Prolonged exposure of *Stenotrophomonas maltophilia* biofilms to trace levels of clofibric acid alters antimicrobial tolerance and virulence

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HIGHLIGHTS

- Non-antibiotic drugs, including clofibric acid (CA) can affect bacterial behavior.
- The presence of CA in water increased *S. maltophilia* tolerance to erythromycin.
- CA exposure increased biofilm tolerance to chorine.
- CA exposure decreased *S. maltophilia* ability to adhere and internalize HT-29 cells.

GRAPHICAL ABSTRACT
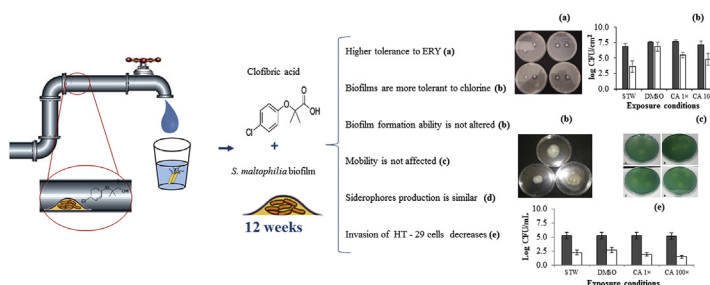
ABSTRACT

The presence of pharmaceuticals in water sources, including in drinking water (DW), is increasingly being recognized as an emerging and global concern for the environment and public health. Based on the principles of the "One Health" initiative, the present work aims to understand the effects of clofibric acid (CA), a lipid regulator, on the behavior of a selected bacterium isolated from drinking water (DW). Biofilms of the opportunistic pathogen *Stenotrophomonas maltophilia* were exposed to CA for 12 weeks at 170 and 17000 ng/L. The effects of CA were evaluated on planktonic *S. maltophilia* susceptibility to chlorine and antibiotics (amoxicillin, ciprofloxacin, erythromycin, kanamycin, levofloxacin, oxacillin, spectinomycin, tetracycline and trimethoprim-sulfamethoxazole), biofilm formation, motility, siderophores production and on the adhesion and internalization of the human colon adenocarcinoma cell line (HT-29). It was found that CA did not affect planktonic *S. maltophilia* tolerance to chlorine exposure. Additionally, no effects were observed on biofilm formation, motility and siderophores production. However, biofilms formed after CA exposure were more tolerant to chlorine disinfection and lower CFU reductions were obtained. Of additional concern was the effect of CA exposure on *S. maltophilia* increased tolerance to erythromycin. CA exposure also slightly reduced *S. maltophilia* ability to invade HT-29 cells. In conclusion, this work reinforces the importance of studying the effects of non-antibiotic contaminants on the behavior of environmental microorganisms, particularly their role as drivers affecting resistance evolution and selection.

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List of abbreviations			
		EA	Ethacrynic acid
		EC	Emerging contaminant
2,4-D	2,4 − dichlorophenoxycetic acid	ERY	Erythromycin
AMO	Amoxicillin	FBS	Fetal bovine serum
ARB	Antimicrobial resistant bacteria	GAC	Granular activated carbon
ARG	Antimicrobial resistant genes	IBP	Ibuprofen
CA	Clofibric acid	KAN	Kanamycin
CAS	Chrome azurol solution	LEV	Levofloxacin
CBZ	Carbamazepine	MBC	Minimum bactericidal concentration
CFU	Colony-forming unit	OXA	Oxacillin
CIP	Ciprofloxacin	PBS	Phosphate buffered saline
CLSI	Clinical and Laboratory Standards Institute	PVC	Polyvinyl chloride
DMSO	Dimethyl sulfoxide	SPE	Spectinomycin
DPD	N,N-diethyl-p-phenylenediamine	STW	Synthetic tap water
DW	Drinking water	TET	Tetracycline
DWDS	Drinking water distribution systems	TMP	SMX − Trimethoprim − sulfamethoxazole
DWTP	Drinking water treatment plant	WWTP	Wastewater treatment plant

1. Introduction

Non-regulated substances are consistently detected in aquatic environments at trace levels and constitute an emerging environmental concern. These substances have been designated as emerging contaminants (ECs) and are typically defined as synthetic or naturally occurring molecules or drug-resistant microorganisms that are not monitored or regulated, but are found in environmental matrices at trace levels (Geissen et al., 2015; Matamoros et al., 2016). These are indispensable for actual society lifestyle and their use and production can hardly be reduced. There is a wide variety of ECs that have been found in the environment such as detergents, fragrances, pesticides, plastics, endocrine disruptors and other pharmaceuticals and many other compounds. Antibiotic resistant bacteria (ARB) and genes (ARG) and disinfection by-products are also considered ECs (Geissen et al., 2015; Matamoros et al., 2016). The presence of ARB and ARG may result from the exposure to other ECs, such as antibiotics or other antimicrobials. Their sub-lethal doses found in the environment can induce antibiotic resistance (Geissen et al., 2015; Matamoros et al., 2016). They may reach water sources by several routes. For example, pharmaceuticals are not completely metabolized by humans and animals being then excreted. Therefore, they enter directly in sewage and soil, and consequently reach wastewater treatment plants and groundwater (Kummerer, 2001). Effluents of fish farms are another direct source of ECs for surface water (Kim et al., 2017). However, conventional wastewater treatment processes fail in removing ECs (Huber et al., 2005; Rigobello et al., 2013; Zhang et al., 2017; Delgado-Moreno et al., 2019; Rizzo et al., 2019). Taking into account that drinking water (DW) is often produced from surface water and groundwater, the efficacy of ECs removal through drinking water treatment plants (DWTPs) is of utmost importance. Some conventional treatments in DWTPs, such as filtration, oxidation and adsorption, may remove some of these contaminants. Several studies demonstrated that oxidative processes particularly the use of chlorine are important strategies to reduce ECs levels in DWTPs (Rigobello et al., 2013; Gabarrón et al., 2016). The use of granular filters, such as sand, is also a common strategy applied in DWTPs with low ability to remove ECs (Pojana et al., 2011; Gabarrón et al., 2016). Gabarrón et al. (2016) demonstrated that high removal rates were obtained only for ECs with log K_w higher than 3. In order to increase the efficiency of the granular filtration process, a new barrier can be applied in DWTPs: biofiltration. The presence of biofilms as filters are potentially important to reduce the presence

of ECs in DW as microorganisms may degrade ECs (Zearley and Summers, 2012) or increase its adsorption on the matrix of extra-cellular polymeric substances of biofilms (Petropavlovskii and Sillanpää, 2013; Torresi et al., 2017). The use of granular activated carbon (GAC) is also a conventional strategy used in DWTPs with important results in the reduction of ECs content (Pojana et al., 2011; Rigobello et al., 2013; Gabarrón et al., 2016). Despite all these barriers presented along conventional DWTPs, the removal of ECs is still inefficient and their presence in final DW remains unavoidable (Tröger et al., 2018).

Biofilm formation along the DWDS is another unavoidable phenomenon that may have significant influence on the spread of ARB and ARG (Simões and Simões, 2013; Zhang et al., 2018). These biofilms are continuously exposed to the ECs persisting in the final DW (Wang et al., 2019). The presence of ECs and biofilms in DWDS should be considered based on a One Health approach aiming to improve public health outcomes, recognizing that human health is connected with animal health and the environment -where both are inserted (Gibbs, 2014; Smith et al., 2019). The One Health approach also focus on antimicrobial resistance transmission between humans and animals and in the potential of the environment as source of ARG and ARB (Smith et al., 2019).

This work aims to understand the effect of a selected EC on the behavior of the opportunistic pathogen *Stenotrophomonas maltophilia* isolated from DW. Clofibric acid (CA) is a lipid regulator used to control cholesterol levels in the blood and has been identified for decades in effluents from domestic wastewater treatment plants (WWTPs), in surface and ground water as well as in DW (Heberer and Stan, 1997; Jux et al., 2002; Zwiener and Frimmel, 2004). CA was found to persist in the environment over 21 years and is still detected in water bodies after being withdraw from the market (Buser et al., 1998; Nega et al., 2019). A previous work evaluated the effects of different ECs (alone and combined) on the behavior of bacteria isolated from a DWDS where it was found that CA was present in all the combinations causing changes in bacterial behavior, particularly biofilm formation ability and tolerance to chlorine disinfection (Gomes et al., 2018a). Therefore, CA was selected for this study, where a prolonged exposure (12 weeks) of *S. maltophilia* to the EC was evaluated in terms of susceptibility to chlorine and antibiotics, biofilm formation and tolerance to disinfection, siderophores production, motility and invasion of the human colon cancer cell line HT-29. To our knowledge, this is the first work studying the effects of ECs and particularly CA on bacterial production of virulence factors, including adhesion and invasion of

human cells, providing preliminary insights on the infectious potential from CA exposure.

2. Materials and methods

2.1. Bacterium

Stenotrophomonas maltophilia previously isolated from a DWDS (Simões et al., 2007), was grown at 25 °C, 120 rpm agitation, using R2A broth [0.5 g/L peptone (Oxoid, Hampshire, England), 0.5 g/L glucose (CHEM-LAB, Zedelgem, Belgium), 0.1 g/L magnesium sulfate heptahydrate (Merck, Darmstadt, Germany), 0.3 g/L sodium pyruvate (Fluka, Steinheim, Germany), 0.5 g/L yeast extract (Merck, Darmstadt, Germany), 0.5 g/L casein hydrolysate (Oxoid, Hampshire, England), 0.5 g/L starch (Sigma-Aldrich, Steinheim, Germany) and 0.4 g/L di-potassium phosphate trihydrate (Aplichem Panreac, Darmstadt, Germany]. *S. maltophilia* was selected for this study as it is an emergent opportunistic pathogen frequently isolated from DW and demonstrated to be tolerant to chlorine exposure (Ortolano et al., 2008; Shih and Lin, 2010; Guyot et al., 2013; Vincenti et al., 2014; Amoli et al., 2017a). A previous study demonstrated that the planktonic and sessile behavior of the selected strain was influenced by the exposure to ECs (Gomes et al., 2018a).

2.2. Substratum

Polyvinyl chloride (PVC) coupons (Neves & Neves, Portugal) with 1 cm × 1 cm were used as substratum for biofilm formation. Before use, the coupons were cleaned and sterilized as described by Gomes et al. (2018a).

2.3. Clofibric acid

Stock solutions of clofibric acid (CA; Acros Organics, New Jersey, USA) were prepared in dimethyl sulfoxide (DMSO) (Fisher Scientific, Leicestershire, UK) and stored at −20 °C until use. Stock solutions were prepared at 0.017 mg/L and 1.7 mg/L in order to use low DMSO volume in biofilms. Stock solutions were diluted 100 times in synthetic tap water (STW) in order to obtain the final concentrations to be tested: 170 and 17000 ng/L. The concentration of DMSO in the final solution was 1%. STW (100 mg/L NaHCO$_3$, 13 mg/L MgSO$_4$·7H$_2$O, 0.7 mg/L K$_2$HPO$_4$, 0.3 mg/L KH$_2$PO$_4$, 0.01 mg/L (NH$_4$)$_2$SO$_4$, 0.01 mg/L NaCl, 0.001 mg/L FeSO$_4$·7H$_2$O, 1 mg/L NaNO$_3$, 27 mg/L CaSO$_4$, 1 mg/L humic acids) was prepared as described by EPA (2011) and Gomes et al. (2018a).

2.4. PVC coupons colonization

The cell density of bacterial suspensions grown as described in section 2.1 was adjusted to 1×10^8 CFU/mL with R2A broth. Then, 1 mL suspension was added to each well of a 48-wells microtiter plate containing sterile PVC coupons. Coupons colonization with *S. maltophilia* occurred for 24 h incubated at 25 °C and under 120 rpm agitation.

2.5. S. maltophilia biofilms exposure to CA

The exposure to CA was performed according to Gomes et al. (2018b), with few modifications. Briefly, colonized PVC coupons were exposed to CA for 12 weeks in 48-wells microtiter plates. Biofilms were formed for 12 weeks. This incubation period was based on the fact that DW biofilms are developed under oligotrophic conditions, taking several weeks/months to reach a mature state (Abe et al., 2012; Abberton et al., 2016). The biofilms were

formed under continuous exposure to DW-containing trace levels of ECs. Two controls were performed: (1) biofilms were grown only in the presence of STW for 12 weeks; (2) biofilms were exposed to 1% DMSO (solvent control) for 12 weeks. The exposure to CA was performed at two concentrations, an environmental concentration similar to those found in DW distribution systems (DWDS) ([DW] - 170 ng/L) (Jones et al., 2005) and a concentration one hundred times higher (100 × [DW] - 17000 ng/L). The solutions were replaced every week, in order to guarantee a constant exposure to trace CA concentrations.

2.6. S. maltophilia characterization after prolonged exposure to CA

After biofilm exposure to CA for 12 weeks, *S. maltophlia* cells from biofilms were recovered in R2A agar (Oxoid, Hampshire, England) plates before further tests to characterize the susceptibility of planktonic cells to chlorine and antibiotics. CA-exposed cells were also used to form biofilms and these were tested on their susceptibility to chlorine. Further experiments were performed to characterize bacterial motility, siderophores production and the adhesion and invasion of HT-29 cells.

2.6.1. Susceptibility to chlorine

The bacterial suspension from CA-exposed biofilms was centrifuged (15 min, 3700 g) and resuspended in STW to a final concentration of 1×10^6 cells/mL, in order to mimic DWDS conditions (Prest et al., 2016). *S. maltohilia* was exposed to different concentrations of free chlorine (1–10 mg/L), prepared with NaOCl (Sigma-Aldrich, Steinheim, Germany) for 24 h in a 96 wells microtiter plate. Free chlorine concentrations were adjusted using a photometer from Hanna Instruments using the N,N-diethyl-p-phenylenediamine (DPD) method (test kit from Hanna Instruments). The minimum bactericidal concentration (MBC) was determined according to Gomes et al. (2018a). Three independent experiments with triplicates were performed for each condition tested.

2.6.2. Susceptibility to antibiotics

Antibiotics from six different classes were prepared using the solvents listed in Table 1: amoxicillin – AMO (Sigma-Aldrich, Steinheim, Germany); ciprofloxacin – CIP (Fluka, Steinheim, Germany); erythromicin – ERY (AppliChem, Darmstadt, Germany); kanamicin – KAN (Eurobio, Courtaboeuf, France); levofloxacin – LEV (Alfa Aesar, Karlsruhe, Germany); oxacillin – OXA (Sigma-Aldrich, Steinheim, Germany); spectinomycin – SPE (Sigma-Aldrich, Steinheim, Germany); tetracycline – TET (Sigma-Aldrich, Steinheim, Germany); trimethoprim - sulfamethoxazole - TMP-SMX (Alfa Aesar, Karlsruhe, Germany). Bacterial suspensions were adjusted to 6×10^8 cells/mL and the disk diffusion test was applied according the Clinical and Laboratorial Standards Institute (CLSI) guidelines (CLSI, 2007) to understand the effects of CA exposure on *S. maltophilia* susceptibility to antibiotics. Negative controls were performed with the solvents used (DMSO, NaOH and HCl). Three independent experiments with duplicates were performed for each condition tested.

2.6.3. Biofilm formation ability of S. maltophilia previously exposed to CA

S. maltophilia from 12 weeks biofilms was grown in R2A broth overnight at 25 °C and 120 rpm. Then biofilms were formed according to Stepanovic et al. (2000). The cell density of each suspension was adjusted to 2×10^8 cells/mL in R2A broth. A volume of 200 μL of bacterial suspension was added to each well of a 96-wells polystyrene (PS) microtiter plate. The plates were further incubated for 24 h at 25 °C and 120 rpm. Sterile R2A broth was used as

Table 1
Antibiotic content per disk and solvent used.

Antibiotic	Disk content (μg)	Solvent	Antibiotic class	Target
AMO	20	NaOH 1 N	β lactam	β lactamase
CIP	5	HCl 0.1 N	Fluoroquinolone	DNA topoisomerase
ERY	15	DMSO	Macrolide	Protein synthesis
KAN	30	Distilled water	Aminoglycoside	Protein synthesis
LEV	5	DMSO	Fluoroquinolone	DNA topisomerase
OXA	1	Distilled water	β-lactam	β-lactamase
SPE	100	Distilled water	Aminoglycoside	Protein synthesis
TET	30	DMSO	Tetracyclines	Protein synthesis
TMP/SMX	1.25/23.75	DMSO	Folic acid inhibitors	Folic acid

negative control. Biofilm production was assessed in terms of culturability (enumeration of colony forming units - CFU) and by crystal violet (Merck, Darmstadt, Germany) staining as described by Borges et al. (2017) and Gomes et al. (2018b).

2.6.4. Chlorine effects on biofilms formed by S. maltophilia exposed to CA

The 24 h-old biofilms (section 2.6.3) were treated with 130 mg/L NaOCl (the minimum bactericidal concentration for S. maltophilia in R2A broth) for 30 min. Bacterial suspension from each well (section 2.6.3) was discarded and the biofilms were washed with saline water (0.85% NaCl − VWR, Leuven, Belgium) in order to remove non-adhered and weakly adhered cells. Afterwards, 200 μL of NaOCl solution (130 mg/L of free chlorine) prepared in STW was added to biofilms for 30 min. Negative controls were obtained from exposure to 200 μL of STW for 30 min. After exposure, chlorine was removed from each well and the remaining biocide was neutralized with sodium thiosulfate at 0.50% (wt/v) for 10 min (Gomes et al., 2018a). Chlorine effects on biofilms were assessed by CFU enumeration and crystal violet staining as previously described by Borges et al. (2017) and Gomes et al. (2018b).

2.6.5. Motility

S. maltophilia motility was assessed after CA exposure for 12 weeks. Swimming, swarming and twitching were the three types of motility evaluated as described by Gomes et al. (2016). Briefly, bacteria isolated from CA-exposed biofilms were grown overnight in LBB (Luria-Bertani broth) and the cell density was adjusted to 5×10^6 cells/mL in fresh LBB. A volume of 15 μL of bacterial suspension was then dropped in the centre of agar plates. These agar plates were prepared with tryptone at 10 g/L (Fisher Bioreagents, New Jersey, USA), NaCl at 2.5 g/L and agar (VWR Chemicals, Leuven, Belgium) at 3 g/L (swimming motility), 7 g/L (swarming motility) or 15 g/L (twitching motility). Plates were then incubated at 25 °C for 72 h. The colony growth was measured at 24 h after incubation. Three independent assays were performed with three replicates.

2.6.6. Siderophores production

Siderophores production was evaluated using chrome azurol S (CAS) plates prepared in R2A. Briefly, 100 μL of a sterilized solution of CAS-iron dye prepared according to Schwyn and Neilands (1987) was added to 900 μL R2A agar (Oxoid, Hampshire, England). A volume of 15 μL of bacterial suspension adjusted to 5×10^6 cells/mL were placed in each plate of CAS agar in three different positions. Plates were incubated for 48 h at 25 °C and siderophores production was evaluated by the formation of an orange halo that was measured (mm). Three independent assays were performed with three replicates.

2.6.7. Adhesion and internalization of S. maltophilia exposed to CA to HT-29 cell line

Human colon adenocarcinoma cell line HT-29 (ATCC® HTB-38™) (kindly provided by the Institute for Research and Innovation in Health Sciences - I3S, Porto) was selected as model to assess the potential effects from the ingestion of DW contaminated with S. maltophilia in the interaction with mammalian intestinal cells. These cells were cultured in RPMI 1640 medium (Biowester, Nuaillé - France) containing 10% (v/v) fetal bovine serum (FBS) (Biowester, Nuaillé - France) and 1% (v/v) antibiotics solution (Biowester, Nuaillé - France). Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere. HT-29 cells were seeded in 24-well plates at concentration of 4.0×10^4 cells per well obtaining a semiconfluent monolayers in 48 h. Cell cultures were replenished with RPMI 1640 without antibiotics 24 h before the assay was performed. All cell cultures were incubated at 37 °C in a 5% CO_2 atmosphere.

The protocol of bacterial adhesion and internalization was performed according to Gagnon et al. (2013). Briefly, S. maltophilia grown as described in section 2.1 were centrifuged at 3777 g for 15 min, washed twice in saline solution (0.85% NaCl). A final concentration of bacteria of 10^6 per mL was prepared in RPMI 1640 medium. A volume of 500 μL of this suspension was then added to each well of the 24-well plate containing a monolayer of HT-29. The procedure was validated with Salmonella sp. (isolated from DW), a bacterium with known ability to internalize human colon cells (Gagnon et al., 2013). Two independent assays were performed with three replicates.

2.6.7.1. S. maltophilia adhesion on HT-29 cells.
To determine bacterial adhesion on HT-29, plates were incubated for 30 min at 37 °C in a 2.5 L AneroJar™ with microaerophilic conditions assured by Oxoid™ CampyGen™ sachet. Afterwards, bacterial suspension was discharged from each well and HT-29 cells were washed twice with phosphate buffered saline (PBS), in order to remove non-adhered cells. A volume of 250 μL of trypsin-EDTA (Sigma-Aldrich, Steinheim, Germany) was added to each well to detach HT-29 cells and adhered bacteria. After incubation for 10 min at 37 °C, trypsin was inactivated by adding 250 μL of RPMI 1640 with fetal bovine serum (FBS). The bottom of each well was scrapped with the pipette to guarantee the maximum removal of HT-29 monolayer. The suspension was then plated in TSA (Merck, Darmstadt, Germany) plates and incubated for 24 h at 37 °C in order to assess the number of bacteria adhered on HT-29 cells.

2.6.7.2. S. maltophilia internalization in HT-29 cells.
Bacteria were incubated with HT-29 cells for 90 min at 37 °C in an 2.5 L AnaeroJar™ with microaerophilic conditions assured by Oxoid™ CampyGen™ sachet, in order to assess the ability of S. maltophilia to invade these cells. After incubation, the supernatant was discharged and cells were washed twice with 500 μL PBS. Afterwards, 1 mg/L of levofloxacin (prepared in RPMI 1640 medium) was added to each

well in order to kill adhered bacteria and incubated for 1 h (time and antibiotic concentration tested for complete inactivation of sessile *S. maltophilia*). Cells were washed twice with PBS after treatment with the antibiotic. A volume of 250 μL of trypsin was added to each well for 10 min, in order to detach cells from the plate (Section 2.6.4.1), followed by 250 μL of ice cold Triton X −100 (Sigma-Aldrich, Steinheim, Germany) at 2% (v/v) in order to lyse the HT-29 cells and release the internalized bacteria. The bottom of each well was scraped with a pipette to guarantee maximum cell removal. Suspensions were collected to 1.5 mL microtubes, vortexed and incubated for 10 min in order to improve homogenization and achieve complete cell lysis. The number of CFU of internalized bacteria were then assessed by plating the suspension in TSA followed by incubation for 24 h at 37 °C.

2.7. Statistical analysis

Statistical analysis of results was performed using the software IBM® SPSS® Statistics (Statistical Package for the Social Sciences) version 24.0. One-way analysis of variance (ANOVA) was applied and the comparisons between and within experimental groups were carried out using Tukey test. Statistical calculations were based on confidence level $\geq 95\%$ ($P < 0.05$) which was considered statistically significant.

3. Results

3.1. Characterization of S. maltophilia from biofilms exposed to CA

S. maltophilia cells were isolated from biofilms exposed to CA during 12 weeks. These cells were characterized in terms of susceptibility to chlorine (Section 3.1.1), susceptibility to nine antibiotics (AMO, CIP, ERY, KAN, LEV, OXA, SPE, TET, TMP-SMX - Section 3.1.2) using planktonic cells. Biofilm formation ability (Section 3.1.3) and susceptibility to chlorine (Section 3.1.4) were further assessed. The effects of CA were also evaluated on bacterial motility (Section 3.1.5), siderophores production (Section 3.1.6) and on the ability of *S. maltophilia* to adhere and internalize the human colon adenocarcinoma cell line HT-29.

3.1.1. Effects of CA exposure on S. maltophilia susceptibility to chlorine

The MBC of chlorine was used as measure of *S. maltophilia* susceptibility to this disinfectant. Results demonstrate that concentrations of free chlorine between 2 and 4 mg/L were bactericidal for *S. maltophilia* obtained from both biofilms grown in the presence of CA at [DW] and at $100 \times$ [DW] for 12 weeks. Significant differences were not observed between bacteria exposed to STW, DMSO and CA at [DW] and at $100 \times$ [DW] ($P > 0.05$).

3.1.2. Effects of CA exposure on S. maltophilia susceptibility to antibiotics

S. maltophilia isolated from 12 weeks biofilms was resistant to AMO, OXA and SPE, regardless the conditions under which biofilms were exposed (Table 2). The exposure to CA did not alter bacterial susceptibility to these antibiotics ($P > 0.05$). *S. maltophilia* was susceptible to CIP, KAN, LEV, TET and TMP-SMX, when bacteria obtained from biofilms exposed to STW, DMSO and CA at [DW] and at $100 \times$ [DW] were used. *S. maltophilia* had intermediate susceptibility to ERY. Bacteria obtained from biofilms exposed to CA at $100 \times$ [DW] had higher tolerance to this antibiotic ($P < 0.05$) (Figure S.1). On the other hand, exposure to CA at $100 \times$ [DW] increased susceptibility to CIP ($P < 0.05$).

Table 2
Zone of inhibition (mm) for the selected antibiotics. Mean values and the corresponding standard deviation are presented.

Antibiotics[a]	Halo inhibition (mm) for each exposure condition			
	STW	DMSO	CA at [DW]	CA at $100 \times$ [DW]
AMO	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
CIP	29.3 ± 1.0	27.0 ± 1.4	28.5 ± 1.9	31.3 ± 1.4[b]
ERY	19.2 ± 2.2	19.7 ± 1.9	16.6 ± 2.7	15.7 ± 0.8[b]
KAN	19.8 ± 3.3	22.3 ± 2.5	21.8 ± 3.0	19.0 ± 4.8
LEV	30.5 ± 2.6	30.7 ± 2.8	31.2 ± 1.3	30.7 ± 1.6
OXA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
SPE	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
TET	14.5 ± 2.0	14.2 ± 2.5	13.7 ± 2.0	13.5 ± 1.8
TMP - SMX	28.3 ± 1.2	28.3 ± 0.8	28.3 ± 1.0	28.2 ± 1.3

[a] AMO- amoxicillin, CIP- ciprofloxacin, ERY — erythromycin, KAN- kanamycin, LEV — levofloxacin, OXA — oxacylin, SPE- Spectinomycin, TET — tetracycline, TMP — SMX— trimethoprim-sulfamethoxazole.
[b] Zone of inhibition statistically different from those obtained after exposure to the solvent (DMSO) − $P < 0.05$.

3.1.3. Effects of CA exposure on S. maltophilia biofilm formation and susceptibility to chlorine

S. maltophilia from CA-exposed biofilms were used to assess their ability to form biofilms and the susceptibility of these biofilms to chlorine in terms of total biomass (crystal violet method — Fig. 1) and culturability (log CFU/cm² — Fig. 2). It was found that exposure to 1% DMSO (solvent control) for 12 weeks increased *S. maltophilia* ability to form biofilms on PS compared to *S. maltophilia* exposed to STW for 12 weeks ($P < 0.05$). However, exposure to CA at [DW] and $100 \times$ [DW] did not cause significant changes in biofilm formation
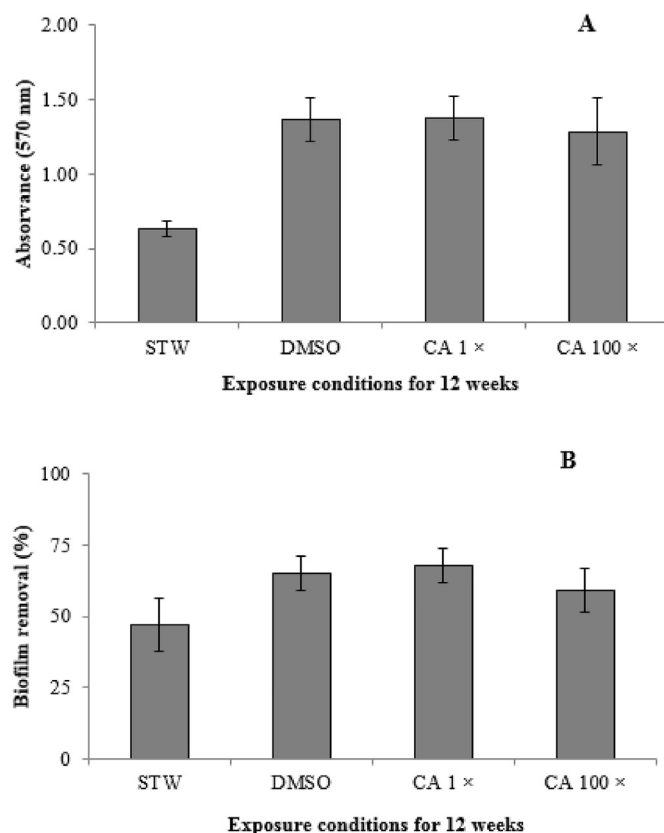

Fig. 1. Biofilm formation for 24 h after *S. maltophilia* exposure to STW, DMSO and CA at [DW] and $100 \times$ [DW] for 12 weeks in terms of total biomass as assessed by crystal violet staining (absorbance at 570 nm) − A. Biofilm removal after treatment with free chlorine (130 mg/L) for 30 min in terms of total biomass − B.
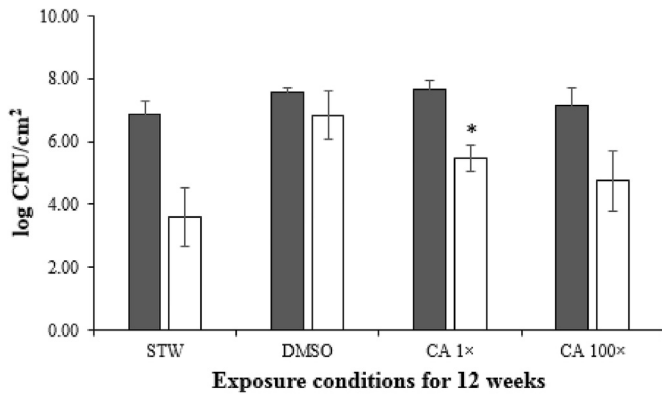
Fig. 2. The effects of the exposure to STW, DMSO at 1%, CA at [DW] and 100 × [DW] for 12 weeks on the ability of *S. maltophilia* to form biofilms, with data presented as log CFU/cm^2 (■). Biofilm susceptibility to free chlorine at 130 mg/L for 30 min, with data presented as log CFU/cm^2 reduction (□).* - mean values statistically different from those obtained after exposure to the solvent (DMSO) − $P < 0.05$.

when compared to the solvent control ($P > 0.05$) (Fig. 1.A).

Complete biofilm removal was not obtained after treatment with 130 mg/L of free chlorine for 30 min (Fig. 1.B). Biofilms formed after exposure to DMSO were removed in a higher extent (65% biofilm removal) than biofilms formed after exposure to STW (47% biofilm removal). *S. maltophilia* exposed to CA at [DW] formed biofilms with similar susceptibility to these obtained from *S. maltophilia* exposed to DMSO ($P > 0.05$). Although, statistically significant differences were not observed, the biofilms formed after *S. maltophilia* exposure to CA at 100 × [DW] showed a decrease in removal from chlorine exposure.

The culturability of *S. maltophilia* biofilms was not significantly affected by the conditions under which the cells were grown, *i.e.* exposed to CA at [DW], 100 × [DW] and solvent control (DMSO at 1%) ($P > 0.05$). A slight increase in CFU numbers was observed for biofilms exposed to DMSO (1%) in comparison to those formed after exposure to STW for 12 weeks ($P < 0.05$). Complete CFU reduction was not achieved after 30 min in the presence of 130 mg/L of free chlorine for any of the biofilms tested. However, reductions of 3.60, 6.84, 5.48 and 4.76 log CFU/cm^2 were obtained for biofilms formed after *S. maltophilia* exposure to STW, DMSO, CA at [DW] and at 100 × [DW], respectively. This means that CFU reduction decreased significantly due to previous bacterial exposure to CA compared to the DMSO control ($P < 0.05$). Moreover, increasing the levels of CA exposure decreased CFU reduction, even if not statistically significant ($P > 0.05$).

3.1.4. Effects of CA exposure on S. maltophilia motility

The exposure to CA at both concentrations tested did not cause significant changes (Table 3) in *S. maltophilia* swimming and twitching motilities ($P > 0.05$). The previous exposure to CA at [DW] slightly increased *S. maltophilia* swarming motility, even if not

Table 3
Motility of *S. maltophilia* from biofilms grown in the presence of CA at [DW], 100 × [DW], DMSO and STW. Motility halos were measured 24 h after incubation. Diameter of the initial drop was 7.2 ± 0.4 mm.

Condition	Colony growth halo (mm)		
	Swimming	Swarming	Twitching
STW	11.0 ± 0.1	8.6 ± 0.5	10.9 ± 0.6
DMSO	8.0 ± 0.0	8.5 ± 0.0	9.8 ± 0.8
CA [DW]	8.1 ± 0.3	8.9 ± 0.1	9.3 ± 0.1
CA 100 × [DW]	8.3 ± 0.8	8.6 ± 0.6	10.0 ± 0.9

statistically significant ($P > 0.05$).

3.1.5. Effects of CA exposure on S. maltophilia siderophores production

S. maltophilia was found to be able to produce siderophores for iron acquision when this metal is limiting in the environment (Table 4). The results further demonstrate that the diameter of the orange halo formed around the growing colony was very similar for all the CA-exposed cells and the control ($P > 0.05$).

3.1.6. Effects of CA exposure on S. maltophilia ability to adhere and internalize within HT-29 cells

S. maltophilia demonstrated to be able to adhere and internalize HT-29 cells (Fig. 3). The exposure to CA did not affect the ability of *S. maltophilia* to adhere on HT-29 cells. For both CA concentrations 5.19–5.29 log CFU of *S. maltophilia* was able to adhere ($P > 0.05$). Following adhesion, *S. maltophilia* was able to invade HT-29 cells. However, internalization was significantly lower for *S. maltophilia* obtained from CA-exposed biofilms ($P < 0.05$) (Fig. 3).

4. Discussion

The effects of the presence of CA on *S. maltophilia* tolerance to chlorine and antibiotics were evaluated under conditions mimicking DWDS. Also, the effects on bacterial virulence factors, such as biofilm formation, motility, siderophores production as well as adhesion and invasion of human colon carcinoma cells were assessed. *S. maltophilia* is an emerging pathogen characterized as a multi-drug resistant and often detected in tap water (Cervia et al., 2008; Amoli et al., 2017a, 2017b). Therefore, following the One Health initiative, it is of utmost importance to understand how ECs may affect bacterial behavior and the potential impacts from such behavioral changes for public health.

CA is a metabolite of clofibrate, etofibrate, and etofyllinclofibrate, pharmaceuticals commonly used as lipid regulators (Stumpf

Table 4
Siderophores production after exposure to CA. Diameter of the orange halo around the grown colony (mm).

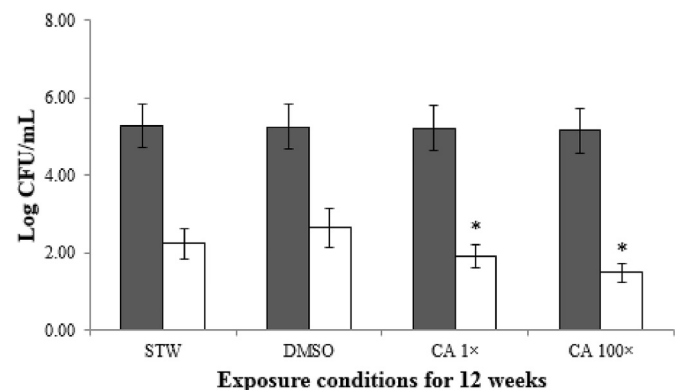| Condition	Siderophores production halo (mm)
STW	1.6 ± 0.3
DMSO	1.6 ± 0.6
CA [DW]	1.4 ± 0.4
CA 100 × [DW]	1.4 ± 0.1

Fig. 3. Effects of CA exposure on *S. maltophilia* interaction with HT - 29 cells. ■ - *S. maltophilia* adhesion on HT-29 cells; □ - *S. maltophilia* internalization in HT-29 cells. * - mean values statistically different from those obtained after the exposure to the solvent (DMSO) − $P < 0.05$.

et al., 1999). CA is also an isomer of the herbicide mecoprop (Buser et al., 1998) and was the first pharmaceutical detected in the environment as contaminant (Evangelista et al., 2010). Although it has been discontinued as pharmaceutical and new drugs have been used to replace it, this molecule is still a concern, due to its widespread occurrence and its environmental persistence (Emblidge and DeLorenzo, 2006). CA was selected for this study based on previous results (Gomes et al., 2018a) on ECs effect on bacteria, where CA was found to be present in all the combinations of ECs causing changes in bacterial behavior. For instance, *S. maltophilia* exposure to CA at 1700 ng/L or to the combination of CA (170 ng/L) and ibuprofen - IBP (3 ng/L) increased biofilm formation ability. On the other hand the exposure to the combination of CA (1700 ng/L) with carbamazepine (CBZ at 2580 ng/L) and IBP (30 ng/L) reduced biofilm productivity (Gomes et al., 2018a). The biofilms formed after exposure to the combination of CA (170 ng/L) and CBZ (258 ng/L) or CA (1700 ng/L), CBZ (2580 ng/L) and IBP (30 ng/L) were also more tolerant to disinfection with chlorine (Gomes et al., 2018b). Based on these results (Gomes et al., 2018a) and on CA environmental persistence (Schmidt et al., 2017), this EC was selected for the present study.

The tolerance of planktonic *S. maltophilia* to free chlorine was not affected from CA exposure. Concentrations of free chlorine, 2—4 mg/L, slightly higher than those recommended for chlorinated DWDS (0.5—1 mg/L) (WHO, 2011) were needed to cause bactericidal effects. Therefore, *S. maltophilia* may be able to survive in chlorinated systems, and other works already described the presence of *S. maltophilia* in chlorinated DWDS (Silbaq, 2009; Amoli et al., 2017a). The use of chlorine is a widespread strategy used to avoid bacterial growth along DWDS. However, it is not completely efficient and the presence of ARBs has been consistently reported (Xi et al., 2009; Li and Gu, 2018). The use of sub-inhibitory concentrations of disinfectants is known to be responsible for the increase of incidence of ARBs and ARGs in DWDS (Li and Gu, 2018). Previous studies propose that the presence of antibiotic residues in the environment are responsible for the selection and spread of ARBs and ARGs (Baquero et al., 2008; Martinez, 2009; Xie et al., 2019). However, information on the effects of non-antibiotic contaminants on bacterial resistance to antimicrobials is scarce. Subirats et al. (2017) studied the effects of contaminated wastewater on the resistome of biofilms. Using a variety of contaminants they found that biofilms exposed to wastewater had higher levels of ARBs and ARGs than biofilms exposed to treated wastewater. Wang et al. (2019) also verified that exposure to ciprofloxacin and sulfadiazine promoted ARGs spread in drinking water biofilms. The present results demonstrated that *S. maltophilia* was resistant to AMO, OXA and SPE, even without being exposed to CA (Table 2). *S. maltophilia* is commonly described as an opportunistic pathogen with intrinsic multidrug-resistance (Zhang et al., 2000; Sánchez, 2015; Adegoke et al., 2017; Rizek et al., 2018). However, it is also known that *S. maltophilia* can acquire resistance (Song et al., 2010; Furlan et al., 2018; Rizek et al., 2018). This study demonstrates that exposure to CA at 100 × [DW] increased *S. maltophilia* tolerance to ERY (Table 2, Figure S.1). Some works described that *S. maltophilia* strains may use efflux pump systems to pump out ERY such as the SmDEF, SmeOP, EmrCABsm and MexCD-OprJ, and in some cases the influx of this antibiotic through the bacterial membrane is not favored due to the reduced permeability of the outer membrane (Alonso et al., 2000; Safdar and Rolston, 2007; Chang et al., 2015; Adegoke et al., 2017). It is possible CA exposure may increase the activity of some *S. maltophilia* efflux systems or alter bacteria membrane permeability, therefore increase tolerance to ERY. Balagué and Véscovi (2001) demonstrated that exposure to CA, ethacrynic acid (EA) and 2,4-dichlorophenoxyacetic acid (2,4-D) altered the outer membrane permeability of *Escherichia coli*. *E. coli*

exposure to these aryloxoalcanoic agents - CA, EA and 2,4-D — caused the induction of the MarAB regulatory system (Balagué and Véscovi, 2001). Balagué et al. (2006) further studied the effects from exposure of *E. coli* to CA, EA and 2,4-D (three structurally analogue compounds) on ERY susceptibility. No significant effects from CA exposure were observed on the antibiotic action of ERY. On the other hand, *E. coli* was more tolerant to ERY when exposed to the other two CA analogs. It is important to emphasize that the concentrations used in that study are the therapeutic doses (CA — 28.6 mg/kg/d, EA — 2.9 mg/kg/d and 2,4-D — 70 mg/kg/d) which are significantly higher than the trace concentrations detected in environment (170—17000 ng/L). TMP-SMX is the most common therapeutic option used to treat *S. maltophilia* infections (Emblidge and DeLorenzo, 2006; Sánchez and Martínez, 2015) and tolerance to this combination of antibiotics was not observed despite the exposure of *S. maltophilia* to CA.

CA had no remarkable effects on *S. maltophilia* ability to form biofilms. Significant changes in biofilm formation ability were observed after exposure to DMSO, particularly the increase in biofilm formation. Lim et al. (2012) also observed an increase in *E. coli* biofilm formation after exposure to 4% DMSO. This emphasizes the importance of adequate control testing for a reliable analysis of results. Biofilms formed by *S. maltophilia* exposed to CA were more tolerant to chlorine action (Fig. 2). The changes in biofilm tolerance to chlorine were mostly in bacterial inactivation (Fig. 2), and not in biofilm removal (Fig. 1). This altered tolerance can result from changes on the bacterial membrane structure or associated proteins caused by the prolonged exposure to CA, as proposed by Balagué et al. (2006). Therefore, it is important to take into account that prolonged exposure of DW bacteria to CA may hinder DW disinfection with chlorine. For a shorter exposure to CA (26 d), Gomes et al. (2018a) did not observe changes in biofilm susceptibility to chlorine.

Siderophores are molecules produced by microorganisms whose main function is to chelate the ferric iron (Fe^{3+}) from different environments making it available for the producer microorganism. Siderophores production is considered a virulence factor as during the infectious process microbes secrete siderophores to acquire and solubilize ferric iron from the host (Wilson et al., 2016). Iron is essential to host cells, being regulated by iron transport systems. Nevertheless, the affinity of bacterial siderophores to iron are generally much higher than those of host proteins/molecules, allowing pathogens to outcompete the host in iron acquisition (Wilson et al., 2016). This study shows that *S. maltophilia* produces siderophores regardless the conditions tested. Nas and Cianciotto (2017) also described the production of siderophores by *S. maltophilia*, suggesting a unique siderophore structure for iron acquisition for this species. However, no changes in siderophores production were observed from CA exposure (Table 4).

S. maltophilia motility was also studied in the present work as it is an important factor affecting bacterial ability to form biofilms as well as the interaction with host cells (Trifonova and Strateva, 2018). In pathogens, motility and virulence are often linked by a complex regulatory system (Josenhans and Suerbaum, 2002). It is known that the ability of *S. maltophilia* to adhere on host tissues and infect host cells is related to motility (Adegoke et al., 2017; Trifonova and Strateva, 2018). Swimming motility is usually related to the ability of bacteria to move in liquid medium due to the presence of functional flagella (Ha et al., 2014). Swarming is related to the flagellum-mediated movement of bacteria, responsible for biofilm spread over a surface (Vanderleyden et al., 2004). In this study, the modest increase in swarming motility caused by CA did not cause changes in biofilm formation. Twitching motility is mediated by type IV pilli, a polar fimbriae responsible for bacterial

adhesion on eukaryotic cells and abiotic surfaces, also allowing its movement over surfaces (Deziel et al., 2001). The presence of fimbriae is a virulence factor described for *S. maltophilia* - essential for colonization of the host and crucial in the infection process (Trifonova and Strateva, 2018). The results demonstrated no significant changes in bacterial twitching motility after CA exposure (Table 3). Therefore, it is possible to predict that *S. maltophilia* adhesion on intestinal cells may not be influenced by CA exposure. In fact, the results corroborate this prediction and no significant effect was observed on the ability of CA exposed *S. maltophilia* to adhere on HT-29 cells (Fig. 3). CA exposure slightly decreased *S. maltophilia* ability to internalize within HT-29 cells. This can be attributed to changes in *S. maltophilia* invasion mechanisms caused by CA. Two mechanisms of bacterial invasion of hosts cells are recognized: the zipper mechanism of cell invasion (outer membrane proteins of bacteria will interact with the host membrane and force the cytoskeletal rearrangement for invasion); the trigger mechanism (is a consequence of the production of effectors that will rearrange the host cytoskeletal structure enabling invasion) (Ribet and Cossart, 2015). Balagué et al. (2004) observed that CA was responsible for a decrease in the expression of membrane proteins and fimbriae in *E. coli*, with significant impact on bacterial ability to invade host cells.

5. Conclusions

The presence of ECs in DW is a problem of potential environmental and public health concern. Their impacts on the DW microbiome and as drivers affecting resistance evolution/selection is far from being understood. This is particularly critical for non-antibiotic drugs. The present work showed that the presence of trace concentrations of CA did not affect planktonic *S. maltophilia* tolerance to chlorine. Nevertheless, the presence of CA in DW was responsible for the formation of biofilms with higher tolerance to chlorine disinfection. *S. maltophilia* ability to produce virulence factors and the ability to form biofilms was also not altered from CA exposure. Of additional concern was the changes observed in *S. maltophilia* susceptibility to antibiotics. After exposure to CA at 17000 ng/L *S. maltophilia* was more tolerant to ERY. Moreover, changes were observed on the ability of *S. maltophilia* to internalize within HT-29 cells. In general, the results propose that CA influenced *S. maltophilia* behavior. It is important to highlight that CA exposure was carried out for 12 weeks. It is expected that real environmental conditions where accumulation and exposure periods higher than 12 weeks can occur will increase the impact of CA on *S. maltophilia* planktonic and biofilm behavior.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.06.184.

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