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The effects of glutaraldehyde on the control of single and dual biofilms of *Bacillus cereus* and *Pseudomonas fluorescens*

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Glutaraldehyde (GLUT) was evaluated for control of single and dual species biofilms of *Bacillus cereus* and *Pseudomonas fluorescens* on stainless steel surfaces using a chemostat system. The biofilms were characterized in terms of mass, cell density, total and matrix proteins and polysaccharides. The control action of GLUT was assessed in terms of inactivation and removal of biofilm. Post-biocide action was characterized 3, 7, 12, 24, 48 and 72 h after treatment. Tests with planktonic cells were also performed for comparison. The results demonstrated that in dual species biofilms the metabolic activity, cell density and the content of matrix proteins were higher than those of either single species. Planktonic *B. cereus* was more susceptible to GLUT than *P. fluorescens*. The biocide susceptibility of dual species planktonic cultures was an average of each single species. Planktonic cells were more susceptible to GLUT than their biofilm counterparts. Biofilm inactivation was similar for both of the single biofilms while dual biofilms were more resistant than single species biofilms. GLUT at 200 mg l^{-1} caused low biofilm removal ($<10\%$). Analysis of the post-biocide treatment data revealed the ability of biofilms to recover their activity over time. However, 12 h after biocide application, sloughing events were detected for both single and dual species biofilms, but were more marked for those formed by *P. fluorescens* (removal $>40\%$ of the total biofilm). The overall results suggest that GLUT exerts significant antimicrobial activity against planktonic bacteria and a partial and reversible activity against *B. cereus* and *P. fluorescens* single and dual species biofilms. The biocide had low antifouling effects when analysed immediately after treatment. However, GLUT had significant long-term effects on biofilm removal, inducing significant sloughing events (recovery in terms of mass 72 h after treatment for single biofilms and 42 h later for dual biofilms). In general, dual species biofilms demonstrated higher resistance and resilience to GLUT exposure than either of the single species biofilms. *P. fluorescens* biofilms were more susceptible to the biocide than *B. cereus* biofilms.

Keywords: antimicrobial resistance; biofilm control; glutaraldehyde; dual species biofilms; post-treatment effects; regrowth

Introduction

Biofilms constitute a protected mode of growth that allows microorganisms to survive in hostile environments. Unwanted biofilm formation causes biofouling of heat exchange systems and marine structures, microbial induced corrosion of metal surfaces, deterioration of dental surfaces, contamination of household products including food and pharmaceuticals as well as the infection of short- and long-term biomedical implants and devices (Hall-Stoodley et al. 2004; Choi et al. 2010; Tang et al. 2011; Teodósio et al. 2011). Antimicrobial agents have been the main weapons used to control biofilms, acting either by interfering with microbial metabolism or by facilitating their detachment from the surface (Chen and Stewart 2000; Faÿ et al. 2010; Wong et al. 2010). The target of an antimicrobial strategy is to inactivate and reduce the number of microorganisms and to control the formation of biodeposits on surfaces (Mun et al. 2009; Simões et al. 2010a).

Aldehydes belong to the group of electrophilic active agents which, due to the electron deficiency at the carbonyl carbon atom, can react with nucleophilic cell entities and thus exert antimicrobial activity (Hugo and Russell 1982). Glutaraldehyde (GLUT) (1,5-pentanedial) is a colorless, oily, liquid with a pungent odor. It has been used extensively for disinfection and sterilization purposes in the food, poultry, leather and cosmetic industries and also for various applications in microbiological and biomedical areas (Laopaiboon et al. 2003). It is also used to control microbial growth in cooling water systems and is probably the most widely used biocide in oilfield operations (Lutey 1995; Kjellerup et al. 2009). Several studies have

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demonstrated the effectiveness of GLUT against different microorganisms (*Bacillus subtilis, Candida albicans, Escherichia coli, Pseudomonas aeruginsa, Pseudomonas fluorescens, Serratia marcescens and Staphylococcus aures*) in suspension (Angelillo et al. 1998; Walsh et al. 1999; Simões et al. 2006). The use of GLUT to control biofilms has also been reported (Takeo et al. 1994; Midelet and Carpentier 2004; Simões et al. 2005a, 2009; Jones et al. 2011). Although the ability of GLUT to inactivate biofilms has been demonstrated, it is also reported to increase their attachment and mechanical stability (resistance to removal) (Takeo et al. 1994; Midelet and Carpentier 2004; Simões et al. 2005a). Thus, new data are required in order to clarify the potential of GLUT to control biofilms.

One of the most significant advantages of GLUT is the fact that it is not corrosive to various substrata including stainless steel, soft metals, rubber and glass (Herbert 1995; Laopaiboon et al. 2003). The biocidal effects of GLUT are attributed to the presence of two aldehyde groups that interact with the microbial cell constituents. These can react with ammonia and primary amines and more slowly with secondary amines, contributing to a strong binding to outer cellular layers (Cloete et al. 1998; Walsh et al. 1999) thus hampering essential cellular functions (McDonnell and Russell 1999).

In the food industry, it is important to disinfect the processing equipment, taking into account the constitutive microflora. In dairy environments, the most commonly encountered bacteria belong to the genera *Enterobacter, Lactobacillus, Listeria, Micrococcus, Streptococcus, Bacillus* and *Pseudomonas* (Sharma and Anad 2002; Dogan and Boor 2003; Shakerifard et al. 2009; Simões et al. 2010a). *Pseudomonas* spp. are some of the most important bacteria causing spoilage of conventionally pasteurized liquid milk products and *Bacillus* spp., particularly *B. cereus*, are implicated in food spoilage. In a commercial dairy plant, *B. cereus* accounted for $>12\%$ of the constitutive microflora of biofilms (Sharma and Anand 2002). As *B. cereus* and *P. fluorescens* are ubiquitously present in nature, they are easily spread through food production systems, and contamination with these species is almost inevitable (Andersson et al. 1995; Dogan and Boor 2003). Inhibiting biofilm formation can be achieved through a better knowledge of the mechanisms that contribute to their formation, development and maintenance.

The aim of the present work was to assess the effects of GLUT on the inactivation, removal and regrowth of single and dual species biofilms formed by *B. cereus* and *P. fluorescens*. The characterization of single and dual species biofilms was performed to assess the potential physiological aspects that determine biofilm susceptibility to GLUT. For comparative purposes, antimicrobial tests with planktonic cells were also performed.

Material and methods

Microorganisms and culture conditions

P. fluorescens ATCC 13525^{T} and a *B. cereus* strain isolated from a disinfectant solution and identified by 16S rRNA gene sequencing were used throughout this study (Simões et al. 2008a). Bacterial growth conditions were $27 \pm 2^{\circ}C$ and pH 7, with glucose as the main carbon source. Bacteria were grown in independent 0.5 l glass reactors (Quickfit, MAF4/41, England), with an air flow rate of $0.425\ l\ min^{-1}$ and continuously fed with a sterile concentrated standard growth medium (glucose, $5\ g\ l^{-1}$, peptone, $2.5\ g\ l^{-1}$ and yeast extract, $1.25\ g\ l^{-1}$, prepared in 0.02 M phosphate buffer, pH 7). Continuous feeding, with the aid of a peristaltic pump (Ismatec Reglo, Germany), was at a rate of $10\ ml\ h^{-1}$ (*P. fluorescens*) or $13\ ml\ h^{-1}$ (*B. cereus*) of sterile medium, in order to have the same cell concentration. Under the experimental conditions used, both bacteria had similar growth profiles and rates (Simões et al. 2008a).

Biocide

The aliphatic aldehyde-based biocide GLUT (Reidel-de-Haën, Germany) at 25% (w/v) was used throughout this study. Solutions of biocide were obtained by dilution with sterile distilled water. Planktonic tests were performed with GLUT at 25, 50, 100, 200, 500 and 1000 mg l^{-1}. Biofilm tests were performed with GLUT at 200 mg l^{-1}.

Biofilm reactor

The bacterial cultures referred above were used to continuously inoculate ($10\ ml\ h^{-1}$ – *P. fluorescens*; $13\ ml\ h^{-1}$ – *B. cereus*) two independent 2 l chemostat systems, fed with a minimal nutrient medium (glucose, $0.05\ g\ l^{-1}$, peptone, $0.025\ g\ l^{-1}$ and yeast extract, $0.0125\ g\ l^{-1}$ in phosphate buffer pH 7) at a flow rate of $1\ l\ h^{-1}$, to obtain a bacterial suspension with 6×10^{7} cells ml^{-1}. Biofilms were grown on 24 ASI 316 stainless steel (SS) slides (2.5 cm $\times$ 2.5 cm and 1 mm thick) that were hung within the chemostat system (Figure 1). The chemostat system was operated at $27 \pm 2^{\circ}C$, with aeration (air flow rate – $0.350\ min^{-1}$) and stirring (VWR, VMS-C7 Advanced; 120 rpm; stirrer length – 2.5 cm), according to previous studies (Pereira and Vieira, 2001; Simões et al. 2003a; Ferreira et al. 2010). The biofilms were allowed to grow for

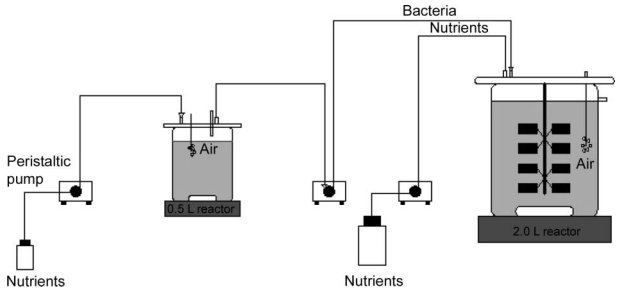

Figure 1. Schematic representation of the experimental system used to form single species biofilms. Dual species biofilms were formed with two independent 0.5 l reactors providing *B. cereus* and *P. fluorescens* to the 2.0 l reactor (chemostat system).

6 days, which was the time needed to reach the steady state in terms of cell density, metabolic activity and mass (Pereira and Vieira 2001). Biofilm formation was performed independently for each bacterium.

For dual species biofilm formation, two independent 0.5 l chemostats were used to grow *B. cereus* and *P. fluorescens*. The 2 l chemostat system was inoculated simultaneously with both strains, and fed with diluted nutrient medium at twice the flow rate (2 l h^{-1}) than the one used for single species biofilm formation, in order to obtain a cell density and residence time similar to that of the single species situation. The experiments were repeated on three different occasions for every scenario tested.

Biofilm sampling for phenotypic characterization

The biofilm (chemically untreated) on the SS slides was removed using a scraper; resuspended in 10 ml of buffer solution (2 mM Na_3PO_4, 2 mM NaH_2PO_4, 9 mM NaCl and 1 mM KCl, pH 7) and then homogenised by vortexing (Heidolph, model Reax top) for 30 s with 100% power input, according to the method described by Simões et al. (2005a). The homogenised biofilm suspensions were characterized in terms of respiratory activity, total and extracellular polymeric substances (EPS) content (proteins and polysaccharides), biomass amount and cell density. The number of spores of *B. cereus* in single and dual species suspensions and in biofilms was assessed by surface plating (300 μl sample) after heat treatment (80°C, 5 min). The plates of solid standard concentrated growth medium (13 g l^{-1} agar) were incubated at 27 $\pm$ 2°C for 72 h.

Respiratory activity

Respiratory activity assays with planktonic cells and biofilm suspensions were performed in a model 53 Yellow Springs Instruments (Ohio, USA) biological oxygen monitor (BOM), as previously described (Simões et al. 2005b). Samples were placed in the temperature-controlled BOM vessel (27 $\pm$ 2°C). Each vessel 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 ($[O_2] = 9.2$ mg l^{-1}, 1 atm). Afterwards, the vessel was closed and the decrease of oxygen concentration monitored over time. The initial linear decrease observed corresponded to the endogenous respiration rate. To determine the oxygen uptake due to substrate oxidation, 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 addition, corresponded to the total respiration rate. The difference between the two respiration rates represented the oxygen uptake rate due to glucose oxidation and was expressed as mg O_2 $g^{-1}_{bacteria}$ min^{-1} or mg O_2 $g^{-1}_{biofilm}$ min^{-1}.

Protein and polysaccharide quantification

Biofilm EPS (proteins and polysaccharides) were extracted using Dowex resin (50X 8, NA^+ form, 20–50 mesh, Fluka-Chemika, Switzerland), according to the methods of Frølund et al. (1996). The separation of the EPS matrix without damaging the cells is an important prerequisite of an extraction procedure. In this study, ATP was used as an indicator of cell lysis (Simões et al. 2005a), and no ATP release was detected during the extraction process. Dowex resin was added to biofilm suspensions. EPS extraction took place at 400 rpm and 4°C, for 4 h. The extracellular components (present in the supernatant) were separated from the cells via centrifugation (3777 *g*, 5 min). The total (before EPS extraction) and extracellular biofilm proteins were determined using the Lowry modified method (Sigma, Portugal), with bovine serum albumin as standard. The procedure is essentially the Lowry method (Lowry et al. 1951) as modified by Peterson (1979). The total and extracellular polysaccharides were quantified through the phenol–sulphuric acid method of Dubois et al. (1956), with glucose as standard.

Biomass quantification

The dry mass of the biofilms was assessed by the determination of the total volatile solids (TVS) of the homogenised biofilm suspensions, according to standard methods (American Public Health Association [APHA], American Water Works Association [AWWA], Water Pollution Control Federation [WPCF] 1995). Following this method, the TVS

assessed at 550 ± 5°C in a furnace (Lenton thermal designs, UK) for 2 h is equivalent to the amount of biological mass. The biofilm mass accumulated was expressed in mg of biofilm per cm^2 of surface area of the slide ($mg_{biofilm}\ cm^{-2}$).

Cell counts

The cells separated through centrifugation from the EPS (present in the supernatant) were stained with 4′,6-diamidino-2-phenylindole – DAPI (Sigma, Portugal, Cat. No. D-9542), a DNA binding stain, as described by Simões et al. (2007a). Cells separated from the extracellular products were diluted to an adequate concentration (in order to have 30–250 cells per microscopic field of view). Thereafter, the bacterial suspensions were filtrated through a Nucleopore® (Whatman) black polycarbonate membrane (pore size 0.22 μm) and then stained with 400 μl of DAPI at 0.5 μg ml^{-1} and left in darkness for 5 min. A Zeiss (AXIOSKOP) microscope fitted with fluorescence illumination was used with a 100 × oil immersion fluorescence objective to visualise the cells. The optical filter combination for optimal viewing of stained preparations consisted of a 359 nm excitation filter in combination with a 461 nm emission filter. Images were obtained using a camera (AxioCam HRC, Carl Zeiss) attached to the microscope and AxioVision software. A program path (Sigma Scan Pro 5) involving object measurement and data output was used to quantify the number of cells. The mean number of cells was determined through counts of a minimum of 20 microscopic fields, per membrane. In dual species biofilms, *B. cereus* and *P. fluorescens* were distinguished by their difference in size (Simões et al. 2007b, 2008a). *B. cereus* biofilm cells were 1.45 ± 0.11 μm compared to *P. fluorescens* cells, which were 0.681 ± 0.09 μm.

Exposure of planktonic cells to glutaraldehyde

A suitable volume of the bacterial cultures was withdrawn from the independent 0.5 l glass reactors, washed with saline (0.85% – w/v NaCl) phosphate buffer (0.02 M) by three consecutive steps of centrifugation (3777 *g*, 5 min) and resuspended in 0.02 M phosphate buffer (pH 7) in order to obtain a suspension with a cell density of approximately 1×10^9 cells ml^{-1}. This bacterial culture was then divided into aliquots of 50 ml which were introduced into several 100 ml sterilised glass flasks. The flasks were incubated on an orbital shaker (120 rpm, 27°C), and exposed to different concentrations of GLUT (0, 25, 50, 100, 200, 500 and 1000 mg l^{-1}) for 30 min. Planktonic cultures of dual species were obtained by the addition of 25 ml of each bacterial culture at a cell density of approximately 1×10^9 cells ml^{-1}.

The antimicrobial action of GLUT against single and dual species *B. cereus* and *P. fluorescens* planktonic cultures was assessed by determining the bacterial respiratory activity through oxygen consumption after neutralizing with sodium bisulphite (Sigma) at a final concentration of0.5% (w/v) (Simões et al. 2006). Bacteria from dual species planktonic cultures were also stained using a Live/Dead *B*acLight bacterial viability kit (Invitrogen) in order to ascertain the proportion of viable cells from each species, as described by Simões et al. (2005b, 2009). At least, three independent experiments were performed for each tested condition.

Exposure of biofilms to glutaraldehyde

The slides covered with biofilms were carefully and aseptically transferred to closed vases containing a 200 mg l^{-1} GLUT solution in phosphate buffer (0.02 M, pH 7) and left under orbital agitation (120 rpm) for 30 min, according to previous studies (Simões et al. 2003a, 2005a, 2008a). The concentration tested was selected based on previous reports (Simões et al. 2003b, 2005a). The biofilms, analyzed immediately after treatment, were subjected to a process of GLUT neutralization with sodium bisulphite. The remaining slides plus biofilm were aseptically transferred, without a GLUT neutralization step (in order to simulate industrial disinfection processes), to the 2 l reactor with fresh medium. Those slides with biofilm were used for post-biocide treatment analysis (for example: regrowth or sloughing events) 3, 7, 12, 24, 48 and 72 h after GLUT exposure. For each conditions tested/sampling time, control (without biocide treatment) experiments were performed.

For analysis, the biofilm was scraped off the metal slides into 10 ml of phosphate buffer pH 7 and vigorously homogenized by vortexing. The homogenized suspensions of biofilm were used to assess the metabolic activity, cell viability (for dual species biofilms) and mass according to the methods above described. After metabolic activity assessment, the EPS from dual species biofilms were extracted as described above and the bacteria were stained with the Live/Dead *B*acLight kit in order to ascertain the proportion of viable cells of each species (Simões et al. 2005b, 2009). At least, three independent experiments were performed for each conditions tested.

Calculations and statistical analysis

The decrease in the respiratory activity (bacterial inactivation) of planktonic cells, as a measure of

GLUT antimicrobial activity, was determined according to the following equation:

$$\text{Bacterial inactivation}\,(\%) = [(\text{RAC} - \text{RAG})/\text{RAC}] \times 100 \quad (1)$$

where RAC is the respiratory activity of the control sample and RAG is the respiratory activity after the treatment with GLUT. Biofilm activity (%) was assessed immediately after biocide exposure, 3, 7, 12, 24, 48 and 72 h after GLUT application.

The results are expressed as the percentage of activity according to the following equation:

$$\text{Biofilm activity}\,(\%) = (A_1/A_0) \times 100 \quad (2)$$

where A_0 is the respiratory activity of the control assay, ie without GLUT treatment, and A_1 is the respiratory activity immediately after the GLUT treatment, 3, 7, 12, 24, 48 and 72 h later.

The percentage of biofilm removal due to GLUT treatment was determined through the following equation:

$$\text{Biofilm removal}\,(\%) = [(W - WB)/W] \times 100 \quad (3)$$

where W is the biofilm mass without biocide application and W_B is the biofilm mass after biocide treatment, 3, 7, 12, 24, 48 and 72 h later.

The data were analysed using the Statistical Package for the Social Sciences, version 17.0 (SPSS, Inc, Chicago, IL). The mean and standard deviation within samples were calculated for all cases. The data were analyzed by the nonparametric Kruskal–Wallis test based on a confidence level $\geq$95%.

Results

Analysis of biofilms formed in the chemostat system showed that *B. cereus* and *P. fluorescens* biofilms oxidized glucose, which was the main carbon source in the growth medium (Table 1). *P. fluorescens* biofilms were found to be three times more metabolically active resulting in higher biomass, cell density, and extracellular proteins and polysaccharides than *B. cereus* biofilms ($P < 0.05$). The biofilm matrix of *P. fluorescens* was highly composed of proteins (31% of total proteins) and polysaccharides (45% of total polysaccharides), while *B. cereus* biofilms had 10% of the total proteins and 8% of the polysaccharides as matrix constituents. Dual species biofilms were about four times more metabolically active than *P. fluorescens* biofilms and had a higher cell density ($P < 0.05$). The mass of dual biofilms was higher than that of *B. cereus* biofilms, but lower than for *P. fluorescens* ($P < 0.05$). The dual species biofilm matrix had a significant proportion of both extracellular proteins (54% of total proteins) and polysaccharides (47% of the total). Moreover, the cell densities (Table 1) of *B. cereus* and *P. fluorescens* in dual species biofilms were similar ($P > 0.05$).

The application of GLUT to planktonic cells (Figure 2) showed that *B. cereus* was more susceptible than *P. fluorescens* at all concentrations tested ($P < 0.05$). The inactivation of dual species planktonic cultures was an average of each single species. However, analysis of cell viability demonstrated that *B. cereus* was more affected by the biocide ($P < 0.05$). GLUT at 200 mg l^{-1} caused inactivation of 95% of *B. cereus* suspensions, 49% of *P. fluorescens* suspensions and 70% of dual species suspensions. In the latter, about 43 ($\pm$7)% of *P. fluorescens* cells and 91 ($\pm$6)% of *B. cereus* cells were non-viable based on Live/Dead *Bac*Light staining. Total inactivation of *B. cereus* was achieved at a concentration of 500 mg l^{-1} while GLUT at 1000 mg l^{-1} caused inactivation of 76% of *P. fluorescens* and 89% for dual species biofilms. Dual species suspensions exposed to GLUT at 1000 mg l^{-1} had 100 ($\pm$0)% non-viable cells of *B. cereus* and 86 ($\pm$3)% non-viable cells of *P. fluorescens*.

The application of 200 mg l^{-1} of GLUT to biofilms promoted a reduction in respiratory activity (Figure 3)

Table 1. Phenotypic characteristics of single and dual species biofilms of *B. cereus* and *P. fluorescens*.

	B. cereus	*P. fluorescens*	Dual
Biofilm mass (mg cm^{-1})	0.106 $\pm$ 0.014	0.220 $\pm$ 0.030	0.145 $\pm$ 0.11
Biofilm activity (mg O_2 $g_{biofilm}^{-1}$ min^{-1})	0.0768 $\pm$ 0.030	0.252 $\pm$ 0.048	0.911 $\pm$ 0.29
Log cell density (cells cm^{-2})	11.4 $\pm$ 0.33	12.5 $\pm$ 0.21	13.1 $\pm$ 0.08[a]
Proteins (mg $g_{biofilm}^{-1}$)			
Total	396 $\pm$ 69	218 $\pm$ 42	413 $\pm$ 25
Matrix	39.3 $\pm$ 1.3	67.6 $\pm$ 15	223 $\pm$ 13
Polysaccharides (mg $g_{biofilm}^{-1}$)			
Total	99.7 $\pm$ 10	69.8 $\pm$ 18	65.3 $\pm$ 15
Matrix	7.48 $\pm$ 1.1	31.6 $\pm$ 2.5	30.5 $\pm$ 5.5

[a]12.8 ($\pm$0.11) of *B. cereus* and 12.8 ($\pm$0.15) of *P. fluorescens*. Mean values $\pm$ SD for at least three replicates are illustrated.

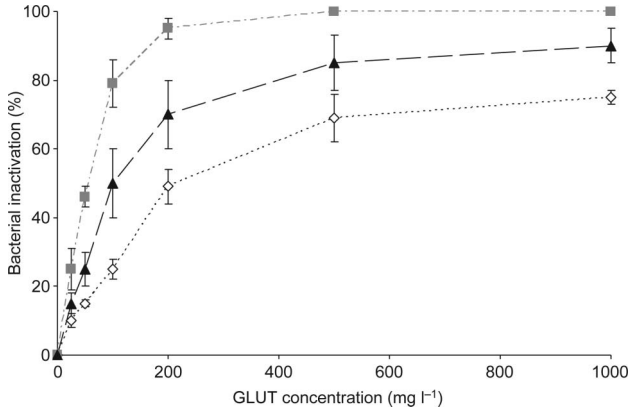

Figure 2. Inactivation of planktonic *P. fluorescens* (◇), *B. cereus* 1 (■) single and dual species (▲) due to exposure to GLUT at 0, 25, 50, 100, 200, 500 and 1000 mg l^{-1} for 30 min. Mean values ± SD for at least three replicates are illustrated.

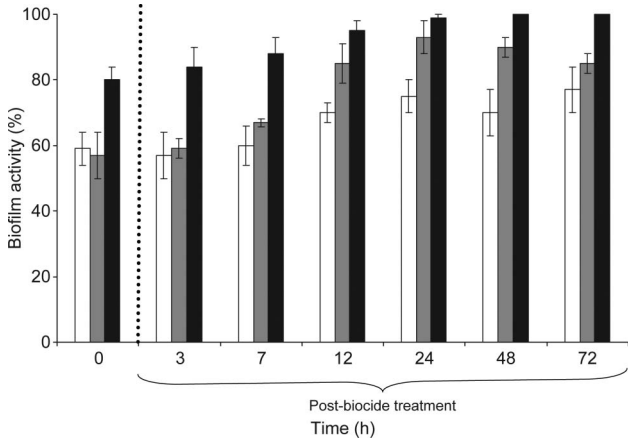

Figure 3. Activity (%) of *P. fluorescens* (□) and *B. cereus* (■) single and dual species (■) biofilms immediately after treatment with GLUT at 200 mg l^{-1} for 30 min and 3, 7, 12, 24, 48 and 72 h later (post-biocide treatment). Mean values ± SD for at least three replicates are illustrated.

>40% for both single species biofilms. The effect of GLUT on the activity of dual biofilms was lower (20% reduction in activity) and the number of non-viable *B. cereus* and *P. fluorescens* in those biofilms was similar ($P > 0.05$). GLUT promoted low biofilm removal (Figure 4) for both single and dual biofilms (removal <10%; $P > 0.05$). The number of spores of *B. cereus* was determined in order to provide information on their density in the total population (spore and vegetative). In both planktonic and biofilm tests with single and dual biofilms with *B. cereus*, spores were never detected at a density of >1 per million vegetative cells.

The post-biocide treatment results (Figures 3 and 4) show that for sampling times of 3 and 7 h, no

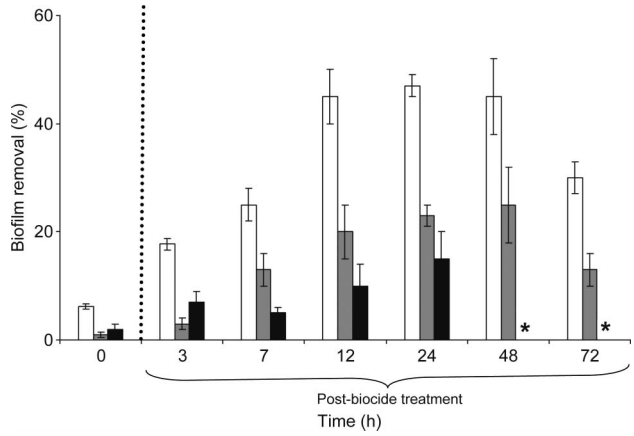

Figure 4. Removal (%) of *P. fluorescens* (□) and *B. cereus* (■) single and dual-species (■) biofilms immediately after treatment with GLUT at 200 mg l^{-1} for 30 min and 3, 7, 12, 24, 48 and 72 h later (post-biocide treatment). * = no biofilm removal compared to the control experiments (without GLUT exposure). Mean values ± SD for at least three replicates are illustrated.

significant regrowth was detected in terms of activity and mass ($P > 0.05$). However, 12 h after GLUT treatment, biofilms recovered their activity (Figure 3). This recovery was more significant for *B. cereus* single species biofilms and for dual species biofilms ($P < 0.05$). The recovery event is particularly relevant for dual species biofilms at 48 h and longer after GLUT exposure. Those biofilms had metabolic activity similar to those that had not been exposed to the biocide ($P > 0.05$). The number of viable *B. cereus* and *P. fluorescens* in dual species biofilms was not statistically different ($P > 0.05$) for several of the post-treatment sampling periods. In terms of the mass results (Figure 4), application of GLUT had a longer term effect on biofilm removal, causing sloughing events. This was found for the several biofilms, being more evident for *P. fluorescens* biofilms and less significant for dual species biofilms ($P < 0.05$). Significant sloughing events (removal > 40% of the total mass) were found for *P. fluorescens* 12, 24 and 48 h after treatment ($P < 0.05$). The application of GLUT had less effect on *B. cereus* single species biofilms (< 25% of biofilm removal) and on dual species biofilms (<10% of biofilm removal) during the post-biocide treatment period. The long-term effects of GLUT on removal of *B. cereus* and *P. fluorescens* single biofilms were attenuated 72 h after biocide application. This effect was verified for dual species biofilms 48 h after exposure to GLUT, being similar (0% biofilm removal) to untreated biofilms. This result demonstrates the higher resistance to removal of dual species biofilms compared to those formed by single species. No significant differences ($P > 0.05$) in

activity and mass were detected with respect to the biofilms used as controls (without biocide treatment).

Discussion

B. cereus and *P. fluorescens* are two major contaminants that cause problems in industrial systems (Dogan and Boor 2003; Shakeifard et al. 2009; Zhao et al. 2009; Lequette et al. 2010). Their undesired effects are accentuated when they form biofilms. Once developed, the biofilms are harder to eradicate and may serve as a chronic source of microbial contamination (Peng et al. 2002; Simões et al. 2008b). GLUT 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 and Russell 1999).

Biofilms were formed in a chemostat system, a bioreactor that offers a simple approach to study and characterize biofilms in a well-controlled, real-time and reproducible manner, and to mimic biofilms formed in industrial processes (Pereira and Vieira 2001; Simões et al. 2003a). Analysis of biofilms formed in the chemostat system showed that dual species biofilms comprised similar numbers of *B. cereus* and *P. fluorescens* cells, although higher cell densities were obtained when compared to single species biofilms. This higher cell density accounts for the higher metabolic activity of dual biofilms. *P. fluorescens* single species biofilms and dual species biofilms contained high amounts of EPS. High EPS production appeared to be associated with high metabolic activity. A previous study demonstrated the correlation between metabolic activity and EPS formation (Simões et al. 2007a). Some of the characteristics studied, namely cell density, metabolic activity and EPS content, are relevant to control of biofilm by conventional disinfectants (Simões et al. 2005a, 2007a).

The association of both species in planktonic culture had no effect on antimicrobial resistance or susceptibility to GLUT compared to the single species situation. *B. cereus* in the planktonic state was more susceptible to the biocide than *P. fluorescens*. This result reflects the expected behaviour of Gram-positive and Gram-negative bacteria when exposed to biocides as the former are more susceptible to biocides than the latter (McDonnell and Russell 1999; Maillard 2002) due to the presence of an outer layer in Gram-negative bacteria (White and McDermott 2001).

The use of a sub-lethal concentration of GLUT (200 mg l^{-1}) allowed the behaviour of biofilms to antimicrobial stress to be assessed. *B. cereus* and *P. fluorescens* biofilms had similar susceptibilities to GLUT; however, this was higher than for dual species biofilms. It was expected that *P. fluorescens* (Gram-negative) biofilms would have higher resistance to the biocide compared to those formed by *B. cereus* due to their cell physiology, higher cell density and the content of the matrix components. Pereira and Vieira (2001) proposed that GLUT applied to biofilms reacts with proteins of the polymeric matrix as well as directly affecting the cells, thereby decreasing their efficacy. There is clear evidence that EPS can decrease biofilm susceptibility to antimicrobial agents (Drenkard and Ausubel 2002; Stewart 2002). EPS is composed mainly of polysaccharides, proteins, nucleic acids and lipids (Flemming and Wingender 2010).

The results showed that planktonic cells were more susceptible to GLUT compared to those in biofilms. This was particularly pronounced for *B. cereus*, when present either as a single or dual species. Several reasons may account for this distinct behaviour of planktonic and biofilm cells, as already reported by several authors. Apart from diffusion limitations that may occur, the penetration of biocide into microbial biofilms is also controlled by the reaction of the antimicrobial chemical with biofilm components, for example, organic matter, inorganic particles and cell debris (Yu and McFeters 1994; Pereira and Vieira 2001; Grobe et al. 2002). Furthermore, the extracellular polymeric matrix is charged and can therefore bind to antimicrobials before they reach the target cell (Stewart 2002; Simões et al. 2008b), while the metabolism of cells within biofilms is different from that of planktonic cells (Lewis 2001; Vilain et al. 2004). In addition to the well-described mechanisms of biofilm resistance to antimicrobials, the associating of two species increased the resistance of the biofilm to inactivation and removal by GLUT. Dual species biofilms were more resistant to GLUT than those of the single species. Therefore, when a biocide is used to control biofilms, the microbial response to the antimicrobial chemical depends not only on the type of microorganisms and on the type of biocide, but also on the complex interactions established between the individual species that may confer increased resistance. In a previous study it was demonstrated that *B. cereus* and *P. fluorescens* had an antagonistic interaction in both planktonic and biofilm systems (Simões et al. 2008a). However, their association enhanced biofilm survival and resilience from GLUT treatment. This interaction may lead to the formation of biofilms with low susceptibility to biocides. Similarly, Leriche and Carpentier (1995) demonstrated that *P. fluorescens* and *Salmonella typhimurium* in biofilm enhanced each other's survival following chlorine treatment. Other apparent protective effects to biocide exposure caused by multispecies association of bacteria in biofilms have

been reported (Whiteley et al. 2002; Leriche et al. 2003; Simões et al. 2010b).

The decreased effects of GLUT in removing biofilms reported in this study concurs with previous reports where it was demonstrated that GLUT reduced removal and increased the attachment strength of biofilms (Midelet and Carpentier 2004; Simões et al. 2005a; Shakeri et al. 2007). This aspect was reinforced in another study with single and dual biofilms of *B. cereus* and *P. fluorescens* (Simões et al. 2009). In a recent study, Jones et al. (2011) studied the viscoelastic properties of bacterial biofilms resulting from chemical and antimicrobial treatments and found that GLUT weakened *P. aeruginosa* biofilms. The differences reported in terms of biofilm cohesion by these studies are apparently related to the microorganism and to the type of system used to form biofilms. Simões et al. (2005a, 2009) used a bioreactor rotating system while Jones et al. (2011) formed biofilms by the colony biofilm method, ie a colony biofilm is grown under static conditions on a semipermeable membrane placed on an agar plate (Merritt et al. 2005).

Understanding how biofilms respond to conditions of external stress is essential for the development of efficient control strategies. The ability of GLUT to inactivate biofilms was greater than its ability to remove biofilms from surfaces. In industrial systems, any biofilm remaining may constitute a source of additional problems including regrowth, development of resistant biofilms, or by providing a haven for other microorganisms, including pathogens (Møretrø and Langsrud 2004; Simões et al. 2008b). This study demonstrates that GLUT affected biofilm proliferation post-biocide treatment. The application of GLUT had a long-term effect on biofilms. This effect was most marked for the biofilm mass data, and was more significant for *P. fluorescens* biofilms then for the dual species biofilms. The post-biocide treatment results for biofilm mass are probably related to the antimicrobial mode of action of GLUT. The high cross-linking effects of GLUT (Walsh et al. 1999) probably influence biofilm development. In other studies (Simões et al. 2005c, 2008b), the application of two biocides, cetyltrimethylammonium bromide and sodium dodecyl sulphate, to biofilms promoted significant regrowth over time both in terms of mass and metabolic activity. In this study, biofilms only recovered in terms of metabolic activity 12 h after treatment, while in terms of mass, recovery was only evident 72 h after treatment for single species biofilms and after 48 h for dual species. Analysis of the post-biocide treatment data reinforces the higher resistance of dual species biofilms to GLUT exposure, compared to single strain *B. cereus* or *P. fluorescens* biofilms. In addition, biofilms of *B. cereus* were more resistant to GLUT treatment than *P. fluorescens* biofilms.

In conclusion, the decreasing rate at which new biocides are being introduced in industrial processes magnifies problems associated with the development of resistance to current biocides. Thus, it has become important to understand all aspects of the interactions between current biocides and biofilms, in order to maximise their efficient use. In this study, planktonic cells of *B. cereus* were more susceptible to GLUT than planktonic cells of *P. fluorescens* and inactivation of planktonic dual species cultures was an average of each single species. GLUT inactivated planktonic cells more efficiently than their biofilm counterparts. *B. cereus* and *P. fluorescens* single and dual species biofilms were different in metabolic activity, cell density and both extracellular protein and polysaccharide content. The role of cell physiology (Gram-negative or Gram-positive) and biofilm characteristics did not alter the susceptibility/resistance when GLUT was applied to biofilms. Single species biofilms of *P. fluorescens* were the most affected by GLUT application, while the species association increased biofilm resistance to GLUT and resilience after treatment. To the authors' knowledge this is the first study demonstrating the long-term effects of GLUT on single and dual species biofilm removal. The study also provides experimental evidence on the role of biofilm diversity in biocide resistance.

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