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Effects of the Interactions Between Glutaraldehyde and the Polymeric Matrix on the Efficacy of the Biocide Against *Pseudomonas fluorescens* Biofilms

MARIA OLIVIA PEREIRA and MARIA JOÃO VIEIRA*

Centro de Engenharia Biológica-IBQF, Universidade do Minho, 4710-057 Braga, Portugal

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Glutaraldehyde (GTA) is a widely used biocide due to its high effectiveness. The experimental work reported here was carried out to assess the effectiveness of GTA in controlling biofilms formed by *Pseudomonas fluorescens* on stainless steel slides, and to compare efficacy against both planktonic and sessile microrganisms. The tests were performed using two concentrations of GTA (50 and 100 mg l^{-1}), biofilms of two ages (7 and 15 d), several pH values (5, 7 and 9) and a range of exposure times (from 0 (control) to 1, 3, 7 and 24 h). The action of GTA on biofilm and planktonic populations was assessed by means of activity tests, zeta potential, and the wet weight of the biofilms. Biofilms were not completely removed after treatment with GTA in any of the conditions studied. The higher GTA concentration was more effective in reducing the bacterial activity of the biofilm. The biocide proved to be more effective for longer exposure times. GTA showed good antimicrobial activity against *P. fluorescens* in suspension, with higher activity at pH 9. The findings of this study suggest that when GTA is used to control biofilms, it reacts with one of the components of the matrix, the proteins, thereby reducing its antimicrobial action.

Keywords: glutaraldehyde; biofilms; *Pseudomonas fluorescens*; polymeric matrix

INTRODUCTION

Biofilms can be described as microsystems adhered to solid surfaces in which the microorganisms are embedded in a matrix of extracellular polymeric substances (EPS) which are responsible for the morphology, structure, coherence and physico-chemical and biological properties of the biofilms (Flemming & Wingender, 1999). The most common practice to elimine unwanted biofilms is the application of antimicrobial chemicals. Such agents may have different modes of action, namely they can adversely affect the initial adhesion of microorganisms by changing the surface free energy of the substratum or the bacterial wall hydrophobicity (Cheung & Beech, 1996), they may also affect cell viability, by short-circuiting the electron transport system, or they may cause changes to the EPS that may affect biofilm structure (Nesaratnam & Bott, 1984).

*Corresponding author. Fax: 00 351 253 678986, e-mail: mjv@deb.uminho.pt

It is known that a biocide is not as effective on biofilm microorganisms as on the same microorganisms found in the planktonic phase. Gaylarde & Gaylarde (1996) found that, while *Pseudomonas fluorescens* in the planktonic state was killed by glutaraldehyde, the agent was less effective on sessile populations. Keevil *et al.* (1993) studied the resistance to biocides of *Legionella pneumophila*, and concluded that, while the pathogen bacteria and other microorganisms present in the planktonic mode were killed when treated with biocides, the same concentration was not sufficient to eradicate the bacteria from biofilms. Blenkinsopp *et al.* (1992) showed that glutaraldehyde, when applied to biofilms formed by *Pseudomonas aeruginosa*, did not have a significant effect on the number of colony formation units per unit area, compared to the ability of the biocide to kill the same planktonic bacteria.

Several hypotheses have been raised to explain the increased resistance of biofilm microorganisms to biocides: (i) the EPS produced by the microorganisms protects them from the action of the harmful agent (Christensen & Characklis, 1990); (ii) the matrix may have degradative enzymes that quench the activity of the biocide (Heinzel, 1998); (iii) reaction between the biocide and community components, such as organic matter, inorganic particles and cell debris (McFeters *et al.*, 1995); (iv) the EPS is a charged matrix, being responsible for binding antimicrobial agents before they reach the target cell (Costerton, 1985); (v) the physiological state of the sessile cells is different from that in the planktonic phase (Morton *et al.*, 1998). Therefore, when a biocide is used to control biofilms, the microbial response to the agent will depend not only on the type of microorganisms and type of chemical agent (Morton *et al.*, 1998), but also on the complex interactions between the biocide and the biofilm matrix.

Glutaraldehyde (GTA) is a biocide widely used in industry and in hospital environments, since it is has a broad range of activity, is non corrosive to metals, rubber and optical lenses (Bott, 1995; Walsh *et al.*, 1999). The biocidal effect of glutaraldehyde is attributed to its two aldehyde groups, which interact with microbial cell constituents, reacting with ammonia and primary amines and more slowly with secondary amines, and also binding strongly to outer cellular layers (Eagar *et al.*, 1986; Paulus, 1993; Russel, 1994; Cloete *et al.*, 1998).

The aim of the work reported here was to clarify the mode of action of GTA, a biocide widely used in industrial environments on biofilms formed by *P. fluorescens*, and specifically interactions with components of the polymeric matrix. *P. fluorescens* was used as a model microorganism since it is found in biofilms formed in industrial equipment (Mattila-Sandholm & Wirtanen, 1992), and in river water. Stainless steel was used to form the biofilm since this alloy is widely used in the construction of industrial equipment. The range of pH used in this work (5, 7 and 9) was chosen as representative of industrial environments, namely the pulp and paper industry where the pH may range from 4 to 10 (Väisänen *et al.*, 1994). Furthermore, pH 7 was used because it is the optimal pH for growth of *P. fluorescens* (Oliveira *et al.*, 1994) and pH 9 since GTA exhibits better antimicrobial activity in the alkaline range (Eagar *et al.*, 1986).

MATERIALS AND METHODS

Microorganism

Pseudomonas fluorescens (ATCC 13525), a Gram-negative aerobic bacterium, was used as a biofilm producer.

Biocide

A non-oxidising biocide solution composed of glutaraldehyde (Riedel-de Haën 62621) in water (25% w v^{-1}) was used as a concentrated stock solution. The GTA concentrations tested (50 and 100 mg l^{-1}) were obtained by dilution with sterile distilled water.

Experiments with Suspended Microorganisms

Bacterial growth

A continuous culture of *P. fluorescens* was grown in a 3 l glass fermenter, at 27 ± 1°C, suitably aerated and magnetically agitated. The fermenter was continuously fed with 0.5 $l\,h^{-1}$ of a sterile 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 (0.2 mol l^{-1} Na_2HPO_4 and 0.2 mol l^{-1} NaH_2PO_4).

Biocide treatment of suspended cultures

Periodically, a volume of the *P. fluorescens* culture was removed from the fermenter, under sterile conditions, centrifuged (3773 g, 10 min) and washed three times with phosphate buffer to remove the growth medium. The pellets thus obtained were resuspended, in order to obtain a bacterial suspension of 10^7 cells ml^{-1}, in one of the following buffers according to the pH value of each assay: phosphate buffer pH 7 (Riedel-de Haën 38746), pH 9 (Riedel-de Haën 38748) and acetate buffer pH 5 (Riedel-de Haën 38744). The bacterial culture obtained was then sub-divided into several sterile glass flasks and incubated in an orbital shaker (120 rpm, 27°C). After 30 min, a known amount of biocide was added to each flask to produce a range of concentrations (50 and 100 mg l^{-1}). One of the flasks without biocide addition was used as a control.

Determination of cellular activity

At known time intervals (immediately after biocide addition, and after 1, 3, and 7 h), samples from each flask was transferred into a respiration chamber to evaluate their cellular activity (through oxygen consumption due to glucose oxidation) in a biological oxygen monitor (BOM) in short-term assays. The assays were performed in a Yellow Springs Instruments BOM (Model 53) as described elsewhere (Nogueira *et al.*, 1998). The results presented are the average of several measurements.

Zeta potential measurements

The effect of the biocide on the electrokinetic potential of bacteria was evaluated at the biocide concentrations stated above. The cells were prepared as described previously to a final concentration of $1 \times 10^9 - 3 \times 10^9$ cells ml^{-1}. The culture was divided into 100 ml flasks and biocide was introduced in to each flask in order to obtain final concentrations of 50 and 100 mg l^{-1}. The zeta potential of each culture was determined according to Loosdrecht *et al.* (1990) using a Zeiss ZetaMeter system 3.0+ (ZetaMeter Incorporated, New York), by applying a potential between 200 and 300 V across the electrophoresis cell. The results expressed in mV are the average of more than 20 measurements.

Experiments with Biofilms

Biofilm set-up

Biofilms were developed in a well stirred continuous reactor at 27 ± 1°C. *P. fluorescens* was grown in a 3 l glass fermenter, suitably aerated and magnetically agitated. The fermenter was continuously fed with 2.41 h^{-1} of a sterile nutrient solution consisting of 40 mg glucose l^{-1}, 20 mg peptone l^{-1} and 10 mg yeast extract l^{-1}, in phosphate buffer at pH 7 (0.2 mol l^{-1} Na_2HPO_4 and 0.2 mol l^{-1} NaH_2PO_4). The bacteria were grown in the fermenter as a batch for approximately 1 d (to reach steady state) before the beginning of the continuous feeding process. The dilution rate of 0.8 h^{-1} ensured that biofilm activity predominated over that of planktonic cells (Vieira *et al.*, 1993).

Twenty four slides (2 cm × 2 cm and 1 mm thick) made of ASI 316 stainless steel were placed within the bacterial suspension for 7 and 15 d for biofilm growth. The slides were degreased, rinsed twice with water and sterilised before they were suspended in the fermenter

using a device that enabled their removal for biofilm sampling. Prior to each experiment, all components of the system were sterilised by autoclaving at 120°C at 1 atm for 25 min. The selection of initial growth conditions and system parameters were based on previous experiments (Vieira *et al.*, 1993).

Treatment of biofilm-covered metal Slides

After biofilm development (7 or 15 d) the biofilm-covered metal slides were carefully transferred to a closed vessel that contained the biocide solution (50 or 100 mg l^{-1} of GTA). The flask was placed in an orbital stirrer throughout the biocide treatment, to ensure the same temperature and agitation conditions as in the fermenter. Some biofilm-covered stainless steel slides were placed in sterile distilled water for control assays. At known time intervals (immediately after biocide addition, and after 1, 3, 7 and 24 h biocide contact time) the stainless steel slides with accumulated biofilm were carefully removed from the biocide-containing flask.

Biocide action on biofilms formed on the metal slides was measured as the variation in the mass deposited during the treatment period, the respiratory activity after biocide treatment and by chemical analysis of the deposit.

Biofilm mass quantification

At the beginning of each experiment, and after been degreased, rinsed and dried, all the stainless steel slides were identified and weighed, to 3 decimal places, before being introduced in the fermenter (W_1). After the period of biofilm formation the wet weight of the slides was determined (W_2). After GTA treatment, the slides plus biofilm were weighed again (W_3).

The wet mass of the biofilm accumulated on each slide was determined as the difference between the two respective weights (W_2 and W_1 or W_3 and W_1), and expressed in g cm^{-2} of surface area of the stainless steel slide. The results are the means of more than three measurements.

The percentage of biofilm removal was determined as:

$$\% \text{ biofilm removal} \quad \frac{(W_2 - W_1) - (W_3 \quad W_1)}{(W_2 - W_1)} \times 100$$

Scraping 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 *ex situ*. The biofilm suspensions that were not treated with GTA were also used to determine the total and extracellular macromolecular composition.

Determination of cellular activity of the biofilm

Biofilm suspensions were transferred into a respiration chamber to evaluate their cellular activity (through oxygen consumption). The respiratory activity of the homogenised biofilm was evaluated by measuring oxygen uptake rates in a Yellow Springs Instruments BOM in short-term assays. The assays were performed as described above. The buffers and the substrate were as described previously, and the temperature in the measuring chamber was 27°C. All tests were carried out in duplicate.

Extraction procedure

Extraction of the extracellular components of the biofilm was carried out using Dowex resin (50X8, Na^+ form, 20–50 mesh, Aldrich-Fluka 44445) according to the procedure described by Jahn and Nielsen (1995). The *P. fluorescens* biofilm samples were suspended in 20 ml of the extraction buffer (2 mmol l^{-1} Na_3PO_4; 4 mM NaH_2PO_4, 9 mmol l^{-1} NaCl and 1 mmol l^{-1} MKCl, pH 7) and 50 g of Dowex resin g^{-1} of

volatile solids were added to the biofilm suspensions and stirred with a paddle at 600 rpm for 2 h at 4°C, thus a mild extraction without cell lysis was ensured (Frolund *et al.*, 1995). Prior to the extraction, the Dowex resin was washed with extraction buffer.

Chemical analysis of the deposit

Chemical analyses were carried out on the homogenized biofilm suspensions, before biocide treatment, and before and after the extraction procedure. Prior to chemical characterisation, the biofilm suspensions were vigorously homogenised in a vortex for 2 min with 100% power input. Protein was determined using the Lowry modified method (SIGMA-Protein Assay Kit n° P5656) with BSA as a standard. Polysaccharides were measured by the phenol-sulfuric acid method of Dubois *et al.* (1956) using glucose standards.

RESULTS

Experiments with Suspended Microorganisms

Table I presents the results of bacterial activity of the suspended cultures over time at different pH values, without biocide application (control) and after treatment with 50 mg l^{-1} and 100 mg l^{-1} of GTA. Bacterial activity, due to glucose oxidation, decreased after GTA application, reaching very low or non detectable values. The action of GTA was more pronounced at pH 9, where no bacterial activity could be detected almost immediately after application.

Table II shows the effect of biocide concentration on the zeta potential of *P. fluorescens* suspensions with and without different concentrations of biocide. The surface charge of the bacteria did not change after biocide application. However, for pH 9 and 100 mg l^{-1} of biocide, the cells became more negative.

Experiments with Biofilms

The characteristics of *P. fluorescens* biofilms before biocide addition are presented in Table III. After 15 d, the mass per cm^2 accumulated on the surface was almost two fold that accumulated after 7 d. The protein content was higher than the polysaccharide content, and the total protein and total polysaccharides were also higher for older biofilms. The matrix of EPS is composed of proteins in addition to polysaccharides, as also reported by Jahn *et al.* (1999) for *P. putida* biofilms.

TABLE I Bacterial activity of suspended cultures of *P. fluorescens*, at different pH values, without biocide application (0 mg l^{-1}) and after treatment with 50 mg l^{-1} and 100 mg l^{-1} of GTA, as a function of time

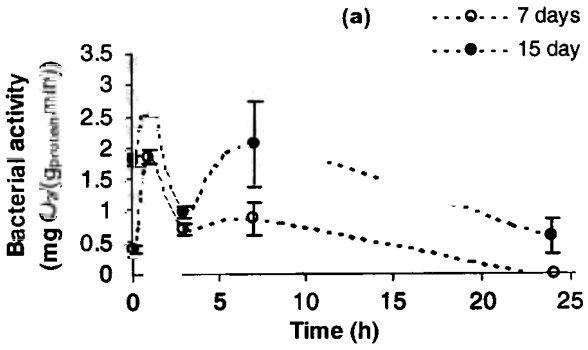
Biocide contact time	Bacterial activity (mg $O_2/(g_{protein}\ min^{-1})$)					
	0 mg l^{-1}		50 mg l^{-1}		100 mg l^{-1}	
(h)	pH5	pH9	pH5	pH9	pH5	pH9
0						
						0
						0
3						
						0
7						

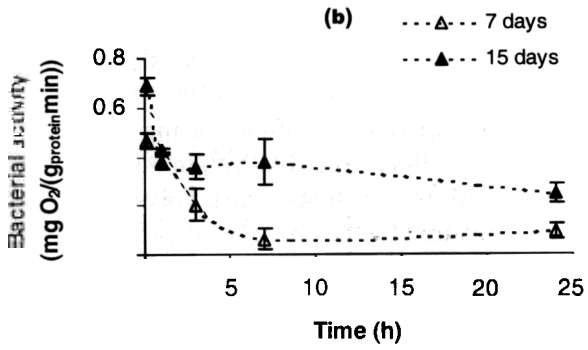
Figures in brackets = SD

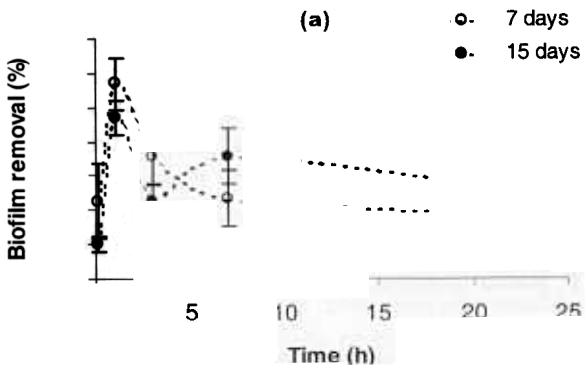
TABLE II Effect of 50 and 100 mg l^{-1} GTA on the zeta potential of suspended cultures of *P. fluorescens*, as a function of pH

	pH	Zeta potential (mV)
	5	
P. fluorescens	7	
	9	
P. fluorescens	5	
+	7	
50 mg l^{-1} biocide	9	
P. fluorescens	5	
+	7	
100 mg l^{-1} biocide	9	

Figures in brackets = SD







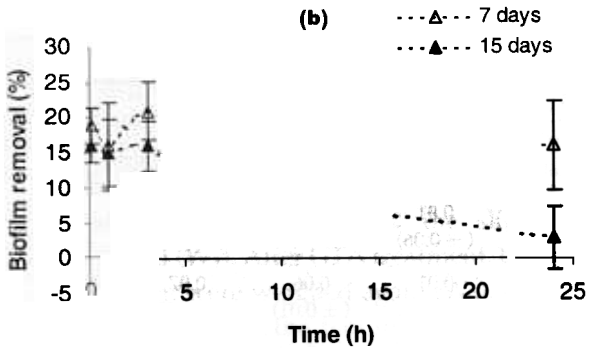


TABLE III Characteristics of *P. fluorescens* biofilms

Biofilm age (d)	Biofilm mass (mg cm^{-2})	Biofilm activity ($mgO_2(g^{-1}_{protein}\ min^{-1})$)	Protein ($mg_{protein}\ g^{-1}_{biofilm}$)			Polysaccharide ($mg_{polysaccharide}\ g^{-1}_{biofilm}$)		
			Total	*Intracellular*	*Extracellular*	*Total*	*Intracellular*	*Extracellular*
7	28.7 (± 8.4)	1.215 (± 0.488)		0.98 (± 0.61)	2.73 (± 0.91)		0.39 (± 0.89)	2.46 (± 1.53)
15	52.9 (± 11.6)	2.884 (± 1.002)		4.03 (± 1.52)	2.96 (± 0.54)		4.65 (± 0.83)	1.67 (± 1.04)

Figures = mean values ± 95% confidence limits

with the biocide, but increased after short contact times (to values even higher than those obtained in the absence of biocide), and decreased for higher contact times; for 100 mg l^{-1} the respiratory activity decreased from the start of the application; (vi) the specific respiratory activity of biofilms treated with 100 mg l^{-1} of GTA was much smaller than for biofilms treated with 50 mg l^{-1}; (vii) the reduction of the specific bacterial activity was higher for younger biofilms.

DISCUSSION

GTA was effective in reducing the respiratory activity of suspended cells of *P. fluorescens*, with a more pronounced effect at pH 9 (Table I). These findings were expected since this biocide is well known for its high reactivity, which increases at alkaline conditions (Bott, 1995). This increase in efficacy for pH values higher than 8 is thought to be due to alteration of the surfaces of the microorganisms (Walsh *et al.*, 1999), rather than to changes in GTA with pH. Under alkaline conditions, the percentage of unprotonated amino groups of the proteins of the bacterial cells increases (Morrison & Boyd, 1987), which is related to the increased activity of GTA.

The action of GTA on the activity of *P. fluorescens* was evaluated by oxygen uptake rate due to glucose oxidation which, according to McFeters *et al.* (1995), is more accurate than the traditional method of colony formation on agar medium. This latter method may underestimate the actual viable population since the bacteria can remain viable after biocide application, but may not grow on solid medium.

The reaction of GTA with the cells did not change the surface charge of the bacteria, as reported for the same bacterial culture, using another biocide (Pereira *et al.*, 1998). As indicated in Table II, GTA did not shift the surface charge towards the positive or negative ranges for the pH range studied. In the case of pH 9 and 100 mg l^{-1} of GTA, the cells became more negative in the presence of biocide, which may be related to the more intense reaction that takes place at alkaline conditions. Regarding biofilms, this result is important since surface charge influences not only the attachment of cells to surfaces, but also detachment of cells from surfaces.

GTA was not efficient at removing all the biofilm from the surface (Figures 1a and 1b), in spite of the fact that this biocide is frequently used to chemically control the accumulation of biofilms. Although GTA controlled the activity of suspended microorganisms it may not be efficient in reducing the amount of biological deposit that remains attached to surfaces. The biofilms are composed not only of microbial cells but also of EPS, which may not be harmed by the presence of the biocide, and may retard penetration of the antimicrobial agent. Dispersants and surfactants often need to be employed in combination with biocides, in order to

increase the amount of biofilm removed from the surface (Paulus, 1993).

The age of the biofilm is important in terms of the amount removed, less being removed in the case of older biofilms, when similar concentrations of biocide are used. The effect of biocide contact time on the amount of biofilm removed from the surface is not very important for younger biofilms. Conversely, older biofilms appear to be more resistant to the effects of GTA, especially when a higher concentration was used. These results are probably due to the different characteristics of biofilms of different ages. Older biofilms are thicker, which renders them more difficult to be penetrated by the biocide, and they have a different macromolecular composition that can influence the action of GTA. When a concentration of 100 mg l^{-1} of GTA was used, less biofilm was removed from the surface than with 50 mg l^{-1}, possibly due to the ability of GTA to attach cells to the surface. GTA is traditionally used for the immobilisation of yeast cells to supports, which after immobilisation become resistant to chemical and physical disruption (Workman & Day, 1984).

Therefore, although GTA removed part of the biofilm, it may have contributed also to the formation of a harder deposit that remained on the surface after treatment. Consequently, it is important to assess if the layer that is not eliminated upon treatment still retains its bacterial activity, since it may contribute to biofilm regrowth.

The trend observed for biofilm respiratory activity was not the same for the two concentrations of biocide used. An increase in the specific respiratory activity of the biofilm occurred for short contact times after the application of 50 mg l^{-1} of biocide (Figure 2a), in contrast to the behaviour after 100 mg l^{-1} (Figure 2b) which always decreased.

These findings may be related to the interaction established between GTA and the polymeric matrix. GTA reacts with amino acids [histidine (Walsh *et al.*, 1999) may be used as GTA neutraliser], decreasing biocidal efficacy. Therefore, when a biofilm is treated with GTA, the biocide will react with proteins of the biofilm. Table III shows that the polymeric matrix of the biofilm had a high content of proteins, in addition to the proteins located at the bacterial cell surface. Hence, GTA reacts not only with cell proteins (the mechanism responsible for the lack of activity of the suspended bacterial cells treated with GTA) but also with proteins in the matrix. The function of proteins in the matrix is not fully understood (Jahn *et al.*, 1999), but may be related with the maintenance of the structure. Therefore, the reaction of GTA with matrix proteins can probably lead to the disruption of the matrix structure, while bacterial cells remain attached to the surface. This argument is reinforced by SEM observation of biofilms treated with GTA, which showed a less evident slimy matrix of EPS than untreated controls. Similar observations were also reported by Azeredo *et al.* (1999).

The specific activity of the biofilm increased, since the mass of biofilm that remained on the surface was mainly cells that were not inactivated and thus were able to oxidise the substrate. For longer contact times, it is not surprising that a decrease in the respiratory activity of the biofilms was observed, since the contact time allowed biocide to act not only on the matrix but also on the bacteria. Thus, when a biofilm is exposed to GTA, the exposure time should be long enough to allow the chemical to penetrate to the bacterial cells. If the biocide application is stopped before the complete inactivation of the cells (for example, in short duration shocks), the biofilm will regrow, possibly faster than before, since bacteria are already adhered to the surface and nutrient availability will be high. Moreover, there is also the possibility that some bacteria resistant to GTA will proliferate.

CONCLUSIONS

The doses of GTA applied here were within the range of concentrations applied in industry.

Although the results obtained showed that the amount of deposit accumulated on the surface decreased with biocide application, biofilm was not removed completely. The large amount of protein found in the biofilm matrix reduced the antimicrobial action of the GTA. These findings indicate that, besides the physical characteristics of the biofilms, the biochemical composition of the matrix should also be taken into account in biocide treatment programs *e.g.* continuous application or intermittent dosing, the duration of the treatment, intervals between applications. Therefore, the efficacy of a chemical as a means of controlling biofilms should not be evaluated through its effect on the activity of planktonic cells.

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