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The effects of sodium hypochlorite against selected drinking water-isolated bacteria in planktonic and sessile states

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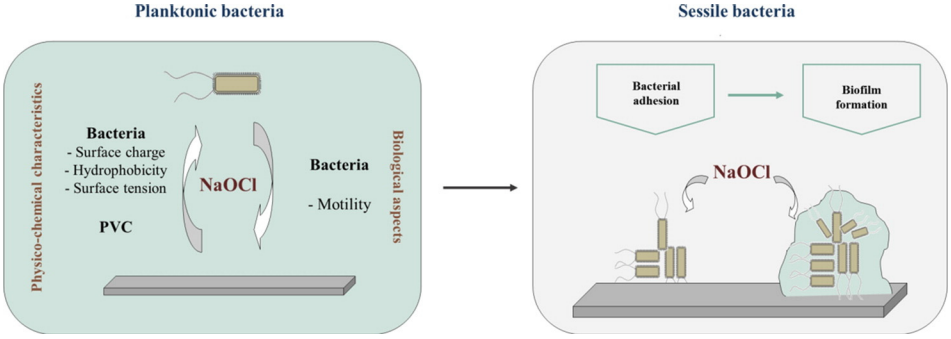
HIGHLIGHTS

- *A. calcoaceticus* was more susceptible to the action of NaOCl than *S. maltophilia.*
- Biofilm removal and killing are distinct phenomena.
- High biofilm killing rates were achieved with NaOCl at residual concentrations.
- High concentrations of NaOCl cause reduced biofilm removal.
- Complementary methods to NaOCl are required for drinking water disinfection.

GRAPHICAL ABSTRACT



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ABSTRACT

Chlorine is the most commonly used agent for general disinfection, particularly for microbial growth control in drinking water distribution systems. The goals of this study were to understand the effects of chlorine, as sodium hypochlorite (NaOCl), on bacterial membrane physicochemical properties (surface charge, surface tension and hydrophobicity) and on motility of two emerging pathogens isolated from drinking water, *Acinetobacter calcoaceticus* and *Stenotrophomonas maltophilia*. The effects of NaOCl on the control of single and dual-species monolayer adhered bacteria (2 h incubation) and biofilms (24 h incubation) was also assessed. NaOCl caused significant changes on the surface hydrophobicity and motility of *A. calcoaceticus*, but not of *S. maltophilia.* Planktonic and sessile *S. maltophilia* were significantly more resistant to NaOCl than *A. calcoaceticus*. Monolayer adhered co-cultures of *A. calcoaceticus-S. maltophilia* were more resilient than the single species. Oppositely, dual species biofilms were more susceptible to NaOCl than their single species counterparts. In general, biofilm removal and killing demonstrated to be distinct phenomena: total bacterial viability reduction was achieved even if NaOCl at the higher concentrations had a reduced removal efficacy, allowing biofilm reseed. In conclusion, understanding the antimicrobial susceptibility of microorganisms to NaOCl can contribute to the design of effective biofilm control strategies targeting key microorganisms, such as *S. maltophilia*, and guarantying safe and high-quality drinking water. Moreover, the results reinforce that biofilms should be regarded as chronic contaminants of drinking water distribution systems and accurate methods are needed to quantify their presence as well as strategies complementary/alternative to NaOCl are required to effectively control the microbiological quality of drinking water.

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1. Introduction

The development of a biofilm occurs in a sequential process that includes transport of microorganisms to a surface, initial (reversible and irreversible) adhesion, cell-cell communication, microcolony formation, production of extracellular polymeric substances (EPS) and biofilm maturation, with a balanced growth and dispersal (Doyle, 2000). Drinking water distribution systems (DWDS) are known to harbor biofilms, even in the continuous presence of a disinfectant. These biofilms can be a reservoir of pathogens (Li et al., 2016; Sun et al., 2014) and are a source of planktonic bacteria, which will remain present when the water is delivered through a consumer's tap (Simões and Simões, 2013). The presence of biofilms in DWDS can cause chronic drinking water (DW) contamination, reduce the esthetic quality and microbiological safety of potable water and increase the corrosion rate of pipes (Niquette et al., 2000; Percival and Walker, 1999; Simões et al., 2006; Tsai, 2005). Therefore, biofilm control is important for technical, esthetic, regulatory, and public health reasons.

Adhesion and consequent biofilm formation gives several advantages to bacteria, particularly the protection against antimicrobial agents and adverse environmental conditions. Therefore, microbial control becomes more difficult when microorganisms are embedded in biofilms (Garrett et al., 2008). Diverse strategies to control microbial adhesion and biofilm formation in DWDS were reported (Simões and Simões, 2013). The most commonly used consists on chemical disinfection, particularly with chlorine (Simões and Simões, 2013). Currently, according to the World Health Organization (WHO) the residual concentration of free chlorine leaving the treatment plant should be <1.0 mg/L and closer to 0.5 mg/L (WHO, 2011). However, the effects of chlorine on biofilm control and its mode of antimicrobial action is still poorly understood.

The use of a material that did not encourage bacterial adhesion is also of utmost importance to control the presence of biofilms in DWDS. In recent years plastic pipes have been applied in DWDS. These plastic materials have advantageous characteristics when compared to metallic and asbestos-cement pipes, particularly its low cost, the simple installation, its external and internal resistance to corrosion and its smooth surface which facilitate the removal of deposits (Rożej et al., 2015; Safe Drinking Water Committee, Board on Toxicology and Environment Health Hazards, National Research Council, 1982). Moreover, several studies demonstrated that metallic and cement-based pipes favored bacterial attachment compared to plastic pipes (Kerr et al., 1998; Niquette et al., 2000). Iron pipes can support 10–45 times more biomass than those plastic, while cement-based materials can harbor 2.6 times more biomass than polyvinyl chloride (PVC) (Niquette et al., 2000). Rożej et al. (2015) compared biofilm formation on different plastic pipes (PVC, silane cross-linked polyethylene-PEX, and high-density polyethylene-HDPE) and demonstrated that the number of bacteria was significantly lower in biofilms formed on PVC than those formed on PEX or HDPE pipes. In this study PVC was selected as substratum for bacterial adhesion due to its broad use in DWDS. For instance, it was reported in 1982 that 80% of the plastic pipes used in DWDS at USA were made of PVC (Safe Drinking Water Committee, Board on Toxicology and Environment Health Hazards, National Research Council, 1982). Two bacteria isolated from DW, *Acinetobacter calcoaceticus* and *Stenotrophomonas maltophilia*, were used due to their increased relevance as opportunistic microorganisms (Gales et al., 2001). These bacteria were already associated with nosocomial infections related to the hospital water supply (Cervia et al., 2008; Talon et al., 1994; Weber et al., 1999). *A. calcoaceticus* is usually present on skin and can act as an opportunistic pathogen with highly variable degree of virulence, depending on a pre-existing break in the normal body defenses (Pal and Kale, 1981). *Acinetobacter* spp. are commonly found in water and are associated with nosocomial infections (Narciso-da-Rocha et al., 2013). In 1996, this species was confirmed as the responsible agent for the death of infants in maternity hospitals in Brazil (Penna et al., 2001). *S. maltophilia* is commonly associated with respiratory infections in humans. The treatment of *S. maltophilia* infections is problematic since it is considered an emerging multidrug resistant organism (Brooke, 2012; Denton and Kerr, 1998). Vincenti et al. (2014) isolated *S. maltophilia* from hospital tap water and 56.3–100% of the isolates were highly resistant to the 14 tested antibiotics (5 beta-lactams, 2 carbapenems, 3 aminoglycosides, ciprofloxacin, colistin, fosfomycin and trimethoprim-sulfamethoxazole). These authors considered *S. maltophilia* potentially more dangerous than other waterborne pathogens commonly described in literature.

The aim of this study was to evaluate the effects of chlorine, as sodium hypochlorite (NaOCl), on the control of bacterial adhesion and biofilms on PVC and to understand how this biocide can act on bacterial surface physicochemical properties, charge and motility.

2. Material and methods

2.1. Bacteria and culture conditions

A. calcoaceticus and *S. maltophilia* were isolated from a DWDS in Braga (Portugal) and identified by 16S rRNA gene sequencing as described previously by Simões et al. (2007a).

Bacterial cells were grown overnight using a synthetic nutrient medium (glucose 5 g/L, peptone 2.5 g/L, yeast extract 1.25 g/L and 0.2 M phosphate buffer at pH 7) (Simões et al., 2009) at room temperature (23 ± 3 °C) and under agitation (120 rpm) in an orbital incubator (New Brunswick Scientific, I26, USA). All growth medium compounds were purchased from Merck (VWR, Portugal). Cells were harvested by centrifugation (Eppendorf centrifuge 5810R) at 3777 *g*, 12 min, washed twice with 0.2 M phosphate buffered saline (PBS) at pH 7 and resuspended in the same buffer or appropriated medium in order to achieve the bacterial concentration required for further experiments.

2.2. Minimum inhibitory concentration of NaOCl

The minimum inhibitory concentration (MIC) of sodium hypochlorite - NaOCl (Sigma, Sintra, Portugal) for *A. calcoaceticus* and *S. maltophilia* was determined by the broth microdilution method according to McBain et al. (2004). A pre-culture grown as described previously was used as inoculum (≈10^8 CFU/mL). MIC was determined in polystyrene 96-well microtiter plates (Orange Scientific, Belgium). NaOCl was prepared at diverse concentrations (0.1–5000 mg/L) from a 10% (v/v) stock solution. A volume of 20 μL of each NaOCl solution was added to each well containing 180 μL of cell culture in synthetic nutrient medium. The optical density was measured in a microtiter plate reader at 610 nm (SpectraMax M2E, Molecular Devices) before and after 24 h incubation at room temperature (23 ± 3 °C) and 120 rpm. The MIC corresponds to the lowest concentration of NaOCl at which no growth was found. Each condition was tested in triplicate with three independent experiments.

2.3. Substratum for bacterial adhesion and biofilm formation

PVC coupons (1 × 1 cm) were used as adhesion substratum. PVC was selected as a representative pipe material from DW networks (Simões et al., 2007a). In order to prepare PVC for further analysis, this material was immersed in a solution of commercial detergent (Sonasol Pril, Henkel Ibérica S. A.) in ultrapure water for 30 min. The coupons were rinsed in ultrapure water and subsequently immersed in ethanol at 96% (v/v) for 1 min in order to remove any remaining detergent (Simões et al., 2007a). Afterwards they were rinsed three times with ultrapure water and dried at 65 °C for 3 h, before being used for contact angle measurements, zeta potential assessment, adhesion and biofilm assays.

2.4. The effect of NaOCl on bacterial surface charge - zeta potential measurement

The zeta potential of PVC and bacteria, before and after contact with NaOCl (0.5 mg/L and MIC for *S. maltophilia* and *A. calcoaceticus*)

for 30 min, was determined using a Nano Zetasizer (Malvern Instruments, UK), according to Ferreira et al. (2011). The bacterial cultures were prepared as described previously. Afterwards, they were washed twice and resuspended in sterile distillated water to a final concentration of 10^8 CFU/mL. Bacterial suspensions without NaOCl were used as control. Each condition was tested in triplicate with three independent experiments.

2.5. The effect of NaOCl on physicochemical properties of bacterial membranes - contact angle measurements

Bacterial lawns were prepared for contact angle measurements as described by Busscher et al. (1984). The bacterial suspensions were exposed to NaOCl at 0.5 mg/L and at MIC of each bacterium for 30 min before contact angle measurements. The surface tension of the bacterial surfaces (with and without NaOCl exposure) and substratum were determined using the sessile drop contact angle method according to Simões et al. (2010a). The measurements were carried out at room temperature (23 ± 3 °C) using three different liquids: water, α-bromonaphthalene and formamide (Sigma). Determination of contact angles was performed automatically using a model OCA 15 Plus (DataPhysics, Germany) video based optical contact angle measure instrument, allowing image acquisition and data analysis. Contact angle measurements, at least 20 determinations, of each liquid were performed for the selected bacteria and for PVC. The reference liquids surface tension components were obtained from literature (Janczuk et al., 1993).

2.6. Determination of hydrophobicity and surface tension parameters

The surface hydrophobicity and the surface tension parameters of bacteria and PVC surfaces were assessed after contact angle measurements according to the procedure described by Simões et al. (2007a). Bacteria and PVC hydrophobicities were assessed using the approach of van Oss et al. (1987, 1988, 1989). In this approach, the degree of hydrophobicity of a given material (i) is expressed as the free energy of interaction between two entities of that material when immersed in water (w) - ΔG_{iwi}. If the interaction between the two entities is stronger than the interaction of each entity with water, $\Delta G_{iwi}^{TOT} < 0$ mJ/m^2, it means that the material is hydrophobic. If $\Delta G_{iwi}^{TOT} > 0$ mJ/m^2 the material is considered hydrophilic. ΔG_{iwi} can be calculated through the surface tension components of interacting entities according to Eq. 1:

$$\Delta G_{iwi}^{TOT} = -2\left(\sqrt{\gamma_i^{Lw}}-\sqrt{\gamma_w^{Lw}}\right)^2 + 4\left(\sqrt{\gamma_i^+\gamma_w^-}+\sqrt{\gamma_i^-\gamma_w^+}-\sqrt{\gamma_i^+\gamma_i^-}-\sqrt{\gamma_w^+\gamma_w^-}\right) \quad (1)$$

where,

γ^{Lw} is the Lifshitz-van der Waals component of the surface free energy;

γ^+ represents the electron acceptor parameter;

γ^- represents the electron donor parameter;

γ^{AB} is the Lewis acid-base component, with $\gamma^{AB} = 2 \times \sqrt{\gamma^+\gamma^-}$.

The surface tension components of bacteria and PVC were obtained by measuring the contact angle of the referred three pure liquids with well-known surface tension components and by simultaneous resolution of three equations of the form of Eq. 2.

$$(1+cos\theta)\gamma_i^{TOT} = 2\left(\sqrt{\gamma_s^{Lw}\gamma_i^{Lw}}+\sqrt{\gamma_s^+\gamma_i^-}+\sqrt{\gamma_s^-\gamma_i^+}\right) \quad (2)$$

where θ is the contact angle and $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$.

2.7. The effect of NaOCl on bacterial motility

Overnight cultures grown in Luria-Bertani broth (LBB) (Merck, Portugal) were used to characterize bacterial motility. Fifteen microliters of these cultures were applied in the center of plates containing 1% tryptone (Merck), 0.25% NaCl (Merck) and 0.3%, 0.7% or 1.5% (w/v) agar (Merck) for swimming/colony spreading, swarming and twitching motilities, respectively (Butler et al., 2010; Stickland et al., 2010). The use of different concentrations of agar enables the characterization of different types of bacterial motility, once the medium porosity is directly related to the concentration of agar. NaOCl were incorporated into the medium obtaining the final concentrations of 0.5 mg/L and MIC, in order to determine if these concentrations affect bacterial motility. Then, the plates (with and without NaOCl) were incubated at 23 ± 3 °C and the diameter (mm) of motility halos was accurately measured at 24, 48 and 72 h. Each condition was tested in triplicate with two independent cultures.

2.8. Monolayer bacterial adhesion on PVC surfaces

Adhesion tests were performed with *A. calcoaceticus*, *S. maltophilia*, and their co-culture, using PVC as adhesion surface. Also, the effect of NaOCl on the control of adhered cells was studied. PVC coupons with 1 cm × 1 cm, prepared as described previously, were inserted in 48-wells microtiter plates (Nunc, Denmark) and 1.2 mL of each cell suspension (10^8 CFU/mL in PBS) was added to each well. In order to test adhesion of *A. calcoaceticus-S. maltophilia* co-culture, 600 μL of each bacterial suspension. Monolayer adhesion on PVC coupons was allowed to occur for 2 h at 23 ± 3 °C in an orbital incubator at 120 rpm according to Simões et al. (2007a). Negative controls were obtained by placing the PVC coupons in PBS without bacterial cells.

2.9. Biofilm formation on PVC surfaces

Biofilms were formed with *A. calcoaceticus*, *S. maltophilia* and their co-culture using PVC as substratum. Cell suspensions, PVC coupons and microtiter plate inoculation were prepared as described previously for cell adhesion assay. Biofilm formation was allowed to occur for 24 h at 23 ± 3 °C, 120 rpm. Negative controls were obtained by placing the PVC coupons in diluted medium without bacterial cells.

2.10. Effects of NaOCl on sessile bacteria culturability and removal

PVC coupons with adhered cells and biofilms were exposed to NaOCl at 0.5 mg/L and at MIC for 30 min. Co-culture adhered cells and dual-species biofilms were exposed to the higher MIC. After biocide exposure, coupons were washed with sodium thiosulphate at 0.5% (w/v) to quench the activity of NaOCl and then with PBS to remove non-adherent and weakly adherent bacteria according to Simões et al. (2010b). Afterwards, the number of cells attached to PVC was assessed by 4′,6-diamidino-2-phenylindole (DAPI, Sigma) staining. Coupons with monolayer adhered bacteria were directly stained with 400 μL of DAPI at 0.5 μg/mL for 10 min in the dark according to Simões et al. (2007b). Biofilms were scraped from the surfaces and resuspended in PBS. These biofilm suspensions, after vigorous vortexing for 1 min (optimized time to ensure the disruption of biofilm aggregates without compromising bacterial viability, allowing further counts of individual cells), were filtered (0.5 mL) using a 0.22 μm black polycarbonate membrane (Nucleopore, UK). The membrane with bacteria was observed by epifluorescence microscopy after staining with 400 μL of DAPI at 0.5 μg/mL. Then, cells were visualized under an epifluorescence microscope Leica DM LB2 (Leica Microsystems, Germany) equipped with a filter sensitive to DAPI fluorescence (359 nm excitation filter in combination with a 461 nm emission filter). Twenty micrographs per coupon were obtained using a microscope camera (AxioCam HRC, Carl Zeiss) in order to calculate the number of cells per cm^2. For dual species biofilms

Table 1
Zeta potential values to *A. calcoaceticus* and *S. maltophilia* after exposure to different concentrations of NaOCl for 30 min. The MIC are 125 mg/L for *A. calcoaceticus* and 175 mg/L for *S. maltophilia*.

[NaOCl] (mg/L)	Zeta potential (mV)	
	A. calcoaceticus	*S. maltophilia*
0	−28.0 ± 0.6	−30.7 ± 1.4
0.5	−24.0 ± 1.1	−20.5 ± 0.7
MIC	−22.0 ± 0.4	−14.6 ± 1.4

the number of cells per cm^2 was counted for each bacterium. This was possible because these bacteria (in the same growth phase) had clear distinct shapes and sizes when observed under the microscope.

For culturability assessment, bacteria (monolayer adhered bacteria and biofilms) were scraped from the surfaces, resuspended in PBS and spread on Plate Count Agar (PCA, MercK, VWR) for colony forming units (CFU) enumeration. In order to break up biofilm clusters, bacterial suspensions were vortexed for 1 min. The numbers of CFU were assessed after 24 h incubation at 23 ± 3 °C. Final results are presented as log CFU per cm^2 of adhesion surface. All the experiments were performed in triplicate with three repeats.

2.11. Statistical analysis

The data were analyzed using the statistical program SPSS version 20.0 (Statistical Package for the Social Sciences). Results were analyzed using One-Way ANOVA test. Statistical calculations were based on a confidence level ≥ 95% ($P < 0.05$ was considered statistically significant).

3. Results

3.1. Minimum inhibitory concentration of NaOCl

Chlorine is commonly used as disinfectant in DWDS. The residual concentrations kept along the DWDS are essential to guarantee DW is microbiologically safe. Chlorine as NaOCl was used as an antimicrobial agent during this study. The MIC for each bacterium was assessed in order to understand the antimicrobial activity of NaOCl. Complete growth inhibition occurred with NaOCl at 125 mg/L and 175 mg/L for *A. calcoaceticus* and *S. maltophilia*, respectively. These concentrations in addition to a residual concentration of 0.5 mg/L were used to assess the effects of NaOCl on bacterial surface physicochemical properties (surface charge, tension and hydrophobicity), on bacterial motility, and on the control of monolayer adhered bacteria and biofilms.

3.2. Physicochemical characteristics and charge of bacterial surfaces and PVC

The surface charge and hydrophobicity of bacteria and the substratum are important aspects that can influence the adhesion process, *i.e.* the first interaction between the bacterium and the substratum. Table 1 shows the surface charge of *A. calcoaceticus* and *S. maltophilia* in the absence/presence of NaOCl. Both bacteria have the surface negatively charged, while PVC surface has positive charge (11.3 ± 0.6 mV). The exposure to increasing concentrations of NaOCl decreased the negativity of the bacterial surface charge. This effect was more significant for *S. maltophilia* than for *A. calcoaceticus* ($P < 0.05$). Concerning to surface tension and hydrophobicity, *A. calcoaceticus* and *S. maltophilia* had both hydrophilic surface properties ($\Delta G_{iwi}^{TOT} > 0$ mJ/m^2) with similar values ($P > 0.05$) of surface tension parameters (Table 2). The PVC surface had also hydrophilic characteristics ($\Delta G_{iwi}^{TOT} = 55.9$ mJ/m^2) and did not have ability to accept electrons ($^{+} = 0$ mJ/m^2). The exposure to NaOCl changed the hydrophobic characteristics of the bacterial surfaces. The ΔG_{iwi}^{TOT} of *A. calcoaceticus* increased significantly ($P < 0.05$) after exposure to increasing concentrations of NaOCl from 17.7 ± 2.8 mJ/m^2 (without treatment) to 29.4 ± 4.0 mJ/m^2 (0.5 mg/L NaOCl) and 72.7 ± 9.0 mJ/m^2 (at 125 mg/L). Also, the electron donor parameter ($^{-}$) of *A. calcoaceticus* increased with the use of NaOCl at MIC (from 44.4 ± 2.1 to 77.1 ± 7.8 mJ/m^2). The electron acceptor parameter ($^{+}$) suffered an opposite effect, decreasing with the residual biocide concentration (from 5.2 ± 0.8 mJ/m^2 to 1.6 ± 0.4 mJ/m^2), and losing all its ability to accept electrons (0 mJ/m^2) with NaOCl at 125 mg/L. The exposure to NaOCl at 0.5 and 175 mg/L did not cause significant changes in *S. maltophilia* hydrophobicity and electron donor values ($P > 0.05$). The electron acceptor parameter decreased moderately ($P < 0.05$) after exposure to both NaOCl concentrations.

3.3. The effect NaOCl on motility of A. calcoaceticus and S. maltophilia

A. calcoaceticus and *S. maltophilia* showed the three studied forms of motility: swimming, swarming and twitching motility (Table 3). The increasing concentrations of NaOCl caused changes in *A. calcoaceticus* swimming and twitching motilities. Swimming motility (after 72 h) of *A. calcoaceticus* decreased ($P < 0.05$) when exposed to NaOCl at MIC. Also, the twitching motility was reduced due to NaOCl ($P < 0.05$), when analyzed at 72 h. *A. calcoaceticus* swarming motility was not significantly affected by NaOCl ($P > 0.05$). *S. maltophilia* motilities were not affected by NaOCl, regardless the concentration used.

3.4. The effect of NaOCl on culturability and removal of monolayer adhered bacteria

The results on the effects of NaOCl on the removal of monolayer adhered bacteria are presented in Fig. 1. *A. calcoaceticus* and *S. maltophilia* single species and co-culture adhered on PVC at similar extents ($P > 0.05$). In the co-culture *S. maltophilia* was predominant compared to the numbers of *A. calcoaceticus* ($P < 0.05$). The use of NaOCl at a residual concentration did not cause bacterial removal for any of the conditions tested ($P > 0.05$). However, the MIC caused modest removal ($P < 0.05$). *S. maltophilia* (maximum removal of 0.69 log) and *A. calcoaceticus* (maximum removal of 0.89 log) monolayer adhered cells demonstrated close susceptibilities to NaOCl ($P > 0.05$). The bacteria in co-culture were even less susceptible to NaOCl. In fact, no significant reduction of *A. calcoaceticus* and *S. maltophilia* in co-culture was observed.

Table 2
Surface tension parameters (γ^{LW}- Lifshitz- van der Waals component; γ_i^+- electron acceptor parameter; γ_i^- electron donor parameter; γ^{AB} - Lewis acid-base component) and hydrophobicity (ΔG_{iwi}^{TOT}) of *A. calcoaceticus* and *S. maltophilia* after exposure to different concentrations of NaOCl for 30 min.

Bacteria	[NaOCl] (mg/l)	Surface tension parameters (mJ/m^2)				Hydrophobicity (mJ/m^2)
		γ^{LW}	γ_i^+	γ_i^-	γ^{AB}	ΔG_{iwi}^{TOT}
A. calcoaceticus	0	25.6 ± 0.9	5.2 ± 0.8	44.4 ± 2.1	30.1 ± 1.8	17.7 ± 2.8
	0.5	35.9 ± 1.3	1.6 ± 0.4	52.2 ± 3.1	18.4 ± 1.7	29.4 ± 4.0
	125	33.3 ± 1.5	0. 0 ± 0.0	77.1 ± 7.8	0.0 ± 0.0	72.7 ± 9.0
S. maltophilia	0	29.8 ± 5.2	5.4 ± 1.3	53.3 ± 2.3	29.2 ± 6.2	27.1 ± 5.4
	0.5	36.4 ± 1.2	1.1 ± 0.8	47.7 ± 7.3	15.5 ± 3.4	29.2 ± 6.4
	175	31.0 ± 1.1	1.8 ± 0.4	49.5 ± 3.6	19.7 ± 2.7	27.1 ± 5.4

Table 3
Motility of *A. calcoaceticus* and *S. maltophilia* in the presence of NaOCl at different concentrations. Values of motility expressed as halos diameter (mm) at different incubation times (24, 48 and 72 h).

A. calcoaceticus									
[NaOCl] (mg/L)	0			0.5			125		
Time (h)	24	48	72	24	48	72	24	48	72
Swimming (mm)	0.0 ± 0.0	10 ± 3	52.0 ± 3.0	1.0 ± 0.0	18.0 ± 6.9	45.0 ± 3.5	2.7 ± 0.5	4.5 ± 0.7	10.0 ± 4.0
Swarming (mm)	2.0 ± 0.0	2.3 ± 0.6	3.3 ± 0.6	1.5 ± 0.7	2.3 ± 1.2	3.0 ± 1.0	3.3 ± 1.5	3.0 ± 1.0	3.2 ± 1.2
Twitching (mm)	3.0 ± 0.0	6.5 ± 0.7	6.7 ± 0.6	2.0 ± 0.0	2.7 ± 0.6	3.0 ± 1.0	2.0 ± 0.0	3.0 ± 1.0	3.3 ± 0.6
S. maltophilia									
[NaOCl] (mg/L)	0			0.5			175		
Time (h)	24	48	72	24	48	72	24	48	72
Swimming (mm)	3.0 ± 1.0	10.3 ± 2.3	20.6 ± 1.5	2.3 ± 0.6	8.0 ± 1.0	18.6 ± 1.5	4.6 ± 1.5	12.3 ± 0.6	20.3 ± 2.9
Swarming (mm)	2.7 ± 1.5	5.6 ± 1.2	7.0 ± 2.0	3.3 ± 1.5	5.3 ± 1.5	7.3 ± 2.4	2.0 ± 1.4	3.5 ± 0.7	5.0 ± 1.4
Twitching (mm)	2.3 ± 1.2	4.0 ± 0.0	4.3 ± 1.0	2.3 ± 0.6	3.3 ± 0.6	4.3 ± 0.6	3.3 ± 0.6	4.3 ± 0.6	5.0 ± 1.0

The results on the effects of NaOCl on CFU numbers of monolayer adhered bacteria are presented in Fig. 2. The number of *A. calcoaceticus* CFU was lower than for *S. maltophilia* in both single species and co-cultures adhered on PVC ($P < 0.05$). The use of NaOCl at 0.5 mg/L and at MIC caused total CFU reduction for all the conditions tested.

3.5. The effect of NaOCl on biofilm culturability and removal

The 24 h-old biofilms were exposed to NaOCl at 0.5 mg/L and at MIC for 30 min (Fig. 3). Both bacteria formed single and dual-species biofilms with similar cell densities ($P > 0.05$). Their exposure to 0.5 mg/L NaOCl did not cause significant removal of single and dual-species biofilms from PVC ($P > 0.05$). NaOCl at MIC caused modest biofilm removal ($P < 0.05$). *S. maltophilia* biofilms were the least susceptible with removal of 0.54 log while *A. calcoaceticus* biofilm removal was 0.88 log. In dual species biofilms, 1.05 log and 0.92 log removal was observed for *A. calcoaceticus* and *S. maltophilia*, respectively.

The results on the effects of NaOCl on CFU numbers of biofilms are presented in Fig. 4. *S. maltophilia* had higher number of CFU on PVC than *A. calcoaceticus* in both single and dual-species biofilms ($P < 0.05$). NaOCl at 0.5 mg/L caused significant CFU reduction of *A. calcoaceticus* biofilms: 3.9 log CFU/cm^2 for single species and 1.8 log CFU/cm^2 for dual-species biofilms. *S. maltophilia* CFU reduction on single species was around 2.1 log CFU/cm^2 and 1.4 log CFU/cm^2 for dual-species biofilms. Therefore, CFU reduction of dual-species biofilms was lower than for single species biofilms. Treatment with NaOCl at MIC caused complete CFU reduction of both bacteria in single and dual-species biofilms ($P > 0.05$).

4. Discussion

The presence of biofilms in DWDS is a public health concern due to their potential to harbor pathogenic microorganisms (Li et al., 2016). Therefore, it is important to understand biofilm formation ability of DW microorganisms and their behavior to control strategies. In this study, two opportunistic microorganisms isolated from a DWDS, *A. calcoaceticus* and *S. maltophilia*, were characterized in terms of surface charge, tension parameters and hydrophobicity, before and after exposure to NaOCl. Similarly to other studies, microtiter plates were used as model system for adhesion and biofilm formation under laboratorial conditions (Johansen et al., 2009; Pompilio et al., 2008; Simões et al., 2010a, 2010b). In fact, microtiter plates are commonly used as the standard bioreactor system for adhesion and biofilm formation of bacteria isolated from many different environments, providing reliable comparative data (Andersson et al., 2008; Djordjevic et al., 2002; Simões et al., 2010a; Suraju et al., 2015).

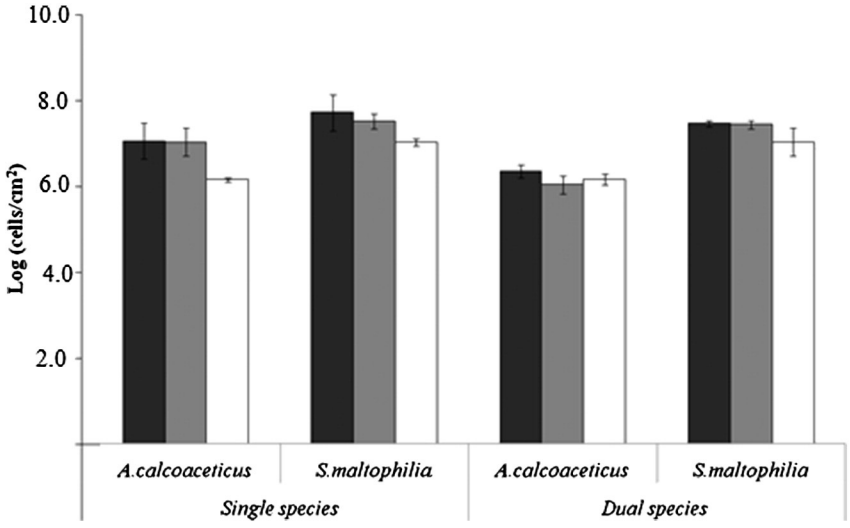

Fig. 1. Effects of NaOCl at different concentrations on the removal of *A. calcoaceticus*, *S. maltophilia* and their co-culture adhered on PVC coupons. ■ - 0 mg/L; ■ - 0.5 mg/L; □ - MIC. The MIC are 125 mg/L for *A. calcoaceticus* and 175 mg/L for *S. maltophilia*. Co-cultures were exposed to 175 mg/L.

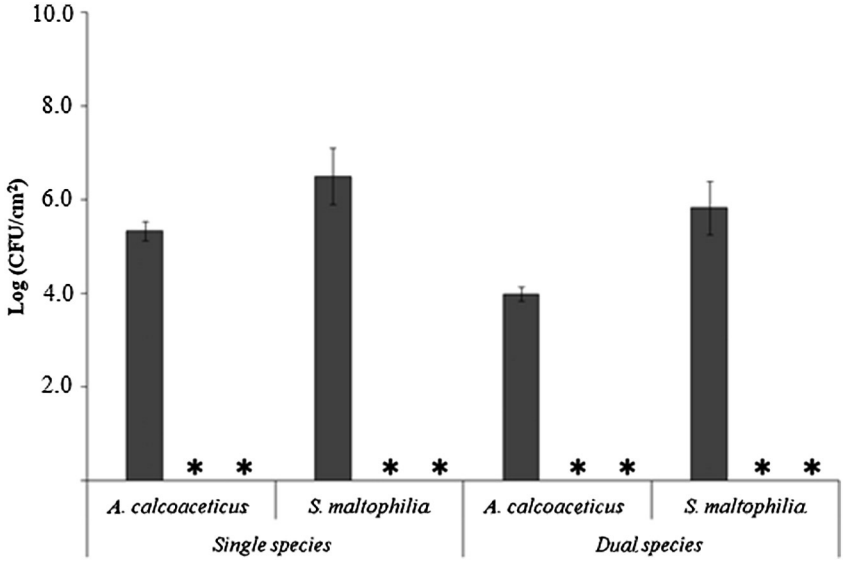

Fig. 2. CFU of monolayer adhered *A. calcoaceticus* and *S. maltophilia* adhered on PVC coupons after exposure to NaOCl at: ■ - 0 mg/L; ■ - 0.5 mg/L; □ - MIC; * - no CFU detected. The detection limit is 2.75 log CFU/cm^2. The MIC are 125 ppm for *A. calcoaceticus* and 175 ppm for *S. maltophilia*. Co-cultures were exposed to 175 mg/L.

According to the MIC values *A. calcoaceticus* was more susceptible to NaOCl than *S. maltophilia*. MIC in the range 100–500 ppm were also described by DeQueiroz (2004) for diverse Gram-negative bacteria. Jaglic et al. (2012) found MIC of NaOCl against diverse Gram-negative and -positive bacteria in the range 1000–4000 ppm. In another study, Seier-Petersen et al. (2014) described MIC of 5000 ppm against *Bacillus subtilis*. The differences in the MIC between the diverse studies is apparently due to the distinct susceptibility of the bacteria but also on the method used to determine MIC, particularly the growth medium. Those growth medium with high organic matter content will require higher NaOCl concentrations to exert antimicrobial effects due to the interaction between the medium constituents and the disinfectant. NaOCl in water exhibits a chemical dynamic balance: $NaOCl + H_2O \leftrightarrow NaOH + HOCl \leftrightarrow Na^+ + OH^- + H^+ + OCl^-$. NaOCl destroys microorganisms by chlorinating the lipid protein substance in the bacterial cell wall to form toxic chloro compounds and induces the leakage of macromolecules from the cells (Kim et al., 2008). Moreover NaOH can neutralize amino acids forming water and salt. Hypochlorous acid (HOCl) when interacting with protein amino groups forms chloramines. Chloramination reaction between chlorine and the amino group forms chloramines that interfere with the cell metabolism (Estrela et al., 2002). HOCl and hypochlorite ions ((OCl^-)) cause the oxidation of some amino acids, namely the sulfhydryl groups or sulfide bridges, present in proteins (Pattison et al., 2007; Rosen et al., 2009). Moreover, they interact strongly with bacterial membranes causing the oxidation of outer membrane proteins and facilitating the oxidation of inner cell proteins with subsequent cell death (Arana et al., 1999). In this study, the effects of NaOCl against planktonic and sessile *A. calcoaceticus* and *S. maltophilia* was tested at 0.5 ppm, a recommended level of free chlorine to keep along the DWDS (WHO, 2011), and at MIC.

Both bacteria had the surface negatively charged possibly due to the presence of anionic carboxyl and phosphate groups in the membrane surface (Ahimou et al., 2002). Similar values of zeta potential were described by other authors for strains of *A. calcoaceticus* and *S. maltophilia*. van der Mei et al. (1991) found that the surface charge of *A. calcoaceticus* RAG1 and *A. calcoaceticus* MR-481 was −20 mV and −33 mV, respectively; van Merode et al. (2007) assessed zeta

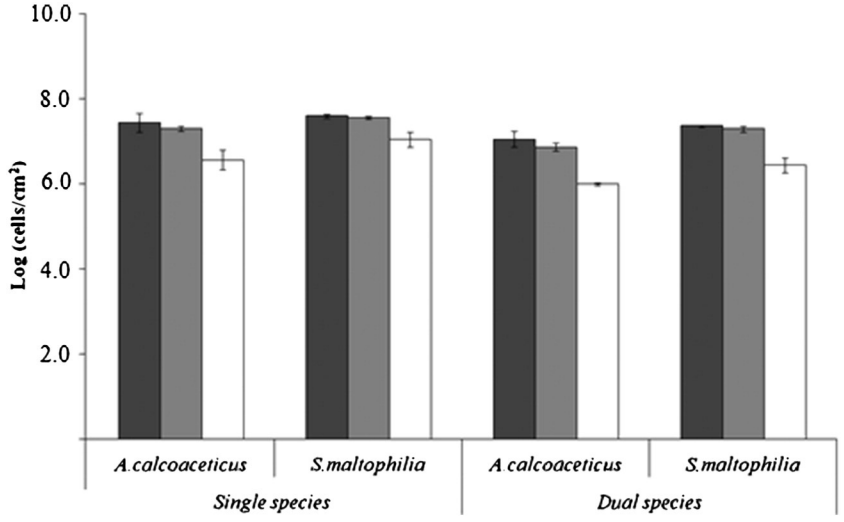

Fig. 3. Effects of NaOCl at different concentrations on removal of 24 h-old single and dual-species biofilms of *A. calcoaceticus* and *S. maltophilia* formed on PVC coupons. ■ - 0 mg/L; ■ - 0.5 mg/L; □ - MIC. The MIC are 125 ppm for *A. calcoaceticus* and 175 ppm for *S. maltophilia*. Dual-species biofilms were exposed to 175 mg/L.

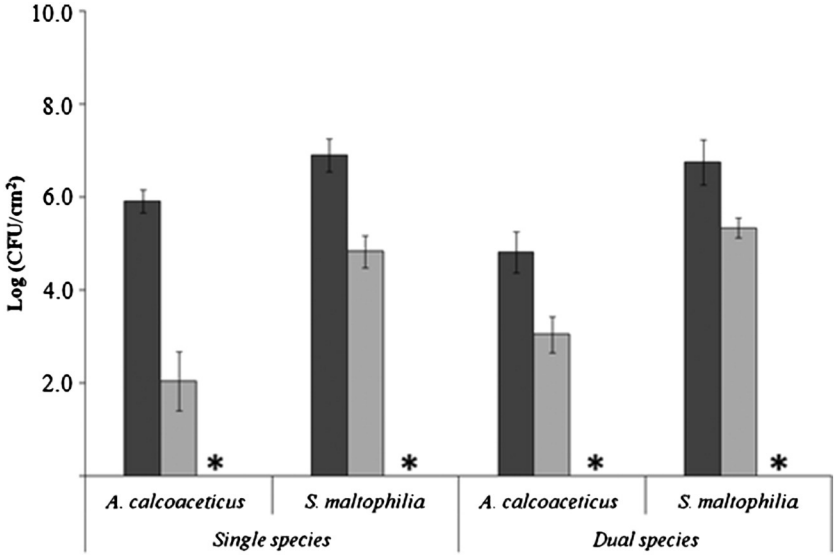

Fig. 4. CFU of *A. calcoaceticus* and *S. maltophilia* biofilms formed on PVC coupons after treatment with NaOCl at: ■ - 0 mg/L; ■ - 0.5 mg/L; □ - MIC; * - no CFU detected. The detection limit is 2.75 log CFU/cm^2. The MIC are 125 ppm for *A. calcoaceticus* and 175 ppm for *S. maltophilia.* Dual-species biofilms were exposed to 175 mg/L.

potential values for *S. maltophilia* in the range of -14 ± 1 and -24 ± 1 mV. As the selected bacteria and PVC had opposite charges it is expected that no electrostatic repulsion will affect the adhesion process. The application of NaOCl changed bacterial surface charge to less negative values. This result proposes that if the bacteria in planktonic state are exposed to NaOCl their ability to adhere on PVC will possibly decrease due to lower electrostatic effects. In fact, NaOCl caused significant changes on the surface charge of both bacteria, particularly of *S. maltophilia*. The formation of HOCl when NaOCl is mixed with water can cause the loss of electrons on the bacterial membrane and consequently alter the surface charge (Peck et al., 2011).

Hydrophobicity is an important property on bacterial attachment but also is essential to ensure the ability of the metabolites to transit through the membrane (Moorman et al., 2008). Bacterial hydrophobicity can be altered by changes in fatty acid and protein composition that occur in response to stress conditions. Changes in fatty acid composition of membrane lipids are the most important reaction of bacteria against membrane active substances (Murínová and Dercová, 2014). Both *A. calcoaceticus* and *S. maltophilia* showed to be hydrophilic as was also reported by Simões et al. (2007a, 2010a). However, the use of NaOCl decreased the hydrophobicity of *A. calcoaceticus*, possibly due to the oxidation and chlorination of membrane unsaturated fatty acids as proposed by Fukayama et al. (1986). The oxidation and chlorination of cell surface molecules could help to explain the changes in the surface tension parameters, particularly the reduction of the electron donor parameter and the increase of the electron acceptor. These results also demonstrate that surface physicochemical surface properties of *A. calcoaceticus* were affected in a higher extent than those of *S. maltophilia*. Bacterial motility is also an important feature that contributes to the adhesion process, helping the microorganism reaching the most favorable environment and to gain advantage when competing with other microorganisms in response to external conditions (Fenchel, 2002; Maes et al., 2013; Stocker et al., 2008). Cell motility is characterized by the presence of structures like flagella, pili or fimbriae that play an important role in microbial initial adhesion to a surface and consequent biofilm formation. These structures are able to overcome the repulsive forces associated with the substratum and can contribute to the overall surface hydrophobicity (Donlan, 2002; Mandlik et al., 2008; O'Toole and Kolter, 1998). Several types of bacterial motility were already defined such as swimming, swarming, gliding, sliding and twitching (Kearns, 2010). In this work swimming, swarming and twitching motility were studied. These types of motility were already described for *A. calcoaceticus* and *S. maltophilia*. Hentuchsen and Blom (1975) found that *A. calcoaceticus* is able to move through twitching motility, while the tests done by Simões et al. (2007c) described also that this bacteria is able to move through swimming motility. Pompilio et al. (2008) showed that most of *S. maltophilia* tested strains are able to move by swimming and twitching motilities, but concluded that these characteristics may not be very influent on initial adhesion but are crucial on biofilm development (Pompilio et al., 2011). Ferrer-Navarro et al. (2013) studied the motility of four strains of *S. maltophilia* and all the strains presented swimming, swarming and twitching motility. Bacterial exposure to NaOCl only affected *A. calcoaceticus* motility, reducing swimming motility which means that the biocide can affect in some way the ability of *A. calcoaceticus* to get closer to the surface and to overcome repulsive forces (Donlan, 2002). Previous studies also described changes in bacterial motility caused by the exposure to some disinfectants. Majtán and Majtánová (2002) described a reduction of *Pseudomonas aeruginosa* motility when exposed to several quaternary ammonium compounds. García-Heredia et al. (2013) observed a reduction of swarming motility of diverse *Salmonella* spp. strains after treatment with a citrus-based disinfectant. However, to our knowledge no studies exist on the effects of NaOCl on bacterial motility.

Both *A. calcoaceticus* and *S. maltophilia* (single and co-culture) adhered on PVC at similar extents as assessed by DAPI staining. Nevertheless, the numbers of CFU of *S. maltophilia* were significantly higher than those of *A. calcoaceticus* in both single and dual-species monolayer adhered cells and biofilms. This difference is arguably related to the limitations of the plate count method (Simões et al., 2005). The underestimation of viable cell can be caused by several reasons, as the presence of starved or injured cells or potentially viable but nonculturable cells, the inadequate culture conditions and the aggregation of bacteria that can lead to the formation of one colony from more than one cell, thereby underestimating the total number of cells (Banning et al., 2002). Nevertheless, in order to avoid the aggregation effect, bacterial suspensions were vortexed for 1 min. The comparison between DAPI and CFU results also reinforce that removal and killing of sessile cells are distinct phenomena. In fact, NaOCl was able to cause total CFU reduction (total CFU reduction was achieved with NaOCl at MIC for all tested conditions) and quite poor removal effects. *S. maltophilia* was more resistant than *A. calcoaceticus*. However, their co-culture increased resilience to

NaOCl. Schwering et al. (2013) also found that chlorine tolerance of pathogenic bacteria increased when they were associated in a multispecies sessile community.

After the adhesion process bacteria begin producing EPS to form mature biofilms. These extracellular substances are recognized as biocide quenching agents increasing substantially biofilm resistance to antimicrobials (Vu et al., 2009). In this study the effects of NaOCl was assessed on the control of 24 h-old biofilms. Both bacteria showed similar ability to form biofilm, with the number of *A. calcoaceticus* in biofilm being similar to those of *S. maltophilia*. The number of culturable *A. calcoaceticus* was lower than those of *S. maltophilia*, as happened in the adhesion assay. *A. calcoaceticus* formed biofilms more susceptible to NaOCl than those of *S. maltophilia*. However, biofilm removal from NaOCl exposure was low for all the conditions. Lenz et al. (2010) reported no CFU on a model DW biofilm after application of diverse disinfectants (free chlorine-electrochemically activated, chlorine dioxide, hydrogen peroxide, silver and fruit acids). However, biofilm remain adhered on the surface. de Souza et al. (2014) also found that NaOCl at 250 mg/L was ineffective on the removal of *Staphylococcus aureus* from food contact surfaces. Davison et al. (2010) tested the efficacy of chlorine at 10 mg/L and 50 mg/L on *Staphylococcus epidermidis* biofilms treatment and found low reduction. Biofilms were more resistant than monolayer adhered cells as evidenced by CFU results, even if NaOCl at a residual concentration was able to cause significant CFU reduction. Further studies were performed with *A. calcoaceticus-S. maltophilia* dual-species biofilms in order to ascertain the role of species association in biofilm susceptibility to NaOCl. It was observed a lower CFU reduction of dual-species biofilms compared to those single species. Species association did not affect the biofilm removal from PVC compared to the single species scenario, demonstrating the resistance and resilience of both single and dual biofilms to removal, even if NaOCl at MIC caused total CFU reduction for all the tested conditions. The biofilm remaining after antimicrobial treatment is of particular concern due to the potential on the colonizing microorganisms to reseed the biofilm (Gagnon et al., 2005; Simões et al., 2009). Moreover, the results on dual-species biofilm removal are intriguing as previous studies report the increased resistance of multispecies biofilms when compared to their single species counterparts (Behnke et al., 2011; Behnke and Camper, 2012; Burmølle et al., 2006; Simões et al., 2009, 2010a). In fact, comparing with the experiments of monolayer adhered bacteria it was expected that the presence of EPS and other biofilm features will increase even more the resistance of dual-species biofilms.

5. Conclusions

The overall results demonstrate that NaOCl interact strongly with *A. calcoaceticus* increasing their cell surface charge, hydrophilicity and electron donor properties. Also, it caused reduction of *A. calcoaceticus* swimming and twitching motilities. *S. maltophilia* was affected by NaOCl exposure in a lower extent: the MIC was higher than for *A. calcoaceticus* and the disinfectant only increased the cell membrane electron donor properties and the surface charge to less negative values. No effects of NaOCl under residual levels and at the MIC were found on *S. maltophilia* motility. The disinfectant had low effects on the removal of monolayer adhered *A. calcoaceticus* and *S. maltophilia* (single species and co-culture) and their biofilms. However, even at a residual concentration NaOCl caused total reduction of monolayer adhered CFU and significant reduction of biofilm CFU. *S. maltophilia* sessile cells were invariably more resistant to removal and killing than *A. calcoaceticus*. This clearly proposes the need for new methods to monitor the microbiological status of a DWDS, considering that biofilms represent 95% of the overall biomass in a DWDS, while only 5% is in the water phase (Flemming et al., 2002). Moreover, alternative and/or complementary methods to NaOCl are required for an effective and environmentally friendly DW disinfection (Meireles et al., 2016). This knowledge on the distinct susceptibilities and behavior of microorganisms can contribute to the design of effective control strategies, able to control the key microorganisms, such as *S. maltophilia*, in the resistance and resilience of a biofilm that will guarantee safe and high-quality drinking water.

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