Surveillance of the behaviour of *pseudomonas fluorescens* biofilms after ortho-phtalaldehyde disinfection

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Abstract A relatively novel antimicrobial agent, ortho-phtalaldehyde (OPA), was tested in the control of biofilms formed by *Pseudomonas fluorescens* on stainless steel surfaces. The toxic action of OPA was assessed by means of activity tests and dry weight of the biofilms. For comparative purposes, the activity of OPA against bacterial suspended cultures was also evaluated. The results showed that concentrations of OPA higher than 15 mg L^{-1} inactivate the planktonic populations while having a lower effect against the *P. fluorecens* biofilms. The inactivation of the biofilm was only attained with the higher OPA concentrations (50 and 100 mg L^{-1}) and for longer exposure times (3 h). The application of OPA appears to cause little effect in the removal of biofilms from the metal slides since the amount of biofilm mass that remained on the surfaces, after biocide treatment, stills to be very high and representative. These results suggest the fact that, with OPA application, biofilms can be inactivated but stay attached to the surfaces, decreasing, by this way, the success of the chemical treatment.
Keywords Ortho-phtalaldehyde; disinfection; biofilm removal; *Pseudomonas fluorescens*.

Introduction

The development of biofilms on wet surfaces are a natural phenomenon that can be noticed in various places in nature as well as in the industrial equipment and in the medical devices. The main strategy of biofilm control is the use of chemical biocides or disinfectants to kill the attached microorganisms and/or remove them from the surface.

Ortho-phthalaldehyde is a new type of disinfectant that is claimed to have a potent bactericidal and sporicidal activity (Mcdonnell and Russell, 1999) and has been suggested as a possible alternative to glutaraldehyde (GTA) for high level disinfection (Walsh *et al*., 1999a) especially in the disinfection of some medical devices as endoscopes (Alfa and Sitter, 1994).

OPA is an aromatic compound with two aldehyde groups. Its antimicrobial action is not well known although some authors (Walsh *et al*., 1999b) suggested an action similar to that of GTA. The strongly reaction of OPA with primary amines and the consequence stabilization of the outer membrane and cell wall of the microorganisms may explain its lethal action.

This preliminary work involves the evaluation of the performance of ortho-phtalaldehyde against both suspended bacterial cultures and biofilms formed by *Pseudomonas fluorescens*, a very common strain in industrial and hospital environments The experimental tests were performed using a range of concentrations of OPA and exposure times, and biofilms formed for 6 days. Tests were also performed with *P. fluorescens* suspensions for comparison purposes. This was considered necessary because disinfectants with high activity in suspension tests are not necessarily as active in biofilms.

Material and methods

Microorganism

Pseudomonas fluorescens ATCC 13525.

Biocide

A solution composed of ortho-phtalaldehyde (Sigma, P-1378) prepared in sterile distilled water.

Suspended tests:

Microorganism growth

A continuous pure culture of the P. fluorescens bacteria as described elsewhere (Pereira et al. 1998)

Biocide treatment: Periodically, a suitable amount of P. fluorescens culture was removed from the fermenter, centrifuged (3777g, 10 min) and washed three times with phosphate buffer. The pellets were ressuspended in phosphate buffer pH 7. The bacterial culture was then divided by several sterilised glass flasks and put in an orbital shaker (120 rpm). The culture was exposed to different biocide concentrations (5, 10, 15, 20 and 50 mg L^{-1}) and the bacterial respiratory activity was assessed over time (5, 60 and 180 min) through oxygen consumption in a respiration chamber.

Experiments with biofilms

Biofilm set-up: Biofilms were grown on ASI 316 stainless steel slides (2.5 cm x 2.5 cm and 1 mm thick) that were hanged within a well stirred reactor containing a batch bacterial culture. The operating conditions of the reactor system have been described previously (Pereira and Vieira, 2001). The fermenter

was continuous fed with a sterile medium consisting of 50 mg glucose L^{-1}, 25 mg peptone L^{-1} and 12.5 mg yeast extract L^{-1}, in phosphate buffer pH 7. The biofilm was allowed to grow for 6 days (the time need to reach the steady state) and was sampled prior to the start of the OPA treatment.

Biocide treatment: The biofilms that covered the metal slides were carefully transferred to closed vases containing the OPA solutions (10. 25, 50 and 100 mg L^{-1}). At known time intervals (5, 60 and 180 min of biocide contact time) the metal slides *plus* biofilm were carefully removed from the biocide-containing flask and reserved for evaluation of the OPA action.

Scrapping and disaggregation of the biofilms: The biofilm that covered the metal slides was completely scrapped from the metal slides into 10 mL of phosphate buffer pH 7 and vigorously homogenised in a vortex. The homogenised suspensions of biofilms were used to assess the bacterial activity of the biofilm. The biofilms suspensions that were not treated with OPA were also analysed for total protein and polysaccharide.

Analytical methods: 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). The dry biofilm mass was assessed by the determination of the total volatile solids (TVS) of the homogenised biofilm solutions, according to the Standard Methods (1989). Biofilm mass accumulated on the several slides was expressed in g of TVS per cm^2 of surface area of the metal slide.

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) and the procedure used was described elsewhere (Nogueira *et al.*, 1998).

Results and Discussion

The effects of OPA on the biofilms were investigated assessing the respiratory activity of the bacterial cells and the variation of the mass of the biofilms during the disinfection period.

The results revealed that OPA reduced the biofilm activity for all the biocide concentrations studied (Figure 1). Nevertheless, the extent of respiratory activity reduction is only significant for higher OPA concentrations and for longer exposure times. The total inactivation of the biofilm is only achieved with 50 and 100 mg L^{-1} of OPA after 3 h of exposure to the biocide. Short exposure periods, as 5 min, seems not to be sufficient for OPA to carry out its antimicrobial action, since only with the higher OPA concentrations a biofilm activity decrease was detected. These remarks pointed out OPA as a disinfectant dependent of exposure time.

The results also demonstrated that OPA seems to have poor removal action since the amount of biofilm mass that remains in the steel surfaces, after biocide treatment, is within the same range of the ones measured in the control assay (Figure 2). Only for OPA concentrations higher than 10 mg L^{-1} it is possible to notice some biofilm removal, this removal being, however, quite independent of the OPA concentration increase. For the higher OPA concentrations (50 and 100 mg L^{-1}) it seems that the removal was dependent on the exposure time since the reduction of biofilm mass increased with time.

The results presented below underscore the fact that biofilm inactivation and biofilm removal are distinct processes. In fact, in this study, OPA causes a significant reduction of biofilm activity conversely to the biofilm removal. Consequently, it is possible to speculate that OPA is more effective in disinfecting biofilms than in promoting their removal from the surfaces. This OPA characteristic supports the need for implementing, together with this biocide, other strategy of biofilm control in order to promote the removal of the inactive biofilm. In many systems where problematic biofilm fouling occurs, the main objective is to have clean surfaces rather than an inactive biofilm attached to the surfaces (Chen and Stewart, 2000).

The application of OPA to the suspended *P. fluorescens* cultures caused the decrease of the bacterial respiratory activity, for all the concentrations tested (Figure 3). However, the bacterial activity decrease was gradually improved by increasing OPA concentration. As can be seen in Figures 1 and 3 the OPA concentration need to cause the total inactivation of the suspended bacterial cultures is lower than the biocide amount required for biofilm inactivation. Conversely to what seemed to happen with biofilms, the action of OPA against the planktonic cultures appears to be more immediate, *i. e*., its toxic action does not significantly increase with the increase of the biocide contact time. These data emphasize that *P. fluorescens*, when grown in biofilm, was less sensitive to the aggressive action of OPA. Some suggestions

have been raised to explain biofilm resistance to biocides (*Morton et al*., 1998) but, in this case, the low OPA efficacy when applied against biofilms may be related to the reaction of OPA with the proteins of the polymeric matrix since it was found (data not shown) that the antimicrobial action of this disinfectant can be totally inactivated in the presence of a protein (BSA).

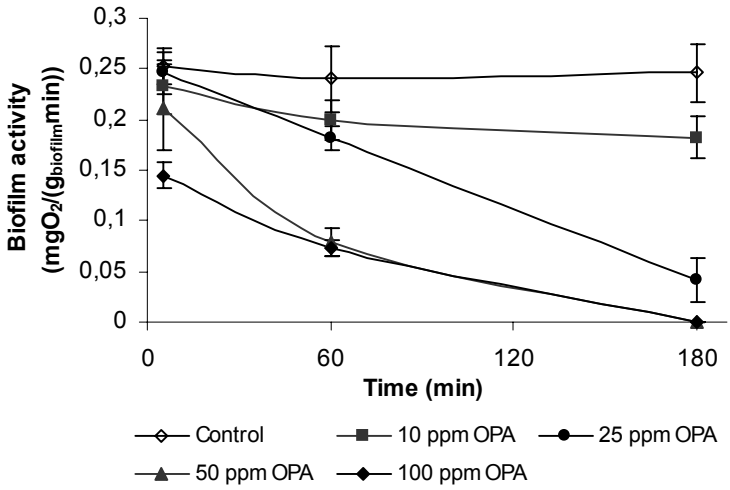

Figure 1 Bacterial activityof the *P. fluorescens* biofilms when treated with several concentrations of OPA. Bars represent the standard deviation of the mean.

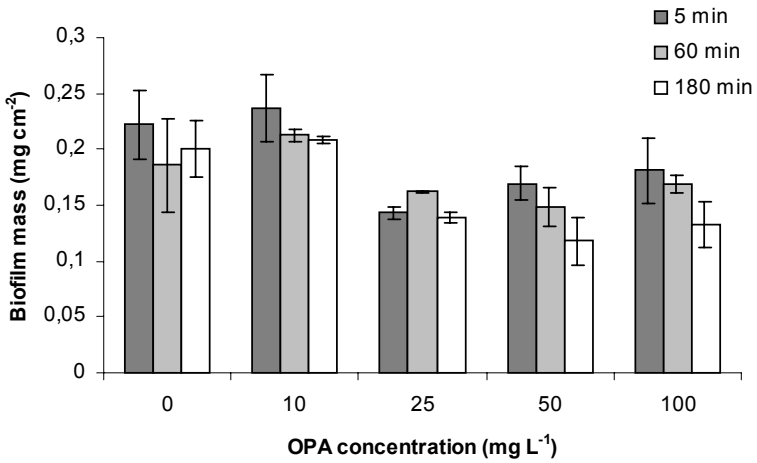

Figure 2 Remained biofilm mass after application of OPA. Mean values ± SD.

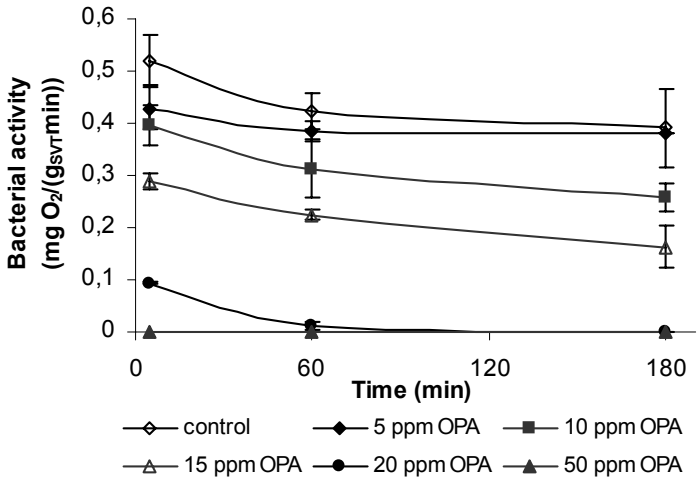

Figure 3 Bacterial activity of the suspended cultures of *P. fluorescens* without biocide application and after treatment with several concentrations of OPA. Mean values ± SD.

Conclusion

This preliminary study showed that the action of OPA, when used to control biofilms formed by *P. fluorescens*, is essentially disinfectant since this chemical agent causes a substantial reduction of biofilm activity but not biofilm removal. Nevertheless, the biocidal efficacy of OPA was improved by increasing the exposure time and the concentration.

These findings highlight that besides the chemical treatment with disinfectants, biofilm control strategies should always comprise practical cleaning formulations for biofilm removal.

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