The effects of selected brominated and chlorinated chemicals on *Pseudomonas fluorescens* planktonic cells and flow-generated biofilms

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Abstract

Sodium hypochlorite (SH) is one of the most widely used disinfectants. However, due to public health and environmental concerns disinfectant alternatives are required. In this study, the control of *Pseudomonas fluorescens* planktonic and biofilm cells was assessed with cetyltrimethylammonium bromide (CTAB), SH, 3-bromopropionyl chloride (BPC) and 3-bromopropionic acid (BPA). Several physicochemical aspects of planktonic cells were assessed, particularly antimicrobial efficiency and effects on bacterial surface. CTAB had the lowest MIC/MBC (20/50 μg/ml). All the chemicals promoted cellular disruption and leakage of essential intracellular constituents. CTAB, BPC and BPA irreversibly changed membrane properties through hydrophobicity modifications and decrease of negative surface charge. The action of selected biocides was also investigated in biofilm control (7-days old) using a flow cell reactor system. Modest killing and removal was achieved (maximum killing of 1 log and 16% removal). In general, both BPC and CTAB had antimicrobial activities similar to SH and therefore demonstrated to be potential alternative biocides to SH to control *P. fluorescens* growth.

Keywords: Antimicrobial activity, Biofilm control, Brominated and chlorinated organics, Mode of action, Sodium hypochlorite

Introduction

The World Health Organization (WHO) mentions food safety as one of the top priorities and challenges of the century (Jahid and Ha, 2012). Nowadays, foodborne diseases are a prime public health concern in developing and developed countries. WHO reported 1.8 million mortality cases of diarrheal diseases worldwide. For instance, in the USA it is estimated that every year, about 48 million people suffer from foodborne diseases (Jahid and Ha, 2012; Scallan *et al.*, 2011; Stein *et al.*, 2007). The Centers for Disease Control and Prevention (CDC) and the US National Health Institute (NIH), documented that biofilms are involved in over 65% of all microbial diseases. In addition, foodborne pathogens can form biofilms, increasing their resistance to commonly used disinfectants (Jahid and Ha, 2012; Potera, 1999). Consequently, the formation of biofilms has severe implications in diverse areas, from industrial processes to health-related fields, with huge economic impacts (Kuehn *et al.*, 2010; Van Houdt and Michiels, 2010).

The sanitizers and biocides used in industry occasionally fail to control microorganisms in biofilms as they are typically 10 - 1000 times more resistant than their planktonic counterparts (Davies, 2003; Simões *et al.*, 2010a). In addition to the resistance mechanisms that exist in planktonic cells (gene transfer from resistant counterparts, efflux pumps, cellular impermeability, enzymes that confer resistance and natural evolutionary mutations) there are six hypothesized mechanisms that help to explain the increased resistance of biofilms to antimicrobial treatments (Simões, 2011). These mechanisms include: the direct interactions between the extracellular polymeric substances (EPS) and antimicrobials by affecting diffusion and availability; altered chemical microenvironment within the biofilm with areas of absence or reduced growth (dormant cells); development of biofilm/attachment specific phenotype and expression of specific antimicrobial resistance genes; possibility of damaged bacteria to undergo apoptosis or programed cell death; and the existence of persister cells (Davies, 2003; Russell *et al.*, 1999; Simões *et al.*, 2011; Tattawasart *et al.*, 1999; Van Houdt and Michiels, 2010).

The efficacy of a disinfectant depends on numerous factors, such as the type of target microorganism and its susceptibility, the adhesion surface, temperature, exposure time, concentration and pH (Araújo *et al.*, 2011). Surfaces that contact with food are normally cleaned and disinfected with agents containing peroxides, chloramines or hypochlorite (Van Houdt and Michiels, 2010). The free chlorine obtained by the use of hypochlorite can be very aggressive to stainless steel, interfering with the surface and thus, facilitating further bacterial adhesion and biofilm formation (Van Houdt and Michiels, 2010; Zottola and Sasahara, 1994). Despite chlorine being the most widely used disinfectant in industry, there is a possibility that during disinfection it may react with natural organic matter or contaminants in surface waters and produce a complex mixture of products which already demonstrated carcinogenic, mutagenic and teratogenic activity in animal studies (European; Ferraris *et al.*, 2005; Gil *et al.*, 2009; Jahid and Ha,

2012; Park *et al.*, 2000). Moreover, SH is included in the indicative list of the Directive on Industrial Emissions as a major pollutant for water emissions (IPCC, 2007/0286 (COD)). Regardless of these disadvantages, chlorine based disinfectants efficacy is also pH dependent and several cases of resistance have been described (Bremer *et al.*, 2002; Gil *et al.*, 2009; Ridgway and Olson, 1982; Sun *et al.*, 2013). Therefore, new biocides, alternative to SH, are required.

Quaternary ammonium compounds (QACs) are cationic compounds with a basic structure (NH_4^+) and a strong antimicrobial potential, are frequently used for disinfection and sanitation in a wide range of fields, like hospitals and food manufacturing (Moore and Payne, 2008; Russell, 2008). Cetyltrimethylammonium bromide (CTAB) is a relatively safe and inexpensive product (Nakata *et al.*, 2011). Nevertheless, it has been shown that intensive use of this compound caused negative impacts, particularly the emergence of resistant bacteria and occasionally multidrug resistance (Bore *et al.*, 2007; Nakata *et al.*, 2011). Comparatively to chlorine, QACs are less affected by organic matter, are not corrosive at low concentrations, are more stable and therefore can be stored for longer periods without compromising their antimicrobial activity (Chaidez *et al.*, 2007).

The formation of biofilms is a microbial community behavior coordinated through cell-to-cell communication mediated by small, diffusible signals, a phenomenon called quorum sensing. Several phenotypes regulated by cell-to-cell communication are implicated in bacterial colonization and virulence (Hentzer and Givskov, 2003). Therefore, eukaryotes have developed defense mechanism based on chemicals, including secondary metabolites that inhibit these phenotypes (Borchardt *et al.*, 2001; Hentzer and Givskov, 2003; Rasmussen and Givskov, 2006). For example, furanones produced by the marine alga *Delisea pulchra* (Givskov *et al.*, 1996), oxidized halogen compounds produced by *Laminaria digitata* or haloperoxidases produced by seaweeds (Borchardt *et al.*, 2001). Haloperoxidases catalyze the production of the hypobromous acid (HOBr) and hypochlorous acid (HOCl) known to have antibiofilm properties (Borchardt *et al.*, 2001). The natural furanones are halogenated at several positions by bromine, iodide or chloride and the concentration of furanones is inversely correlated with the degree of bacterial colonization (Hentzer and Givskov, 2003). Stabilized halogen antimicrobials are extensively used to control biofouling in industry and they have been shown to be more effective in penetrating and disinfecting biofilms than free halogen (Borchardt *et al.*, 2001). Little is known about the usefulness of bromide as a disinfectant for the food industry. Previous studies demonstrated that dibromodimethyl hydantoin was as effective as chlorine against *Streptococcus faecalis* cells (Ortenzio and Stuart, 1964), but was less effective against *Bacillus cereus* spores (Cousins and Allan, 1967). As with free chlorine, there are safety concerns about the production of brominated organic compounds and their impact on human and environmental safety (WHO, 2009).

This study aims to assess the antimicrobial action of selected halogen-based chemicals against planktonic cells and biofilms of *Pseudomonas fluorescens*. This bacterium is commonly found in drinking water (Penna *et al.*, 2002; Wong *et al.*, 2011) and is considered a major contaminant in food industry causing produce spoilage and foodborne illnesses (Mundo *et al.*, 2004; Simões *et al.*, 2009).

Materials and Methods

Antimicrobial agents

Sodium hypochlorite solution (SH) and cetyltrimethylammonium bromide (CTAB) were purchased from Sigma (Portugal); 3-bromopropionic acid (BPA) was purchased from Merck (VWR, Portugal); 3-bromopropionyl chloride (BPC) was purchased from Alfa Aesar (VWR, Portugal). All dilutions were done using sterile distilled water.

Microorganism and culture conditions

The bacterium used in this study was *Pseudomonas fluorescens* ATCC 13525. Bacterial growth was obtained from overnight cultures (16 h) in 250 ml flasks with 100 ml of culture medium (5 g l^{-1} glucose, 2.5 g l^{-1} peptone and 1.25 g l^{-1} yeast extract in 0.025 M phosphate buffer, pH 7) and incubated at 30 ± 3°C, under 150 rpm of agitation (Simões *et al.*, 2007b).

Antibacterial susceptibility testing – minimum inhibitory and bactericidal concentrations

The minimum inhibitory concentration (MIC) of each agent was determined by the microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012). Bacteria at a density of 10^8 colony forming units (CFU) per ml were inoculated into fresh culture medium and a volume of 200 µl per well was inserted in 96-well microliter plates, along with the different concentrations of the chemicals (10% v v^{-1}). The bacterial growth was determined at 640 nm using a microplate reader (Spectramax M2e, Molecular Devices, Inc.). The MIC was determined as the lowest concentration that inhibited microbial growth (Ferreira *et al.*, 2011). A volume of 10 µl per well was plated in plate count agar (PCA, Merck, Germany) and incubated overnight at 30 ± 3°C, after a neutralization step to quench the chemicals antimicrobial activity by dilution to sub-inhibitory concentrations (Johnston *et al.*, 2002). The minimum bactericidal concentration (MBC) was considered the lowest concentration of the antimicrobial chemical were no growth was detected on the solid medium (Ferreira *et al.*, 2011). Three independent experiments were performed for each chemical.

Physicochemical characterization of the bacterial surfaces

The physicochemical properties of *P. fluorescens* cell surface were assessed by the sessile drop contact angle measurement on bacteria lawns, performed as described by Busscher *et al.* (1984) (Busscher *et al.*, 1984). Contact angles were determined using an OCA 15 Plus (DATAPHYSICS) video-based optical measuring instrument, allowing image acquisition and data analysis. The measurements (≥ 15 per liquid and chemical) were performed according to Simões *et al.* (2007) (Simões *et al.*, 2007a), after bacteria incubation (one hour) with the chemical at the MBC. The liquid surface tension components reference values were obtained from the literature (Janczuk *et al.*, 1993). Hydrophobicity was assessed after contact angle measurement, following the van Oss method (van Oss *et al.*, 1987; van Oss *et al.*, 1988; van Oss *et al.*, 1989). The liquids with known surface tensions used to determine the surface tension components were α-bromonaphtalene (apolar), formamide (polar), and water (polar).

Bacterial surface charge - Zeta potential

An overnight culture of *P. fluorescens* was used to prepare a bacterial suspension with an optical density of 0.2 (640 nm). The zeta potential of bacterial suspensions was determined in sterile water using a Nano Zetasizer (Malvern Instruments) according the procedure described by Ferreira *et al.*, (2011) (Ferreira *et al.*, 2011). This determination was performed before and after bacterial exposure to the chemical at the MBC, for one hour. Three independent experiments were performed for each chemical.

Potassium (K^+) leakage

The quantification of K^+ in bacteria solutions, before and after one hour exposure to the MBC of each biocide, was determined by flame emission and atomic absorption spectroscopy according to Ferreira *et al.*, (2011) (Ferreira *et al.*, 2011). Samples were filtrated (pore size 0.2 µm) and analyzed in a GBC AAS 932 plus device using GBC Avante 1.33 software. Three independent experiments were performed for each chemical.

Colony biofilm formation and antimicrobial penetration tests

Colony biofilms were developed according to the methods of Anderl *et al.* (2000) and Singh *et al.* (2010) (Anderl *et al.*, 2000; Singh *et al.*, 2010). Briefly, biofilms were grown in Mueller-Hinton agar plates (24 hours, 30 ± 3 °C). For this purpose, a volume of 40 µl of cell suspension was placed on a 13 mm polycarbonate membrane, pore size 0.2 µm (Merk, Millipore) originating colony biofilms. Afterwards, the membranes with biofilms were transferred to a fresh plate seeded with *Staphylococcus aureus* CECT 976 at a McFarland Standard of 0.5 (Anderl *et al.*, 2000; Singh *et al.*, 2010). Another polycarbonate membrane was placed on top of the biofilm. The antimicrobial discs (Biochemica) were impregnated with 15 µl of each chemical from a stock solution (a mass of 350 µg of CTAB, BPA, BPC or SH was provided in the 15 µl) and placed over the polycarbonate membrane, avoiding direct contact with the biofilm. The positive controls were obtained in the absence of biofilm. The plates were incubated for 24 hours at 30 ±

3 °C before the assessment of the inhibition halos. The positive controls were taken as 100% penetration and used to assess the mass transfer limitations when biofilms were present. Three independent experiments were performed for each chemical.

2.8 Biofilm formation in a flow cell system

The flow cell system used in this study was similar to the one used on the study of Teodósio *el al.* (2011) (Teodósio *et al.*, 2011). The system consists of a recirculating bioreactor, two vertical polymethyl methacrylate (PMMA) flow cells operating in parallel, one 0.5 l bioreactor, peristaltic and centrifuge pumps. A pure culture of *P. fluorescens* was used to inoculate the 0.5 l bioreactor that operated continuously, dripping into the 5 l bioreactor at a flow rate of 10 ml h^{-1}. This larger bioreactor was fed with 100 times diluted culture medium (0.05 g l^{-1} glucose, 0.025 g l^{-1} peptone and 0.0125 g l^{-1} yeast extract in 0.025 M phosphate buffer, pH 7) at a flow rate of 0.833 l h^{-1}. The dilution rate of 0.28 h^{-1} ensured that biofilm formation was stimulated over planktonic growth (Simões *et al.*, 2003). The flow cells were designed so that stainless steel coupons AISI 316 (1 cm × 2 cm) could be applied into structures that were inserted into the flow cells, in order to facilitate biofilm sampling. The bacterial suspension from the 5 l bioreactor was allowed to recirculate in the flow cells in order to form biofilms on the stainless steel coupons at a flow rate of 3.4 l h^{-1} (Reynolds number of 4000).

Biofilm control using different chemicals

The flow-generated biofilms of *P. fluorescens* were submitted to a one hour disinfection process of the selected chemicals (CTAB, BPC and SH) at MBC. The flow cell was carefully emptied and the disinfection of the biofilms was made by recirculation of the chemical at MBC, at 3.4 l h^{-1} flow rate for one hour. After that period, the initial conditions were restored in the flow cell system. Control experiments with 0.025 M phosphate buffer (pH 7) were also performed. Four coupons, two from each flow cell were removed at different time periods: before chemical exposure, immediately after the antimicrobial exposure and 2, 12 and 24 hours post-antimicrobial treatment. After biofilm chemical exposure, a neutralization step by dilution to sub-inhibitory concentrations was performed according to Johnston *et al.* (2002) and as applied previously by Simões et al. (2009) (Johnston *et al.*, 2002; Simões *et al.*, 2009). The analysis 2, 12 and 24 hours were performed to assess the biofilm regrowth potential post-antimicrobial treatment.

Biofilm analysis

P. fluorescens biofilms grown on stainless steel were characterized in terms of mass and colony forming units (CFU). The stainless steel coupons were removed from the flow cell and the biofilms that covered the coupons surface were completely scraped using a sterile scalpel and resuspended in 10 ml of phosphate buffer. The biofilm suspensions were vortexed (IKA TTS2) for 30 s with maximum power to disrupt cell aggregates. The biofilm mass was determined according to standard methods (American Public Health Association [APHA], American Water Works Association [AWWA], Water Pollution Control Federation [WPCF]) (APHA *et al.*, 1989). The CFU, expressed as log (CFU cm^{-2}), were assessed in Plate Count Agar (Merck, Portugal), according to Simões *et al.* (2005) (Simões *et al.*, 2005b).

Statistical analysis

Data were analyzed applying the parametric paired t-test using the statistical program SPSS version 22.0 (Statistical Package for the Social Sciences). The average and standard deviation (SD) within samples were calculated for all cases. Statistical calculations were based on confidence level ≥ 95% ($P < 0.05$) which was considered statistically significant.

Results

The MIC and MBC values obtained for the tested chemicals (Fig. 1) against *P. fluorescens* are presented in Table 1. In general, the MBC was always slightly higher than the MIC, with the exception of SH (MIC

= MBC). The results showed that CTAB was the most efficient biocide with MBC values 10 to 20 times lower compared to the other chemicals.

The parameters of the surface tension for *P. fluorescens* before and after the treatment with each chemical were determined to ascertain their effects on the bacterial surface properties (Table 2). *P. fluorescens* is naturally hydrophilic ($\Delta G_{sws} > 0$ mJ m^{-2}). However, this property was less pronounced ($P < 0.05$) when the cells were in contact with BPA and CTAB (Table 2). Regarding the apolar (γ_s^{LW}) parameter, only CTAB caused changes, *i.e*. a decrease compared to the untreated cells. The polar (γ_s^{AB}) parameter of the bacterium was enhanced with the application of CTAB and decreased due to SH exposure ($P < 0.05$). The treatment with SH decreased significantly the cell surface ability to accept (γ_s^+) or donate (γ_s^-) electrons ($P < 0.05$), while BPA and CTAB increased the value of the electron acceptor component of *P. fluorescens* surface ($P < 0.05$).

P. fluorescens presented a negative surface charge of -13.53 mV with a conductivity of 0.05 mS cm^{-1} (Table 3). The exposure to CTAB, BPC or BPA made *P. fluorescens* surface charge less negative and increased its conductivity ($P < 0.05$), with the exception of CTAB that had no effects on the cell surface conductivity ($P > 0.05$). Conversely, SH enhanced cell surface conductivity ($P < 0.05$), without interfering with the charge ($P > 0.05$).

The intracellular K^+ leakage was assessed in order to ascertain the effects of the chemicals in the cell integrity. The K^+ concentration in solution before and after exposure to several chemicals is presented in Table 4. All tested chemicals promoted an alteration in the cytoplasmic membrane permeability, causing K^+ leakage ($P < 0.05$). Additionally, the expression of outer membrane proteins (OMPs) was assessed by SDS-PAGE, before and after biocide exposure for one hour. No relevant differences in the expression of major OMPs were found after exposure to the selected biocides (online resource 1).

The percentage of retardation gives an estimation of the efficacy of chemical products to penetrate the biofilm (Table 5). In this case, BPC and SH were the most efficient since no retardation was observed**.** Contrarily, CTAB was completely ($P < 0.05$) and BPA partially (15%) retarded by the biofilm.

BPC was selected over BPA for biofilm assays (Figure 2) due to its higher antimicrobial activity (lower MIC and MBC), significant effects on the surface properties of *P. fluorescens* and the ability to penetrate the biofilm without retardation. Therefore, BPC, CTAB and SH were tested against 7-days old flow-generated biofilms formed under a flow rate of 3.4 l h^{-1}. The effectiveness of the chemicals was assessed in terms of CFU (Fig. 2a) and biomass (Fig. 2b).

The results obtained for the number of biofilm CFU revealed a reduction after one hour exposure to the MBC of CTAB and SH (Fig. 2). This effect was more pronounced for SH with 1 log reduction ($P > 0.05$). For CTAB 0.4 CFU log reduction was achieved and for BPC CFU log reduction was almost negligible. In order to ascertain the role of the biocides tested on biofilm regrowth, CFU were determined 2, 12 and 24 h after chemical exposure. Two hours after this treatment the number of CFU increased for the three chemicals tested, reaching values similar to those before the treatment ($P > 0.05$, Fig. 2a). The number of biofilm CFU, remained constant overtime for all the conditions tested, except for the 24 h BPC-treated biofilms. For this case the number of biofilm cells increased significantly ($P < 0.05$, Fig. 2a) when compared to the control values (untreated biofilms).

In terms of biofilm mass, the three chemicals promoted similar biofilm biomass removal (average of 16%, Fig. 2b) immediately after treatment. These values remained unchanged 2 h after the treatment for all the conditions tested. When analyzing the period of 12 h after the treatment, no significant biomass changes were found for the biofilms treated with CTAB and BPC, compared to the biofilms immediately after exposure. The SH treated biofilms recovered significantly ($P < 0.05$). However, 24 h after treatment the biomass values obtained for these biofilms were as low as those found immediately after the treatment ($P > 0.05$). After this regrowth period the biomass of CTAB treated biofilms was also similar to the values immediately after treatment, while significant regrowth was found for the BPC treated biofilms ($P < 0.05$).

Discussion

In the last decades, due to the high food demands to match population needs there have been increasing concerns on outbreaks of foodborne pathogens (Bhat, 2008). Thus, the food industry requires major investments in produce decontamination and facilities sanitation in order to prevent and control microbial contaminants (Todd *et al.*, 2010). However, resistance to disinfectants and sanitizers has been increasing, urging the necessity for the development of new formulations (Bore *et al.*, 2007; Bremer *et al.*, 2002; Fraise, 2002; Nakata *et al.*, 2011; Simões *et al.*, 2011). In this work, the antimicrobial activity and capacity for biofilm control of chlorine (as sodium hypochlorite) and three brominated and chlorinated chemicals (CTAB, BPC and BPA) against *P. fluorescens* was studied. In addition, several aspects of their interaction with bacteria were assessed. The three brominated chemicals were selected based on their structure, particularly the presence of bromine (Petruso *et al.*, 1994; Ward *et al.*, 1981), chlorine (Gottardi and Nagl, 2005; Ward *et al.*, 1981) and carboxyl groups (Kabara *et al.*, 1972) that are known for their antimicrobial properties. *P. fluorescens* was chosen as it is a well-studied Gram-negative bacterium, ubiquitous in natural, medical and industrial environments that can cause serious problems in its planktonic and biofilm states (Ferreira *et al.*, 2011; Simões *et al.*, 2010b; Wong *et al.*, 2011). In addition, this bacterium is known to form biofilms resistant to disinfectants (Lindsay *et al.*, 2002; Selvaraju *et al.*, 2005; Simões *et al.*, 2008). Several bacterium physiological characteristics were assessed such as MIC, MBC, hydrophobicity, K^+ leakage, and surface charge.

The MIC and MBC values of CTAB against *P. fluorescens* were 20 and 50 μg ml^{-1}, respectively (Table 1). The previous use of CTAB, in other studies, revealed MICs values of 4 μg ml^{-1} against the Gram negative *Salmonella typhimurium* and *Pseudomonas aeruginosa* or 18 μg ml^{-1} for the yeast *Candida albicans* (Lindstedt *et al.*, 1990). The MIC and MBC of SH was 500 μg ml^{-1}. A range of SH concentrations from 50 to 5000 μg ml^{-1} has been determined by several authors for a variety of conditions and bacteria (Maillard, 2011; Perez *et al.*, 2005; Rasmussen *et al.*, 2013; Rutala and Weber, 1997). Differences between the values obtained from the previous studies and the present can be explained by the different methods and bacteria used to determine these parameters. Moreover, it is known that no strain can mimic the behavior of a species (Fux *et al.*, 2005).

To our knowledge, this is the first study reporting the antimicrobial properties of BPA and BPC. It was found that both chemicals possess antimicrobial activity, with a MIC and MBC against *P. fluorescens* of 650 and 700 μg ml^{-1} for BPC and 850 and 900 μg ml^{-1} for BPA. The lower MIC and MBC of BPC can be possibly due to the presence of chlorine, known for its antimicrobial properties (Gottardi and Nagl, 2005; Winter *et al.*, 2008).

In order to understand the action of the selected chemicals on *P. fluorescens*, diverse aspects of the interaction between the chemicals and the bacterial cells were assessed particularly the surface physicochemical properties, charge (Table 3) and K^+ leakage.

CTAB is a compound that binds to the negative cell surface of bacteria due to electrostatic attraction by chemisorption (Azeredo *et al.*, 2003; Neu, 1996; Rodrigues *et al.*, 2013). Azeredo *et al.* (2003) proposed that when a concentration of CTAB higher than the MBC is used, hydrophobicity and surface charge properties can be enhanced and bacteria become hydrophilic and positively charged (Azeredo *et al.*, 2003). Upon interaction with the surface, CTAB promotes cell membrane disorganization (McDonnell and Russell, 1999) or even disruption (Simões *et al.*, 2005a). In this work, this effect of cell disruption was verified by the amount of K^+ leakage as this assay is usually the primary indicator of gross and irreversible cytoplasmic membrane damage (Borges *et al.*, 2013; Carson *et al.*, 2002).

Conversely to QACs, *P. fluorescens* exposure to SH decreased the bacterial polar (γ_s^{AB}) character and, consequently the capacity to accept (γ_s^+) or donate (γ_s^-) electrons. According to Gottardi and Nagl (2005), the action of active chlorine (hypochlorous acid - HOCl) in bacteria can be divided in two effects, non-lethal and lethal (Gottardi and Nagl, 2005). The first implies reversible chlorination of the bacterial surface and the second is based on penetration into the bacteria combined with irreversible alterations. HOCl can also promote aggregation of essential proteins (Winter *et al.*, 2008). The present study corroborates the findings of Winter *et al.* (2008) on the membrane destabilization effects (Winter *et al.*, 2008). In fact, K^+ leakage is a good indicator of cytoplasmic membrane damage (Codling *et al.*, 2003; Lambert and Hammond, 1973). The interaction of active chlorine did not interfere with the cell surface

charge possibly suggesting covalent links between the biocide and the bacterial membrane (Gottardi and Nagl, 2005). Moreover, SH dissociation in ions can originate salt formation that can help to explain the observed increase in the cell surface conductivity.

BPA decreased bacterial hydrophilic characteristics and improved electron acceptance (γ_s^+). The membrane interaction with the chemical may promote a destabilization and consequently K^+ leakage into solution. In addition, previous reports suggest the reaction between BPA and sulfhydryl groups of proteins (Bradbury and Smyth, 1973; Chadha and Plapp, 1984). The effects of BPC were less noticeable on the hydrophobicity values, but this chemical was the one that most affected the charge of the bacterial cells. This difference in the antimicrobial activity and mode of action of BPA and BPC can be due to the presence/absence of OH^- or Cl^-. It seems that the presence of Cl^- may improve the antimicrobial activity of the molecules, causing a significant decrease in the cell surface charge and the leakage of intracellular K^+. These antimicrobial effects were lower when BPA was applied (higher MIC and MBC values). Even if significant effects on the cell surface properties and charge were promoted by the biocides, no changes were induced on OMP expression (online resource 1). This may indicate that these compounds may not potentiate antimicrobial resistance, regarding OMP expression. This hypothesis is based on the knowledge of OMP importance in bacterial resistance to biocides and antibiotics (Cha and Cooksey, 1991; Delcour, 2009; Masuda *et al.*, 1995; Walsh *et al.*, 2003; Winder *et al.*, 2000).

The penetration of CTAB through the *P. fluorescens* biofilms was totally retarded. In fact, bacteria grown as a biofilm exhibit less susceptibility to antimicrobials due to spatial heterogeneity in its structure which consequently originates nutrient depletion within the biofilm, reduced access of the chemicals to the bacteria inside the biofilm, biocide interaction with extracellular polymeric substances (EPS) and the existence of degradative enzymes and neutralizing chemicals (Brown and Gilbert, 1993; Fazlara and Ekhtelat, 2012; Simões *et al.*, 2010b). A previous study supports the accessibility hypothesis as it was verified that low mass biofilms were more susceptible to biocides than high mass biofilms (Simões *et al.*, 2006).

Biofilms are organized cell aggregates in a self-produced extracellular matrix and can be formed on living or inert surfaces, which can create serious problems in several fields if the disinfection protocols fail (Simões *et al.*, 2010b). As biofilms are a major problem in industry, it is important to understand the effects of biocides in their control. In this study, BPC was selected over BPA to control the flow-generated biofilms. This selection was based on the similar chemical structures and the most promising antimicrobial effects of BPC.

P. fluorescens biofilms were exposed to the chemicals at their MBC values, for one hour. Only modest reductions in the log CFU cm^{-2} were obtained. This fact reinforces the higher resistance of biofilm cells compared to their planktonic counterparts (Davies, 2003; Simões *et al.*, 2010a). In terms of biofilm mass removal the use of BPC, CTAB or SH promoted low removal of the total biofilm mass ($\leq$ 16%). It can be hypothesized that CTAB acts by eroding the biofilm, which may consist on eradication of the superficial bacteria and disruption of the EPS matrix, in which they are embedded. This assumption is based on the fact that CTAB is the most efficient of the chemicals tested despite lacking the ability to cross the biofilm layers. BPC and SH pass through the biofilm but are not effective. This may be the consequence of an intrinsic or acquired resistance of biofilms (Gibson *et al.*, 1999; Winter *et al.*, 2008). Furthermore, the mechanism of action of these chemicals did not interfere with quorum sensing (online resource 1). A screening protocol developed by McLean *et al.* (2004) was used for the detection of quorum signal inhibition (targeting acylated homoserine lactones dependent signaling) (McLean *et al.*, 2004). No effects were found other than antimicrobial action (online resource 1).

In order to ascertain the ability of the biofilms to regrow after the chemical treatment the conditions existing prior to exposure were reestablished, therefore mimicking a disinfection practice in industry. Biomass regrowth was observed mostly for SH treated biofilms (12 hours) and BPC treated biofilms (24 hours). In terms of CFU, regrowth was verified for all biofilms upon 2 hours after treatment, being the CFU counts for the other sampling periods within the values of untreated biofilms (control). This result can be explained by the possible presence of starved or injured cells or potentially viable but not culturable cells (Banning *et al.*, 2002; Simões *et al.*, 2011). Also, Pereira *et al.* (2002) found that 7-days old *P. fluorescens* biofilms formed in a flow cell system are in the stationary or stabilization phase (Pereira *et al.*, 2002). This means that the loss of biomass due to physical stresses is balanced by the

growth of new cells at the edge of the biofilm (Capdeville *et al.*, 1992). The results obtained show low/modest effects of the selected biocides on biofilm removal and killing and the rapid regrowth to the stabilization phase. These results may indicate that the selected biocides, at the concentrations tested, had no significant effects on the dynamic behavior of the biofilms. Moreover, this preservative recovery showed by the post-antimicrobial effect evaluation could lead to populations of resistant bacteria, which may be recalcitrant to disinfection (Stewart, 2003).

It is apparently clear that the promising results obtained with the tests on planktonic cells did not provide relevant insights on their application in biofilm control, even if the same strain and antimicrobial concentrations were used in both tests. Moreover, in this study, it seems that the chemical nature of the biocide was not relevant on biofilm control and the brominated and chlorinated products had no clear advantage on biofilm control over SH. In fact, the results obtained with SH and the brominated-products are comparable. At their MBC, the selected chemicals promoted similar effects on the biofilms. This proposes that the CTAB and BPC can be alternatives to SH for biofilm control. Further studies will be performed on the effects of the combination between CTAB and BPC with SH. In fact, the combination of SH with biocides already proved to potentiate their effects as antimicrobials and biofilm control agents (DeQueiroz and Day, 2007; Dutta and Kundabala, 2014; Luddin and Ahmed, 2013). Pioneer studies (Kristoffersen, 1958; Shere *et al.*, 1962), demonstrated synergistic antimicrobial relationship when bromine was added to chlorine solutions. However, as with free chlorine, there are safety concerns about the production of brominated organic compounds and their impact on human and environmental safety, even if bromide ion has a low degree of toxicity (WHO, 2009).

Conclusions

SH, BPA, BPC and CTAB promoted cellular disruption and leakage of essential intracellular constituents of planktonic *P. fluorescens*. CTAB had the lowest MIC and MBC values. All the chemicals promoted cellular disruption and leakage of essential intracellular constituents. CTAB, BPC and BPA irreversibly changed membrane properties through hydrophobicity modifications and decrease of negative surface charge. *P. fluorescens* biofilms formed in a flow cell system under turbulent flowing conditions were highly resistant to control by the selected chemicals. CTAB, BPC and SH at their MBC promoted similar biofilm removal and killing. However, total biofilm control was not achieved and the biofilms were able to recover from the antimicrobial exposure. The overall results also propose that CTAB, BPA and BPC had antimicrobial effects comparable to SH and therefore they can be used as alternative biocides.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JM and PA performed the experiments for this study, participated in acquisition, analysis and interpretation of data, drafting and revising critically the manuscript. IM and ML participated in the analysis and interpretation of data, and contributed in the writing and editing of the manuscript. FM and LM participated in drafting and revising critically the manuscript. MS did the experimental design of the study and participated in drafting the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 – Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the selected biocides against *P. fluorescens*

	MIC (µg mL^{-1})	MBC (µg mL^{-1})
BPC	650	700
BPA	850	900
CTAB	20	50
SH	500	500

Table 2 – Surface tension parameters, hydrophobicity (ΔG_{sws}), apolar (γ_s^{LW}) and polar (γ_s^{AB}), of untreated *P. fluorescens* (control) and of cells exposed to the selected biocides. The mean ± SD is presented

	Surface tension parameters (mJ m^{-2})				ΔG_{sws}(mJ m^{-2})
	γ_s^{LW}	γ_s^{AB}	γ_s^{+}	γ_s^{-}	
Control	22.77 ± 4.43	30.27 ± 4.79	4.14 ± 1.25	56.96 ± 4.71	30.71 ± 6.34
BPC	19.36 ± 0.43	31.45 ± 2.92	4.40 ± 1.07	57.20 ± 3.96	29.79 ± 5.54
BPA	20.32 ± 0.80	34.13 ± 3.61	6.04 ± 1.25	53.09 ± 3.51	23.39 ± 4.90
CTAB	11.97 ± 1.35	46.99 ± 7.06	10.36 ± 2.98	54.00 ± 0.81	14.02 ± 5.04
SH	29.49 ± 4.68	13.27 ± 1.83	0.89 ± 0.33	51.36 ± 5.93	33.53 ± 8.73

Table 3 – Zeta potential and conductivity of *P. fluorescens* before (control) and after 1 hour exposure to the selected biocides. The mean ± SD is presented

	Zeta Potential (mV)	Conductivity (mS cm^{-1})
Control	-13.53 ± 2.32	0.05 ± 0.02
BPC	-2.88 ± 0.66	2.25 ± 0.06
BPA	-4.96 ± 0.95	0.46 ± 0.09
CTAB	-8.14 ± 0.42	0.05 ± 0.01
SH	-13.00 ± 1.41	31.10 ± 0.14

Table 4 – Concentration of K^+ in solution before and after 1 hour exposure to the selected biocides. The mean ± SD is presented

	Concentration of K^+ in solution (µg ml^{-1})
Control	1.21 ± 0.08
BPC	1.99 ± 0.21
BPA	1.96 ± 0.25
CTAB	2.09 ± 0.28
SH	2.07 ± 0.26

Table 5 –Retardation caused by *P. fluorescens* biofilms, for the selected biocides. Data are presented as mean ± SD of the percentage of diameter measurements for halo readings as compared with controls (without chemical exposure)

	Retardation (%)
BPC	0.0 ± 0.0
BPA	15.7 ± 4.4
CTAB	100 ± 0.0
SH	1.90 ± 3.2

Figures

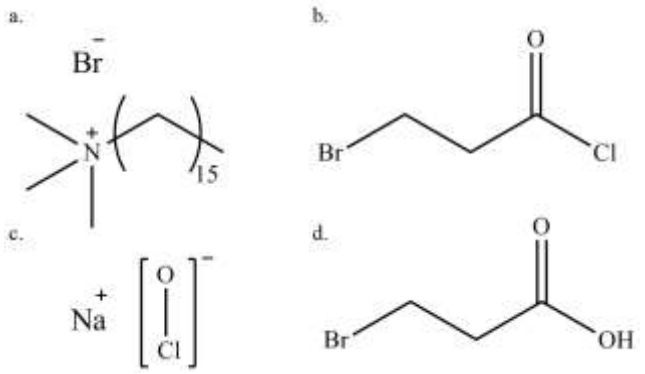

Fig. 1 – Chemical structures of the chemicals used: a. cetyltrimethylammonium bromide (CTAB); b. 3-bromopropionyl chloride (BPC); c. sodium hypochlorite (SH) and d. 3-bromopropionic acid (BPA).

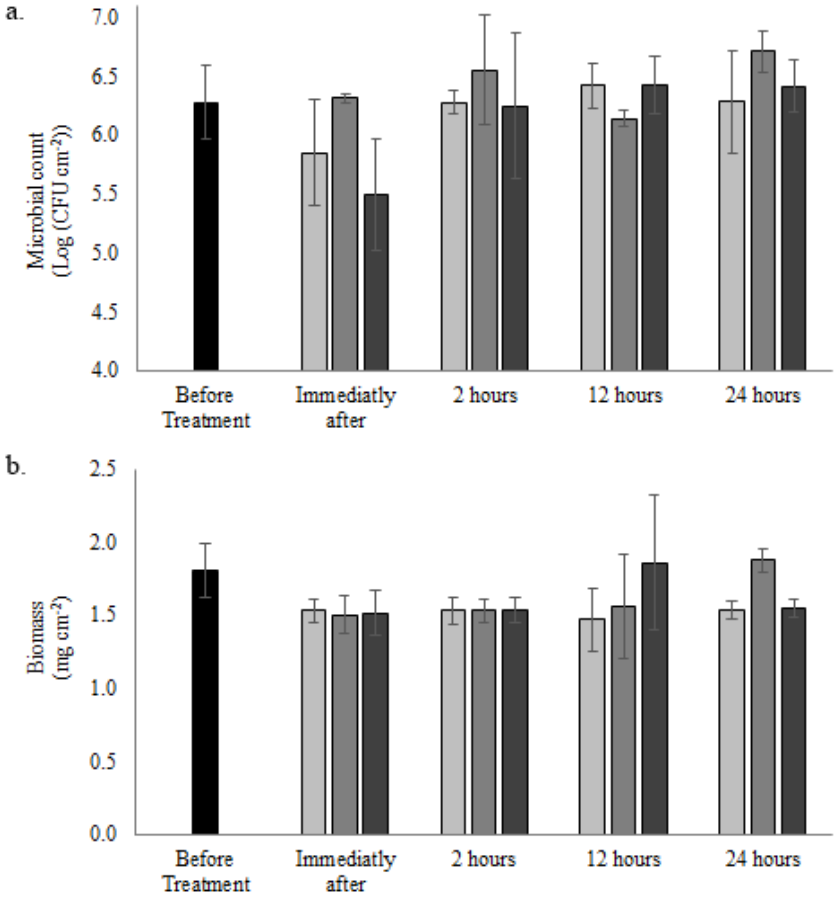

Fig. 2 – *P. fluorescens* biofilm cell density (log CFU cm^{-2}) (a) and mass (b) before and after treatment with CTAB (□), BPC (■) and SH (■). Samples were collected before treatment (■), immediately after treatment and 2, 12 and 24 hours following the biocide treatment. Values are mean ± SD.