens* on stainless steel slides, in laminar and turbulent flow, was investigated. Tests were performed using a concentration of the biocide of 200 mg L^{-1} and a range of exposure times. The cohesion of the biofilm before and after exposing the biofilm to different biocide concentrations was also studied. The GTA action on biofilm was assessed by means of activity tests and wet weight of the biofilms.
The results showed that biofilms were not controlled after the treatment with biocide, in all the situations studied, and that the cohesion of the biofilms increased with biocide application.
Keywords Glutaraldehyde; biofilm characteristics; Pseudomonas fluorescens; laminar flow; turbulent flow; cohesion

Introduction

The unwanted accumulation of biofilms in industrial equipment – biofouling – is a natural occurrence, due to the favorable conditions. Biocides still represent the more significant countermeasure to control biofouling formation. However, these chemical substances are not fully effective to remove the biofilm. On that account, to increase the biocide concentration applied, to use new protocols of application or to change to more aggressive agents are the more frequent practices to overcome the problem.
Glutaraldehyde (GTA) is one of the biocides widely used in industry (Bott, 1995). Its biocidal effect is attributed to its two aldehyde groups, that interact with microbial cell constituents, reacting with ammonia and primary amines and more slowly with secondary amines, binding strongly to outer cellular layers (Eagar et al., 1986; Cloete et al., 1998).

The aim of this work was to clarify the way of action of the glutaraldehyde (GTA) on biofilms formed by Pseudomonas fluorescens, an abundant bacteria in biofilms formed in industrial equipment, under laminar and turbulent flow. The experimental tests were performed using 200 mg L^{-1} of GTA and a range of exposure times.

Material and methods

Microorganism

Pseudomonas fluorescens ATCC 13525 was used throughout this work.

Biocide

A non-oxidising biocide solution of glutaraldehyde (Reidel–de Haën 62621) in water was used as astock solution. The biocide concentration tested was 200 mg/l.

Experiments with biofilms

Flow cell reactor: A flow cell described elsewhere (Pereira, 2001) was used as a biofilm formation apparatus. Two flow cells were operating in paralel, to obtain one cell working under laminar flow and the other cell with turbulent flow. Biofilms were formed on stainless steel plates (1.75 cmx 1.25 cm) inserted into the flow cells. The system was continuously fed with a sterile medium containing 50 mg/l glucose, 25 mg/l peptone and 12.5 mg/l yeast extract in phosphate buffer.
Biocide treatment: After 7 days of conyinuous operation two coupons of each cell were removed from the flow cells and were used as a control. The system was then exposed to the biocide during a certain interval of time (1 hour, 2 hours, 2 times 1 hour and 2 times ½ hour). These multiple applications were spaced for 2 hours. The biofilms were sampled in the end of the exposure period to the biocide and after 3 hours.Each experiment was repeated three times.
Biofilm mass quantification: Biofilm mass quantification: Before being inserted in the flow cells, and after been degreased, rinsed and dried, the metal slides were identified and weighed. After 7 days of biofilm formation, and as soon as they were removed, the wet mass of the slides plus attached biofilm was also determined. After the GTA treatment, the slides were weighed again. Biofilm mass accumulated on the several slides was thus calculated as the difference between the two respective weights and expressed in kg per m^2 of surface area of the slide.
Scrapping and disaggregation of the biofilms: The biofilm that covered the metal slides was completely

scraped off the metal and ressuspended into 10 mL of phosphate buffer pH 7. The homogenised suspensions of biofilms were used to assess the cellular activity of the biofilm. The biofilms suspensions that were not treated with GTA were also used to determine the total and extracellular macromolecular composition.

Chemical analysis of the biofilm: The chemical analyses were carried out on the homogenised biofilm suspensions, before biocide treatment. The proteins were determined using the Lowry modified method (SIGMA-Protein Assay Kit nº P5656) and the polysaccharides by the phenol-sulfuric acid method of Dubois et al. (1956).

Oxygen Uptake Rates Measurement: 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) and the procedure used was described elsewhere (Pereira et al., 1998).

Cohesion assay: Three stainless steel cylinders were inserted in a 3 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 while the others were immersed in reactors containing a 200 mgL^{-1} glutaraldehyde solution (volume of the reactor 170 mL). After the treatment, the cylinders rotated at 300 rpm during 30 s. The cylinders were subjected to serial velocities of rotation - 500, 1000, 1500 and 2000 rpm-for periods of 30 seconds. The weighed of the cylinder was determined before the immersion and after each rotation. The experiment was repeated twice for each biocide treatment (the cylinders were immersed in the biocide solution for ½ hour, 1 hour and 2 hours).

Results

The GTA action on biofilms formed on the metal slides was studied by the assessment of the variation of the mass of the deposit experienced during the treatment period, the determination of the respiratory activity after the GTA treatment and the cohesion of the deposit after biocide addition.

Figure 1 presents potographs of coupons with stainless steel slides with biofilms formed under turbulent (a) and laminar (b) flow.

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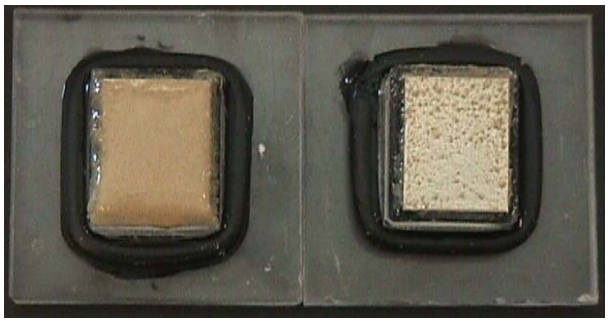

A B

Figure 1 Photograph of the coupons with biofilms formed in turbulent (a) and laminar flow (b)

Table 1 presents the characteristics of Pseudomonas fluorescens biofilms grown under turbulent and laminar flow.

Table 1 Characteristics of Pseudomonas fluorescens biofilms grown under turbulent and laminar flow Mean values ± confidence interval of 95%.

Flow	Biofilm mass (mg/cm2)	Biofilm activity (mg O2/g biofilm.min)	Total protein (mg/g biofilm)	Total polysaccharide (mg/g biofilm)
Turbulent	1,55 (±0,199)	0,273(±0,0916)	261 (±40,9)	151(±29,1)
Laminar	0,775 (±0,154)	0,0611(±0,0177)	128(±22,5)	196(±76,4)

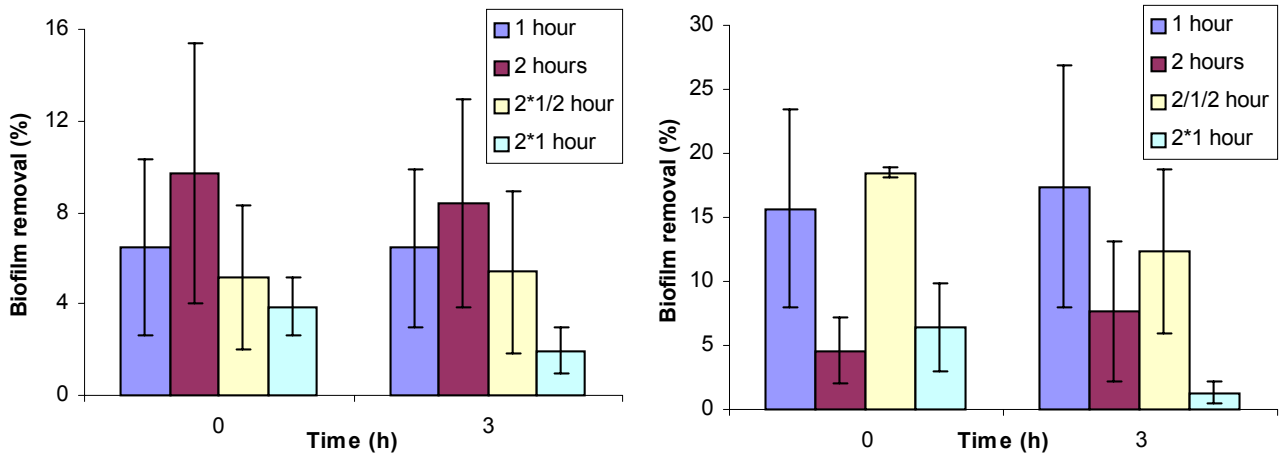

Figure 2 Biofilm removal after GTA application as function of time (biofilm grown under turbulent flow-bars represent the standard deviation) - (a) Turbulent flow, (b) Laminar flow

The results obtained showed that:
i) the structure of biofilms depend on the flow condition: while the biofilm formed under turbulent flow is homogeneous and slimy, the one formed under laminar flow is scattered on the surface; ii) the macroscopic composition of a P. fluorescens biofilm (Table 1) shows that the biofilms have a high content of proteins iii) biofilms were not removed after the GTA treatment, in all the situations studied; iv) the effect of the biocide is dependent on the flow regime under which the biofilm was formed; v) the the biocide proved to be more effective for longer exposure times; vi) the cohesion of the biofilm increases with the exposure time to the biocide.

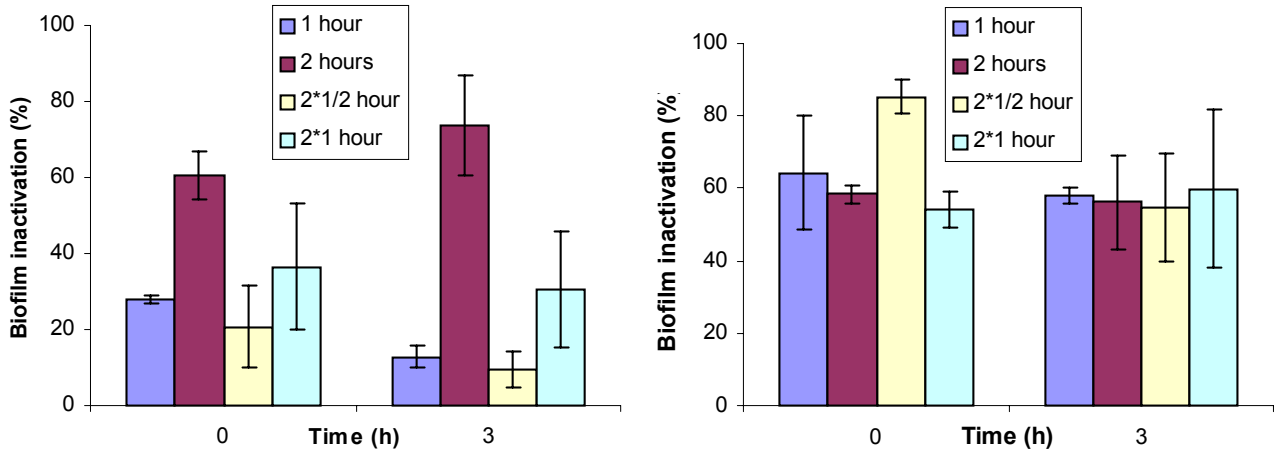

Figure 3 Biofilm inactivation after GTA application as function of time (turbulent flow - bars represent the standard deviation)

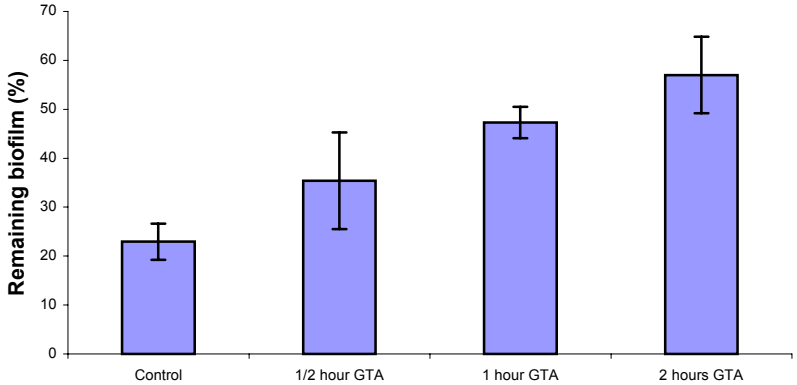

Figure 4 Percentage of biofilm remaining on the surface after the cohesion test

Discussion and Conclusions

The results presented show that the flow regime under which the biofilm is formed has a strong impact on biocide action: biofilms formed under laminar flow are more easily inactivated that the ones formed under turbulent flow. Biofilms formed under turbulent flow are denser, they have a stronger matrix of EPS; conversely, laminar biofilms appear scattered on the surface, they have protuberances and they are easily sloughed off (Vieira et al., 1995). As a consequence, GTA may penetrate more easily the biofilms formed under laminar flow. Additionally, biofilms formed in turbulent flow have a higher content of proteins that can react with the GTA, lowering the GTA available to inactivate the bacterial cells. In a previous work Pereira and Vieira (2001) noticed that when GTA was applied to control biofilms it reacted with the proteins of the polymeric matrix, leading to some disruption of the matrix, besides to the reaction with the bacterial cells. As a consequence, the concentration of biocide available for killing the cells is reduced and thus, the action of the biocide is lower than in suspension tests as pointed out previously (Pereira and Vieira, 2001).

An important conclusion is that it was not possible to reduce the mass of biofilm accumulated on the surface, and that the cohesion of the biofilms increased with biocide application. This fact can be explained by the ability of GTA to cross-link proteins, expressed as a fixative action of the biofilm to the surface. This is not surprising since GTA is used as fixation agent in microscopy.

It is surprising to notice that, in general, higher biofilm removal from the surface is obtained for shorter exposure times to the biocide. This result may be associated the higher cohesion of the biofilms obtained after the application of the biocide, related, probably, with the fixative action of GTA.

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