Science of the Total Environment 643 (2018) 1348–1356

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

The effects of emerging environmental contaminants on *Stenotrophomonas maltophilia* isolated from drinking water in planktonic and sessile states

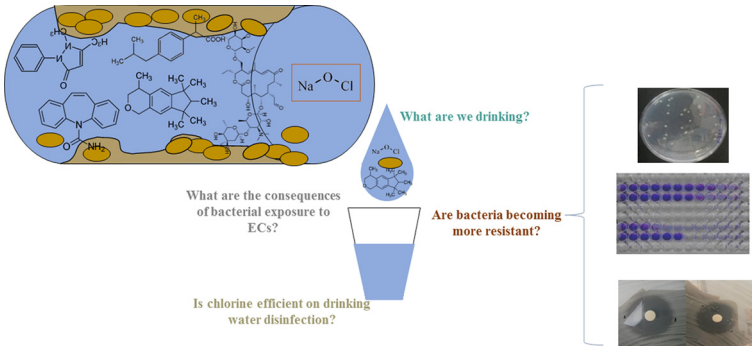
Inês B. Gomes [a], Lúcia C. Simões [a,b], Manuel Simões [a,*]

[a] LEPABE, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, s/n, 4200-465 Porto, Portugal
[b] CEB - Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

HIGHLIGHTS

- Exposure to ECs had no significant effect on *S. maltophilia* tolerance to antibiotics.
- The combination of CA, CBZ and/or IBP affected biofilm production.
- The combination of CA, CBZ and/or IBP increased biofilm tolerance to NaOCl.
- The exposure to ECs may hinder chlorine efficient in terms of biofilm control.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 5 February 2018
Received in revised form 2 June 2018
Accepted 21 June 2018
Available online xxxx

Keywords:
Biofilm control
Chlorine
Drinking water disinfection
Emerging contaminants
Stenotrophomonas maltophilia

ABSTRACT

Concerns on the presence of emerging contaminants (ECs) in water sources have increased in recent years. The lack of efficient technologies to remove ECs from residual waters contributes for their appearance in drinking water distribution systems (DWDS). Therefore, sessile microorganisms on DWDS pipes are continuously exposed to trace concentrations of ECs. However, no data exists on the role of ECs on the resident microbiota. The present work aims to understand the effects of prolonged exposure of a bacterial strain of *Stenotrophomonas maltophilia*, isolated from a DWDS, in both planktonic and biofilm states, to trace concentrations of selected ECs (antipyrine–ANTP; diclofenac sodium salt–DCF; ibuprofen–IBP; galaxolide–GAL; tonalide–TON; carbamazepine–CBZ; clofibric acid–CA; tylosin–TY) on its tolerance to sodium hypochlorite (NaOCl) and resistance to antibiotics. Pre-established *S. maltophilia* biofilms were exposed to ECs for 26 d. Subsequently, the planktonic behaviour of the biofilm cells grown in the presence of ECS was characterized in terms of susceptibility to NaOCl and to selected antibiotics (levofloxacin and trimethoprim-sulfamethoxazole). Moreover, *S. maltophilia* was tested on its biofilm productivity in the presence of ECs (alone and mixed). These biofilms were challenged by NaOCl in order to assess the role of ECs on biofilm susceptibility. The results did not evidence remarkable effects of ECs on planktonic *S. maltophilia* susceptibility to NaOCl and antibiotics. However, *S. maltophilia* biofilm production and susceptibility to NaOCl was affected from ECs pre-exposure, particularly by the combination of different ECs (CA + CBZ, CA + IBP, CA + CBZ + IBP). *S. maltophilia* biofilms became more resistant to removal by NaOCl when developed in the presence of mixtures of CA + CBZ and CA + CBZ + IBP. Also, biofilm production was significantly affected. CA was present in all the combinations that altered biofilm behaviour. The overall results propose that exposure to ECs for 26 days had not a huge impact on *S. maltophilia* planktonic antimicrobial

* Corresponding author.
E-mail address: mvs@fe.up.pt (M. Simões).

https://doi.org/10.1016/j.scitotenv.2018.06.263

susceptibility. Nevertheless, the prolonged exposure to some ECs altered biofilm production and tolerance to NaOCl, with a potential practical outcome of hindering DWDS disinfection. The simultaneous presence of different ECs in the environment may amplify biofilm resilience.

1. Introduction

Emerging contaminants (ECs), also defined as micropollutants, are a vast and expanding array of anthropogenic and natural substances that have been recently found in the environment. These ECs are continuously released into the environment and have been found in surface water, wastewater, groundwater and drinking water (DW), at very low concentrations (from ng/L to μg/L) (Petrie et al., 2015; Richardson and Kimura, 2017). ECs include pharmaceuticals and personal care products (PPCPs), steroid hormones, industrial chemicals, pesticides and many other emerging substances (Luo et al., 2014). Current wastewater treatment plants (WWTPs) are unable to completely remove ECs. Therefore, these compounds arrive to water typically from wastewater treatment plants that treat domestic sewage, wastewater from hospital effluents and chemical manufacturing plants, as well as from livestock and agriculture (Cunningham et al., 2009; Pal et al., 2010; Zhang et al., 2014). Water effluents are discharged into rivers and watercourses, while sludge is spread on the soil as fertilizers, enabling ECs to reach all the environmental water sources (Vulliet and Cren-Olive, 2011). Furthermore, some ECs are environmentally persistent, bioactive and have the potential for bioaccumulation (de Solla et al., 2016; Sui et al., 2015). The worldwide detection of ECs in DW took the attention of the World Health Organization (WHO), who published a report exclusively about the presence of pharmaceuticals in DW and the potential risks for human health and for the environment (WHO, 2011). Regardless the limited knowledge on the ECs toxicity to human health, DW is a major focus of consumers concern as it is a direct route for ECs to the human body (Jones et al., 2005). In fact, the presence of ECs in the aquatic environment has been frequently associated to diverse problems: toxicity, endocrine disrupting effects and antibiotic resistance of microorganisms. For example, pharmaceutical compounds which are specially designed to produce biological response in a target organism may also produce this response in non-target organisms when chronically exposed to trace concentrations of these compounds (Wilkinson et al., 2016). Some ECs, namely endocrine disrupting chemicals, are known to affect the endocrine function of non-target organisms disrupting the estrogenic hormonal pathways (Fisher and Eugster, 2014; Sarria et al., 2011; Vajda et al., 2011). Some anti-inflammatory drugs, such as diclofenac and naproxen, can cause impairment of health of non-target organisms found in stream biofilms (Corcoll et al., 2014). Schwaiger et al. (2004) found that the extended exposure to trace levels of diclofenac caused alterations in rainbow trout kidney and gills. Naproxen at sublethal concentrations was responsible for oxidative stress and genotoxicity in *Hyalella azteca*, an amphipod crustacean (Lucero et al., 2015). Some other ECs as plasticizers or musk fragrances were detected in human tissues and fluids. Galaxolide and tonalide are two polycyclic musk fragrances whose ecological and human toxicity is still unknown. Nevertheless, these compounds have been detected in human blood, adipose tissue and breast milk (Wombacher and Hornbuckle, 2009). Duedahl-Olesen et al. (2005) also found galaxolide in trout samples. A possible estrogenic activity of musk fragrances was described by Bitsch et al. (2002), who observed an increase in the proliferation rate of human MCF-7 breast cancer cells related with the presence of two nitro musks (musk xylene, musk ketone), *p*-amino musk xylene (a major metabolite of musk xylene) and tonalide. The exposure to ECs is also known to be responsible for changes in the composition and behavior of microbial communities. Of particular concern is the development of resistance to antibiotics and the spread of antibiotic resistant bacteria (ARB) and genes (ARG), which can be a consequence of the prolonged presence of antibiotics in aquatic environments (Baquero et al., 2008; Berglund, 2015; Martinez, 2009). However, not only antibiotics may be responsible for bacterial resistance to antibiotics. Several works demonstrated that water pollution with ECs, including non-antibiotic drugs, has an important role on the acquisition and spread of bacterial resistance determinants (Corcoll et al., 2014; Subirats et al., 2017, 2018). As most of ECs are used worldwide and are essential for modern society (Gavrilescu et al., 2015) it is of utmost importance to better understand their possible effects on the exposed microbiota.

Despite the multiple disinfection processes used in drinking water distribution systems (DWDS) to keep the DW microbiologically safe for consumers, biofilm development in pipe walls and other fittings is unavoidable (Simões and Simões, 2013). The presence of biofilms constitutes a global concern for DW companies. In fact, biofilms can act as reservoir for pathogenic microorganisms that, if released to the bulk water, may be a source of waterborne diseases (Simões and Simões, 2013). Microorganisms in biofilms are protected from environmental stresses due to the production of an extracellular polymeric matrix that hinders the penetration of disinfectants and other antimicrobials. The sophisticated biofilm structure causes the failure of conventional DW disinfection strategies in effective biofilm control (Gomes et al., 2016, 2018).

The aim of the present work was to understand the role of prolonged exposure to selected ECs on biofilm formation and antimicrobial tolerance of a *Stenotrophomonas maltophilia* strain isolated from DW. *S. maltophilia* is an emerging opportunistic bacterium characterized by its multi-drug resistance to antibiotics (Brooke, 2012). Moreover, *S. maltophilia* strains have been found in DWDS (Guyot et al., 2013; Vincenti et al., 2014) and shown to be resistant to chlorine disinfection (Gomes et al., 2016). Eight ECs, including pharmaceuticals and fragrances were used in the present study. The main classes of pharmaceuticals detected in water sources (Fatta-Kassinos et al., 2011) were tested: NSAIDs (ibuprofen, diclofenac, antipyrine), lipid regulators (clofibric acid) and antibiotics (tylosin). Carbamazepine was selected attending its high recalcitrance and the difficulty to remove this compound from water sources (Golan-Rozen et al., 2011). Galaxolide and tonalide were selected as representative musk fragrances, commonly found in water sources and already detected in human tissues (Golan-Rozen et al., 2011; Wombacher and Hornbuckle, 2009). The main results of the present work propose that exposure to ECs for 26 d had not a huge impact on *S. maltophilia* planktonic antimicrobial susceptibility. Nevertheless, the prolonged exposure to some ECs altered biofilm production and tolerance to NaOCl.

2. Materials and methods

2.1. Bacteria and culture conditions

Stenotrophomonas maltophilia was used as a model microorganism from DW (Simões et al. (2007a). Bacterial cells were grown overnight at 25 °C and under agitation (120 rpm) in R2A broth medium: 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) and 0.4 g/L di potassium phosphate trihydrate (Applichem Panreac, Darmstadt, Germany).

2.2. Selected emerging contaminants

Three nonsteroidal anti-inflammatory drugs (NSAIDs) (antipyrine - ANTP - from Alfa Aesar (Karlsruhe, Germany); diclofenac sodium salt - DCF - from Fluka Steinheim, Germany) and ibuprofen – IBP - from Alfa Aesar, two musk fragrances (galaxolide – GAL - and tonalide - TON - both from Sigma-Aldrich), one neuro-active drug (carbamazepine - CBZ - from Acros Organics, New Jersey, USA), one lipid regulator (clofibric acid - CA - from Acros Organics, New Jersey, USA) and one veterinary antibiotic (tylosin - TY - from Sigma-Aldrich, Steinheim, Germany) were selected as emerging contaminants for the experiments. Stock solutions (from 800 to 1000 mg/L) were prepared in dimethyl sulfoxide (DMSO) (Fisher Scientific, Leicestershire, UK) and stored at −20 °C until use. The concentrations tested were prepared from the stock solutions using synthetic tap water (STW) (EPA, 2011): 100 mg/L $NaHCO_3$ (Fisher Scientific, Leicestershire, UK), 13 mg/L $MgSO_4.7H_2O$ (Merck, Darmstadt, Germany), 0.7 mg/L K_2HPO_4 (Applichem Panreac, Darmstadt, Germany), 0.3 mg/L KH_2PO_4 (CHEMLAB, Zedelgem, Belgium), 0.01 mg/L $(NH_4)_2SO_4$ (Labkem, Barcelona, Spain), 0.01 mg/L NaCl (Merck, Darmstadt, Germany), 0.001 mg/L $FeSO_4.7H_2O$ (VWR PROLABO, Leuven, Belgium), 1 mg/L $NaNO_3$ (Labkem, Barcelona, Spain), 27 mg/L $CaSO_4$ (Labkem, Barcelona, Spain), 1 mg/L humic acids (Sigma-Aldrich, Steinheim, Germany) (EPA, 2011). DMSO final concentration in each solution was 1% (v/v).

Table 1 shows information on the ECs tested and the respective concentrations found in DWDS and used in the present study. The concentration of each EC in combinations was the same used for the tests using single compounds ([DW] and 100 × [DW]).

2.3. Experimental set-up

The experimental set-up is constituted of four main steps (Fig. 1). Firstly, *S. maltophilia* biofilms were developed on PVC coupons for 24 h using 48 wells microtiter plates (Section 2.4 – Fig. 1.A). Afterwards, coupons with biofilms were exposed to ECs for 26 d in a new 48 wells microtiter plate (Section 2.6 – Fig. 1.B). Biofilms exposed to ECs were characterized in terms of numbers of colony forming units (CFU) (Section 2.6). Moreover, these coupons were used to inoculate fresh R2A broth (Section 2.6 – Fig. 1.C) to characterize the bacteria exposed to ECs (Section 2.6 – Fig. 1.D) in terms of planktonic behaviour (determination of the minimum bactericidal concentration of NaOCl - Section 2.6.1; susceptibility to antibiotics - Section 2.6.2), biofilm productivity (Section 2.6.3); and susceptibility to NaOCl (Section 2.6.4).

2.4. Biofilm pre-establishment

S. maltophilia biofilms were formed on polyvinyl chloride (PVC) for 24 h. PVC coupons (1 × 1 cm) were used as substratum for biofilm formation. PVC was selected as a representative pipe material from DW networks (SDWC et al., 1982). In order to clean and sterilize PVC for further analysis, coupons were immersed in a solution of a commercial detergent (Sonasol Pril, Henkel Ibérica S.A.) in distilled water for 30 min. Afterwards, the coupons were rinsed in distilled water and subsequently immersed in ethanol at 70% for 30 min. After that, coupons were rinsed three times with distilled sterile water and dried overnight at 60 °C (Simões et al., 2007a). Then PVC coupons were placed in 48 well microtiter plates and exposed to ultra-violet (UV) light for 30 min before being used for biofilm formation. After sterilization, 1 mL of bacterial suspension in R2A broth (2×10^8 CFU/mL) was added to each well. Microtiter plates were incubated for 24 h at 25 °C and under agitation (120 rpm) – Fig. 1.A.

2.5. S. maltophilia biofilms exposure to ECs

After 24 h, colonized PVC coupons, prepared as described in Section 2.4, were carefully removed from the wells and placed in new microtiter plates with STW in order to remove the weakly and non-adherent bacteria. Then, *S. maltophilia* biofilms were exposed to ECs for 26 days (Fig. 1.B). For that, colonized coupons were inserted in other microtiter plates with the ECs solutions (prepared as described previously) using STW at environmental concentrations found in DWDS ([DW]) and at 100 times the [DW] (100 × [DW]). ECs solutions were renewed every 2 days, in order to ensure the continuous exposure to a constant amount of ECs for 26 days. Before being placed in new ECs solutions, colonized coupons were carefully washed in STW to remove non-adherent bacteria and the ECs solution. Two controls were performed: biofilm in contact with STW and biofilm in contact with 1% DMSO (v/v) in STW. After 26 days of continuous exposure to ECs, the numbers of CFU of *S. maltophilia* biofilms were determined. The planktonic behaviour of bacteria grown from biofilms exposed to ECs was also evaluated. For that, colonized coupons were used to inoculate R2A broth (Fig. 1.C) in order to characterize bacterial susceptibility to NaOCl and antibiotics (levofloxacin and trimethoprim-sulfamethoxazole) and its ability to form biofilms.

Bacterial adaptation to ECs exposure for 26 days was performed in duplicate with three independent assays.

2.6. Characterization of bacteria exposed to ECs

Coupons with biofilms exposed to ECs were inserted in 50 mL falcon tube containing 10 mL of R2A broth (Fig. 1.C). Bacterial cells were naturally released from coupons to the bulk medium and were grown overnight at 25 °C and 120 rpm for further evaluation of the effects of ECs on *S. maltophilia* behaviour. These bacteria were subsequently characterized in terms of susceptibility to NaOCl (Section 2.6.1) and to antibiotics (Section 2.6.2), as well as biofilm formation ability (Section 2.6.3) and biofilm susceptibility to NaOCl (Section 2.6.4) – Fig. 1.D.

2.6.1. Minimum bactericidal concentrations

A pre-culture inoculated from a PVC coupon colonized with *S. maltophilia* biofilms exposed to ECs and grown as described previously was centrifuged (12 min, 3777*g*) and resuspended in STW to achieve a concentration of 3×10^5 CFU/mL in order to mimic the number of cells present in DWDS (Prest et al., 2016). NaOCl (Acros Organics,

Table 1
Selected emergent contaminants (ECs) and respective concentration detected in DWDS. The concentration detected in DWDS ([DWDS]) and a concentration 100 times higher was tested in the present work.

Class of contaminants	ECs	Abbreviation	[DWDS] (ng/L)	References
Nonsteroidal anti-inflammatory drugs (NSAIDs)	Antipyrine	ANTP	400	Reddersen et al. (2002)
	Diclofenac sodium salt	DCF	6	Jones et al. (2005)
	Ibuprofen	IBP	3	Jones et al. (2005)
Musk fragrances	Galaxolide	GAL	2.2	Wombacher and Hornbuckle (2009)
	Tonalide	TON	0.51	Wombacher and Hornbuckle (2009)
Neuro-active drug	Carbamazepine	CBZ	24–258	Jones et al. (2005)
Lipid regulator	Clofibric acid	CA	5.3–170	Jones et al. (2005)
Veterinary antibiotic	Tylosin	TY	1.7	Jones et al. (2005)

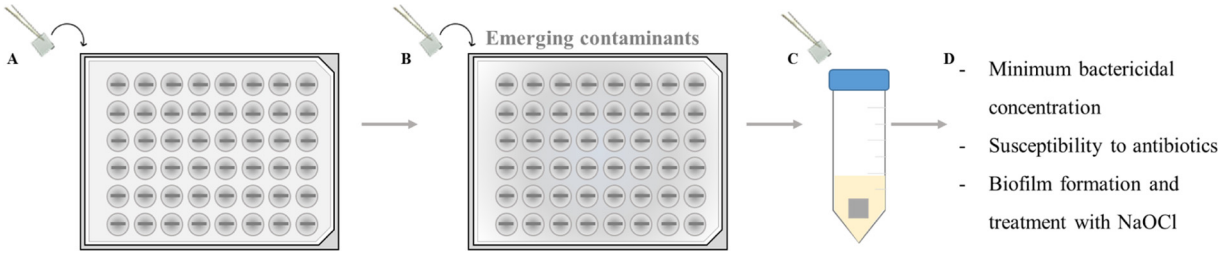


Fig. 1. Experimental set-up. A – Biofilm formation for 24 h in PVC coupons (25 °C, 120 rpm). B – Biofilm exposure to ECs for 26 days in STW (25 °C, 120 rpm). C – Inoculation of R_2A using a colonized coupon exposed to ECs (25 °C, 120 rpm, overnight). D – Characterization of bacteria after biofilms exposure to ECs.

New Jersey, USA) was prepared at different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 and 150 mg/L) in sterile distilled water. A volume of 20 μL of each NaOCl solution was added to each well of a 96-well microtiter plate containing 180 μL of bacterial suspension in STW. The microtiter plate was incubated for 24 h at 25 °C and 120 rpm. Then, 180 μL of each well was discarded and 180 μL of sodium thiosulfate (0.5% w/v) was added to quench NaOCl action (Gomes et al., 2016). From each well, 20 μL was spread on R2A agar plates and incubated for 48 h at 25 °C. The minimum bactericidal concentration (MBC) corresponds to the lowest concentration of NaOCl at which no growth was found. Each condition was tested in triplicate in three independent experiments.

2.6.2. Susceptibility to antibiotics

S. maltophilia grown as described in Section 2.6. were characterized on its susceptibility to antibiotics by the disk diffusion susceptibility methods according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2007). Levofloxacin (LEV) at 5 μg and trimethoprim-sulfamethoxazole (TMP-SMX) at 1.25/23.75 μg were the selected antibiotics, according to the CLSI guidelines for *S maltophilia* (CLSI, 2007). A bacterial inoculum (6×10^8 CFU/mL), prepared from coupons colonized with biofilms, was exposed to ECs and spread on Mueller-Hinton agar (MHA). MHA plates were incubated at 37 °C for 24 h before measuring the diameter of growth inhibition. Each condition was tested in duplicate with three independent experiments.

2.6.3. S. maltophilia biofilm formation after exposure to ECs

Bacteria obtained from biofilms exposed to ECs were characterized in terms of biofilm production. Biofilms were developed according to the modified microtiter plate test proposed by Stepanović et al. (2000). Briefly, sterile 96-wells microtiter plates were filled with 200 μL of bacterial suspension (2×10^8 CFU/mL in R2A broth). Negative control wells were filled with sterile R2A. The plates were incubated for 24 h at 25 °C and agitated at 120 rpm. Biofilm production was assessed in terms of CFU and by crystal violet (Merck, Darmstadt, Germany) staining.

2.6.3.1. CFU enumeration. After 24 h of incubation, the bulk suspension was discarded and each well was washed with 200 μL of NaCl solution at 8.5 g/L in order to remove the non-adhered and weakly adhered bacteria. Afterwards, biofilms were scrapped for 1 min with the pipette tip and resuspended two times in 250 μL of NaCl solution. The number of CFU was assessed in R2A agar. CFU were determined 48 h after incubation at 25 °C and the results are presented in terms of log CFU/cm^2.

2.6.3.2. Crystal violet staining. After 24 h of incubation the bacterial suspension in the microtiter plate was discarded and each well was washed with 200 μL of sterile water in order to remove the non-adhered and weakly adhered bacteria. Afterwards, biofilms was fixed with ethanol (Fisher, Leicestershire, UK) for 15 min and stained with crystal violet (1% v/v) for 5 min. Acetic acid (33% v/v) was used to elute the crystal violet from the stained biofilm. Absorbance was measured in microtiter plate reader at 570 nm, according to Simões et al. (2007b).

2.6.4. NaOCl effects on biofilms formed by bacteria exposed to ECs

Biofilms developed as described previously (Section 2.6.3) were treated with NaOCl to evaluate biofilm susceptibility to this disinfectant. After 24 h of biofilm formation, the bulk suspension was discarded and each well was washed with NaCl solution at 8.5 g/L. Then, 180 μL of STW and 20 μL of NaOCl solution at 10 × MIC (minimum inhibitory concentration) were added to each well for 30 min at 25 °C and 120 rpm. NaOCl was used at a final concentration of 130 mg/L as this value corresponds to the minimum inhibitory concentration for *S. maltophilia* in R2A broth (data not shown). Negative controls corresponded to the use of 200 μL of STW. After 30 min exposure NaOCl was removed from each well and the remaining biocide was neutralized with sodium thiosulfate at 0.50% (*w*/*v*) for 10 min (Gomes et al., 2016). NaOCl effects on biofilms were assessed by CFU enumeration and crystal violet (Merck, Portugal) staining according to Sections 2.6.3.1 and 2.6.3.2.

2.7. Statistical analysis

Data were analysed applying the one-way analysis of variance (ANOVA) and the comparisons between and within experimental groups were carried out using Tukey test. The software used for statistical analysis was IBM® SPSS® Statistics (Statistical Package for the Social Sciences) version 24.0. Statistical calculations were based on confidence level ≥ 95% ($P < 0.05$) which was considered statistically significant.

3. Results

3.1. Effect of ECs on pre-established S. maltophilia biofilms

Fig. 2 presents the log CFU/cm^2 of *S. maltophilia* biofilms grown on PVC coupons in the presence of ECs (alone and combined) for 26 days. The exposure to GAL slightly increased log CFU/cm^2, even if no significant differences were observed from the other situations ($P > 0.05$). In general, no statistical significant differences were observed on *S. maltophilia* log CFU/cm^2 from the exposure to ECs at [DWDS] and at 100 × [DWDS] ($P > 0.05$).

3.2. Effects of ECs on NaOCl action against planktonic S. maltophilia

Bacteria from the 26 days ECs exposed biofilms were evaluated for their susceptibility to NaOCl (Table 2). The MBC of NaOCl varied from 2.0 to 5.0 mg/L. The presence of 1% (v/v) of DMSO did not cause significant differences in the MBC (3.5–5.0 mg/L), showing that the solvent at the concentration used had no influence on *S. maltophilia* tolerance to NaOCl ($P > 0.05$). CBZ at [DWDS] and the combination of CBZ and IBP at [DWDS] caused a slight increase of MBC, however, not statistically significant ($P > 0.05$). The increase of ECs concentration to 100 × [DWDS] caused no significant changes in *S. maltophilia* susceptibility to NaOCl ($P > 0.05$).

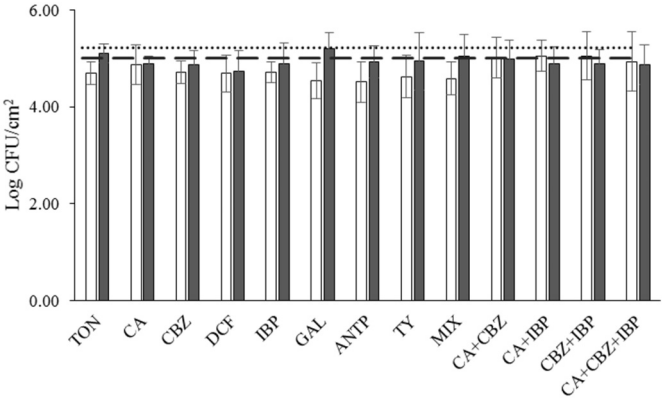


Fig. 2. Log CFU/cm^2 of *S. maltophilia* biofilms after growing for 26 days in the presence of the selected ECs at [DWDS] (□) and 100 × [DWDS] (■). … – biofilm not exposed to ECs (only in STW), ——— Solvent control (biofilms only exposed to DMSO at 1% (v/v)). * TON – tonalide, CA – clofibric acid, CBZ – carbamazepine, DCF – diclofenac, IBP – ibuprofen, GAL – galaxolide, ANTP – antipyrine, TY – tylosin, MIX – Mixture of all compounds.

3.3. Effects of ECs on S. maltophilia susceptibility to antibiotics

S. maltophilia was susceptible to the selected antibiotics (inhibition halo ≥1.7 cm for LEVO and ≥1.6 cm for TMP-SMX) (Table 3). No significant differences in antibiotic susceptibility were observed when using *S. maltophilia* grown in the absence and presence of ECs at [DWDS] or 100 × [DWDS] ($P > 0.05$).

3.4. Effect of ECs on S. maltophilia biofilm production

S. maltophilia from biofilms exposed to ECs were resuspended and used to assess its ability to produce biofilms (Figs. 2 and 3). This was performed to ascertain the influence of ECs pre-exposure in biofilm production. A slight increase in biofilm production was observed when *S. maltophilia* was exposed to the combinations MIX and CA + IBP (Fig. 3). However, in general, the selected ECs (alone and combined) at [DWDS] had no remarkable effects on the ability of *S. maltophilia* to form biofilms ($P > 0.05$). On the other hand, the exposure to ECs at 100 × [DWDS] caused some changes in biofilm formation. *S. maltophilia* exposed to the combination CA + CBZ + IBP at 100 × [DWDS] reduced biofilm production (Fig. 3 and Fig. 4) ($P < 0.05$). The exposure to DCF, IBP and TY at 100 × [DWDS] increased biofilm production, particularly in terms of total biomass (Fig. 4 – $P < 0.05$). These differences were not observed in terms of biofilm CFU (Fig. 3 - $P > 0.05$). Only a modest increase in biofilm CFU was observed from the exposure to CA and MIX at 100 × [DWDS] ($P > 0.05$).

Table 2
Minimum bactericidal concentration of NaOCl (mg/L) after *S. maltophilia* exposure to ECs for 26 days.

	MBC (mg/L)	
	[DWDS]	100 × [DWDS]
STW	2.0–5.0	
DMSO	3.5–5.0	
EC[a]		
TON	3.0–6.0	3.0–4.5
CA	3.0–5.0	3.0–5.0
CBZ	4.5–6.0	2.0–6.0
DCF	3.0–5.0	3.0–5.0
IBP	3.0–5.0	3.0–5.0
GAL	3.0–5.0	3.0–5.0
ANTP	2.5–6.0	3.0–6.0
TY	2.0–5.0	3.5–4.0
MIX	4.0–5.0	3.5–5.0
CA + CBZ	4.0–5.5	4.5–5.0
CA + IBP	4.0–5.5	2.5–5.5
CBZ + IBP	4.0–6.5	2.5–5.5
CA + CBZ + IBP	4.0–5.0	3.5–4.0

[a] DMSO – dimethylsulfoxide, STW – synthetic tap water, Ton – tonalide, CA – clofibric acid, CBZ – carbamazepine, DCF – diclofenac, IBP – ibuprofen, GAL – galaxolide, ANTP – antipyrine, TY – tylosin, MIX – mixture of all compounds.

Table 3
Inhibition halo diameter (cm) for LEVO and TMP-SMX, after *S. maltophilia* exposure to ECs for 26 days.

	Inhibition halo diameter (cm)			
	LEVO		TMP-SMX	
	[DWDS]	100 × [DWDS]	[DWDS]	100 × [DWDS]
STW	2.90 ± 0.21		2.30 ± 0.08	
DMSO	2.83 ± 0.21		2.26 ± 0.13	
EC[a]				
TON	2.81 ± 0.16	2.95 ± 0.15	2.31 ± 0.06	2.23 ± 0.09
CA	2.78 ± 0.20	2.99 ± 0.08	2.33 ± 0.07	2.38 ± 0.14
CBZ	2.73 ± 0.20	2.96 ± 0.03	2.23 ± 0.19	2.20 ± 0.20
DCF	2.77 ± 0.15	2.93 ± 0.13	2.29 ± 0.16	2.32 ± 0.19
IBP	2.74 ± 0.13	2.94 ± 0.12	2.22 ± 0.14	2.34 ± 0.16
GAL	2.77 ± 0.06	2.96 ± 0.02	2.22 ± 0.18	2.31 ± 0.13
ANTP	2.82 ± 0.09	3.09 ± 0.16	2.31 ± 0.24	2.52 ± 0.37
TY	2.84 ± 0.16	2.99 ± 0.03	2.17 ± 0.14	2.36 ± 0.11
MIX	2.84 ± 0.26	2.99 ± 0.18	2.12 ± 0.21	2.43 ± 0.16
CA + CBZ	2.84 ± 0.09	2.92 ± 0.04	2.35 ± 0.26	2.38 ± 0.13
CA + IBP	3.03 ± 0.07	3.01 ± 0.15	2.28 ± 0.23	2.43 ± 0.26
CBZ + IBP	2.90 ± 0.11	2.87 ± 0.07	2.24 ± 0.23	2.32 ± 0.15
CA + CBZ + IBP	2.85 ± 0.07	2.96 ± 0.11	2.19 ± 0.26	2.36 ± 0.20

[a] DMSO – dimethylsulfoxide, STW – synthetic tap water, Ton – tonalide, CA – clofibric acid, CBZ – carbamazepine, DCF – diclofenac, IBP – ibuprofen, GAL – galaxolide, ANTP – antipyrine, TY – tylosin, MIX – Mixture of all compounds.

3.5. Effect of ECs on the susceptibility of S. maltophilia biofilms to NaOCl

The susceptibility of *S. maltophilia* biofilms to NaOCl was evaluated in terms of CFU (Fig. 3) and biomass removal (Fig. 5). The treatment with NaOCl at 130 mg/L for 30 min was not enough to completely inactivate or remove biofilms. NaOCl had no effects on biofilm CFU reduction, regardless the ECs and the concentration under which the biofilms were grown (Fig. 3). In terms of removal, the biofilms formed by bacteria grown in the presence of CA + CBZ at [DWDS] and to CA + CBZ + IBP at 100 × [DWDS] were more resistant to removal by NaOCl ($P < 0.05$). The exposure to TY and MIX at [DWDS] and at 100 × [DWDS] slightly decreased biofilm removal, although without statistical significance ($P > 0.05$).

4. Discussion

Recent studies have demonstrated the presence of ECs in DWDS highlighting their potential public health risks. In fact, there is the potential toxicological risk from the intake of chemically contaminated water (Schriks et al., 2010). However, DWDS are highly colonized by microorganisms. This is particularly relevant when looking to the surface materials of DWDS where the microorganisms can account for 95% of the cells present in the system (Flemming et al., 2002). These cells are inevitably exposed to trace concentrations of ECs. Nevertheless, to our knowledge no studies are available on the effects of ECs on the behaviour of DW bacteria and on their susceptibility to disinfection. The study of ECs mainly focuses human health and aquatic life, including the study of fluvial biofilms (Brodin et al., 2014; Lei et al., 2015; Luis et al., 2016). In this study, a *S. maltophilia* strain isolated from a DWDS (Simões et al., 2007a) was selected to assess the role of ECs on biofilm formation and antimicrobial susceptibility. *S. maltophilia* is considered an emerging pathogen and strains of this species are encountered in DW (Brooke, 2012; Guyot et al., 2013; Vincenti et al., 2014).

This study demonstrates that ECs (alone and combined) at the concentrations used had no antimicrobial effects on *S. maltophilia* biofilms. Also, they had no noticeable action in stimulating biofilm development

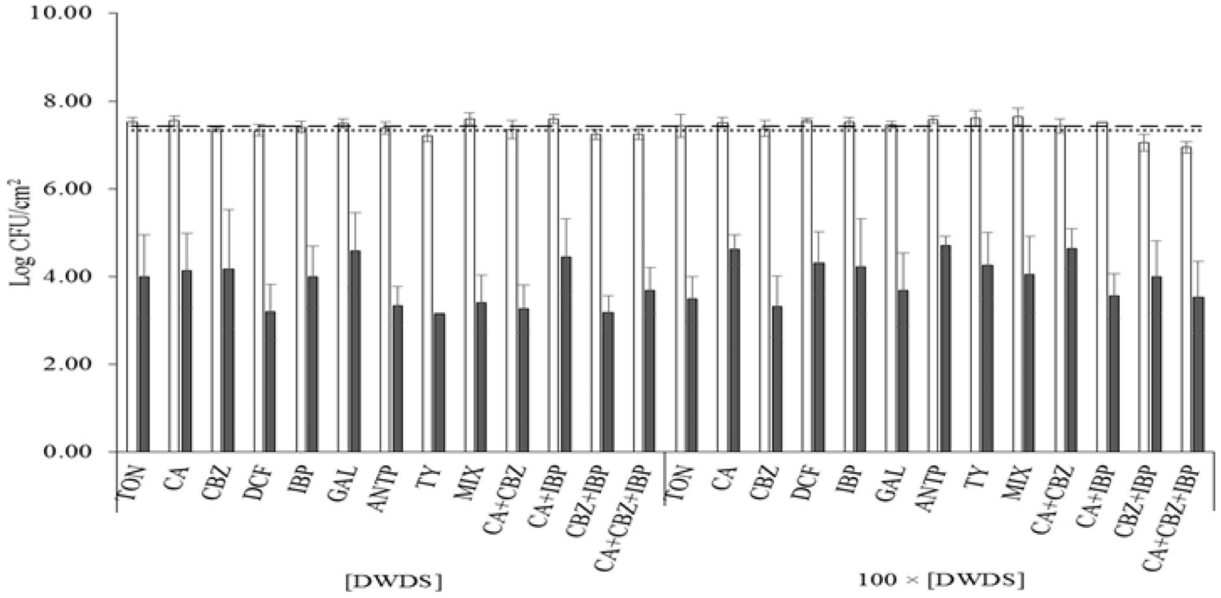


Fig. 3. Log CFU/cm^2 of 24 h *S. maltophilia* biofilms formed after the previous exposure to ECs at [DWDS] and 100 × [DWDS] for 26 d to form biofilms and treated with NaOCl at 130 mg/L for 30 min. □ – 0 mg/L of NaOCl (biofilm formation); ■ – 130 mg/L of NaOCl (biofilm inactivation); … – biofilm not exposed to ECs (only in STW), ——— Solvent control, biofilms only exposed to DMSO. * Ton – tonalide, CA – clofibric acid, CBZ – carbamazepine, DCF – diclofenac, IBP – ibuprofen, GAL – galaxolide, ANTP – antipyrine, TY – tylosin, MIX – Mixture of all compounds.

when *S. maltophilia* was inoculated with ECs (biofilm CFU counts were not affected after 26 d exposure to ECs). Nevertheless, the presence of ECs at higher concentrations in rivers was found to change the composition of biofilm communities, depending on the ECs present in the environment (Bonnineau et al., 2010; Corcoll et al., 2015; Proia et al., 2011; Proia et al., 2013; Shaw et al., 2015). For example, Bonnineau et al. (2010) exposed fluvial biofilms to β-blockers at different concentrations (0.9 μg/L to 9000 μg/L for propranolol and metoprolol, 0.9 μg/L to 900,000 μg/L for atenolol) and described that higher concentrations of metoprolol were toxic for bacteria and propranolol was responsible for the inhibition of algal photosynthesis. On the other hand, Proia et al. (2013) studied the effect of polluted and highly polluted water on fluvial biofilms and concluded that the presence of high concentrations of ibuprofen and paracetamol in river waters may be responsible for a decrease in algal photosynthetic capacity. Other studies reported bacterial death on fluvial biofilms caused mainly by the presence of antimicrobial contaminants, particularly antibiotics (erythromycin, trimethoprim and clindamycin) (Waiser et al., 2016) and triclosan (Proia et al., 2011; Ricart et al., 2010; Shaw et al., 2015; Waiser et al., 2016). Also, Osorio et al. (2014) observed a decrease of bacterial viability when biofilms were translocated from a less to a more polluted site of the river. Therefore, biofilms can be an important way to evaluate the impact of ECs on water systems (Aubertheau et al., 2017). Nevertheless, it is important to mention that fluvial biofilms are significantly different from DWDS biofilms, as they are composed by algae, bacteria, protozoa, cyanobacteria and fungi and are not formed in the presence of chronic residual concentrations of disinfectant (Corcoll et al., 2015). These differences limit any accurate comparison between the results obtained in the present study and the available literature.

The use of chlorine is the most commonly used strategy for DW disinfection. However, the existence of DW bacteria resistant to chlorine is a public health concern and different works already reported the

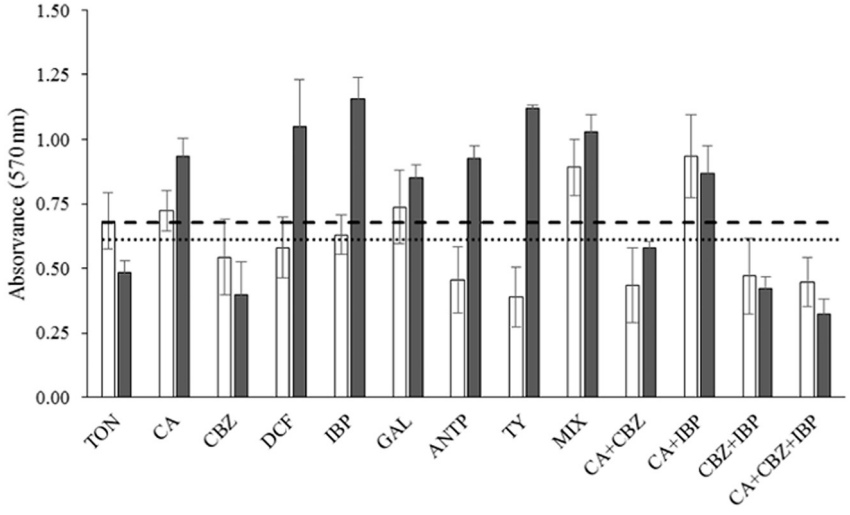


Fig. 4. Biofilm formation for 24 h after *S. maltophilia* exposure to ECs for 26 days in terms of total biomass assessed by crystal violet staining (absorbance at 570 nm). □ – [DWDS]; ■ – 100 × [DWDS]; … - biofilm not exposed to ECs (only in STW), ——— Solvent control, biofilms only exposed to DMSO. * Ton – tonalide, CA – clofibric acid, CBZ – carbamazepine, DCF – diclofenac, IBP – ibuprofen, GAL – galaxolide, ANTP – antipyrine, TY – tylosin, MIX – Mixture of all compounds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

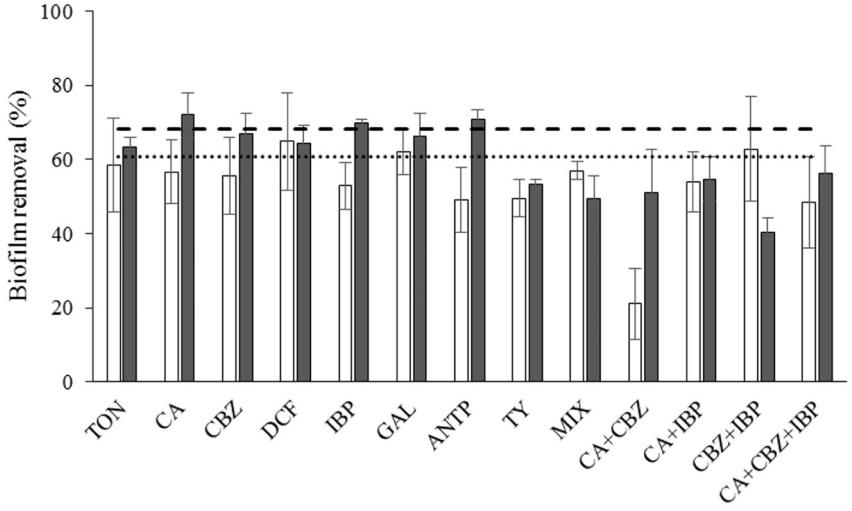


Fig. 5. Biofilm removal after treatment with NaOCl at 130 mg/L for 30 min. □ – [DWDS]; ■ – 100 × [DWDS]; … – biofilm not exposed to ECs (only in STW), ——— Solvent control, biofilms only exposed to DMSO. * TON – tonalide, CA – clofibric acid, CBZ – carbamazepine, DCF – diclofenac, IBP – ibuprofen, GAL – galaxolide, ANTP – antipyrine, TY – tylosin, MIX – Mixture of all compounds.

presence of chlorine resistant bacteria in DWDS (Khan et al., 2016; Sun et al., 2013). Sun et al. (2013) identified and characterized a new chlorine resistant bacterium isolated from a model DWDS (*Sphingomonas* TS001), which survived to 4 mg/L of chlorine for 240 min. Khan et al. (2016) concluded that the presence of chlorine-resistant bacteria surviving in DWDS may carry additional risk of antibiotic resistance. To our knowledge, no previous work was done regarding the effects of ECs on bacterial susceptibility to chlorine. This study shows that CBZ slightly increased planktonic *S. maltophilia* tolerance to chlorine. It is possible that the presence of ECs in chlorinated DWDS may reduce chlorine levels due to ECs degradation by chlorination (Snyder et al., 2003; Weng et al., 2014; Westerhoff et al., 2005). Therefore, in this work the mixture of ECs with chlorine was avoided in order to understand the individual effects of ECs on bacterial susceptibility to chlorine.

DWDS are a recognized pool of antibiotic resistant bacteria and their genes (Bergeron et al., 2015; Schwartz et al., 2003; Xi et al., 2009; Xu et al., 2016). In this study, LEVO and TMP-SMX were tested on their antimicrobial action against cells of *S. maltophilia* obtained from biofilms grown in the presence for ECs for 26 d. The development of antibiotic resistance by bacteria found in aquatic environments exposed to trace levels of antibiotics has been already reported (Baquero et al., 2008; Hong et al., 2013; Martinez, 2009). More recently, Subirats et al. (2017, 2018) demonstrated that water sources polluted with ECs are responsible for bacterial antibiotic resistance in stream biofilms. However, reduced information is available about the possible effects of non-antibiotic compounds on bacterial tolerance to antibiotics. In the present study, trace levels of TY (a veterinary antibiotic) did not cause significant changes on *S. maltophilia* tolerance to LEVO and to TMP-SMX. It is important to highlight that studies correlating the exposure to antibiotics in the environment with the spread of resistance used antibiotics at least at 1 μg/L (Bengtsson-Palme et al., 2016; Henderson-Begg et al., 2006; Jutkina et al., 2016; López and Blázquez, 2009; Lundstrom et al., 2016), which is a concentration significantly higher than these tested in the present work (1.7 to 10.7 ng/L for TY - the only antimicrobial EC tested). Also, the short-term exposure of *S. maltophilia* to non-antibiotic compounds did not alter significantly bacterial tolerance to LEVO and TMP-SMX.

The exposure to ECs at [DWDS] did not alter the ability of *S. maltophilia* to form biofilms in a significant manner. Nevertheless, the exposure to TY, MIX and CA + CBZ at [DWDS] led to the formation of biofilms more tolerant to NaOCl. The exposure to higher concentrations of ECs (DCF, TY and CA + CBZ + IBP at 100 × [DWDS]) altered biofilm formation. After being exposed to CA + CBZ + IBP at 100 × [DWDS] *S. maltophilia* formed lower amounts of biofilm. Moreover, this biofilm was more tolerant to NaOCl. On the other hand, the exposure to DCF and TY at 100 × [DWDS] increased *S. maltophilia* biofilm production. The biofilms formed after exposure to TY and CA + CBZ and MIX were also more tolerant to NaOCl. It is known that some compounds, even when not specific to target bacteria may change bacterial behaviour, as happened in the current study. In fact, previous works reported that NSAIDs such as DCF and IBP are responsible for changes in the gut microbiome (Guslandi, 2012; Rogers and Aronoff, 2016). Cycoń et al. (2016) also found that NSAIDs are responsible for biochemical and microbiological changes in soil.

The results was shown that the simultaneous presence of different ECs (CA + CBZ, CA + IBP, CA + CBZ + IBP) have changed planktonic and sessile bacterial behaviour. CA was the single compound present in all the combinations that altered bacterial behaviour, increasing the ability of *S. maltophilia* to form biofilms and/or increasing biofilm tolerance to NaOCl. DeLorenzo and Fleming (2008) also found that the combination of CA with simvastatin was more toxic for phytoplankton than the exposure to these compounds individually. In another study, Balague et al. (2004) found that CA prevented the assemblage of the fimbria subunits or/and cause genetic control inhibition of fimbriae expression in *Escherichia coli*. Combinations of ECs were also found to cause toxic effects in non-target aquatic organisms (Cleuvers, 2003; Schnell et al., 2009).

5. Conclusions

The presence of ECs in DWDS can constitute a cause of concern for consumers and DW companies regarding their effects on the behaviour of the DW-colonizing microbiota. In this study, it was found no clear evidence of the exposure to ECs and changes in planktonic *S. maltophilia* susceptibility to NaOCl and antibiotics. Nevertheless, some ECs (DCF, IBP, TY and CA + CBZ + IBP at 100 × [DWDS]) were responsible for changes in *S. maltophilia* ability to form biofilms and on their tolerance to NaOCl (CA + CBZ at [DWDS] and CA + CBZ + IBP at 100 × [DWDS]). Therefore, the simultaneous presence of different compounds, even if at trace concentrations, altered *S. maltophilia* biofilm behaviour and can potentially hinder the disinfection of biofilms in DWDS.

Funding

This work was the result of the projects: POCI-01-0145-FEDER-030219; POCI-01-0145-FEDER-006939 (Laboratory for Process

Engineering, Environment, Biotechnology and Energy – UID/EQU/00511/2013) funded by the European Regional Development Fund (ERDF), through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI) and by national funds, through FCT - Fundação para a Ciência e a Tecnologia. NORTE-01-0145-FEDER-000005 – LEPABE-2-ECO-INNOVATION, supported by North Portugal Regional Operational Program (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). Grants attributed by Portuguese Foundation for Science and Technology – FCT – to Inês Gomes (SFRH/BD/103810/2014) and Lúcia Simões (SFRH/BPD/81982/2011).

Disclosure statement

Authors declare that there is no conflict of interest.

References

Aubertheau, E., Stalder, T., Mondamert, L., Ploy, M.-C., Dagot, C., Labanowski, J., 2017. Impact of wastewater treatment plant discharge on the contamination of river biofilms by pharmaceuticals and antibiotic resistance. Sci. Total Environ. 579, 1387–1398.

Balague, C.E., de Ruiz, C.S., Rey, R., de Duffard, A.M., Nader-Macias, M.E., 2004. Clofibric and ethacrynic acids prevent experimental pyelonephritis by *Escherichia coli* in mice. FEMS Immunol. Med. Microbiol. 42, 313–319.

Baquero, F., Martínez, J.-L., Cantón, R., 2008. Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotechnol. 19, 260–265.

Bengtsson-Palme, J., Hammaren, R., Pal, C., Ostman, M., Bjorlenius, B., Flach, C.F., et al., 2016. Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. Sci. Total Environ. 572, 697–712.

Bergeron, S., Boopathy, R., Nathaniel, R., Corbin, A., LaFleur, G., 2015. Presence of antibiotic resistant bacteria and antibiotic resistance genes in raw source water and treated drinking water. Int. Biodeterior. Biodegrad. 102, 370–374.

Berglund, B., 2015. Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics. Infect Ecol. Epidemiol. 5, 28564.

Bitsch, N., Dudas, C., Korner, W., Failing, K., Biselli, S., Rimkus, G., et al., 2002. Estrogenic activity of musk fragrances detected by the E-screen assay using human mcf-7 cells. Arch. Environ. Contam. Toxicol. 43, 257–264.

Bonnineau, C., Guasch, H., Proia, L., Ricart, M., Geiszinger, A., Romani, A.M., et al., 2010. Fluvial biofilms: a pertinent tool to assess beta-blockers toxicity. Aquat. Toxicol. 96, 225–233.

Brodin, T., Piovano, S., Fick, J., Klaminder, J., Heynen, M., Jonsson, M., 2014. Ecological effects of pharmaceuticals in aquatic systems-impacts through behavioural alterations. Philos. Trans. R. Soc., B 369, 20130580.

Brooke, J.S., 2012. *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. Clin. Microbiol. Rev. 25, 2–41.

Cleuvers, M., 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol. Lett. 142, 185–194.

CLSI, 2007. Performance standard for antimicrobial susceptibility testing. In: Institute CaLS (Ed.), Seventeenth Informational Supplement. 17. Clinical and Laboratory Standards Institute, Pennsylvania, USA.

Corcoll, N., Acuna, V., Barcelo, D., Casellas, M., Guasch, H., Huerta, B., et al., 2014. Pollution-induced community tolerance to non-steroidal anti-inflammatory drugs (NSAIDs) in fluvial biofilm communities affected by WWTP effluents. Chemosphere 112, 185–193.

Corcoll, N., Casellas, M., Huerta, B., Guasch, H., Acuña, V., Rodríguez-Mozaz, S., et al., 2015. Effects of flow intermittency and pharmaceutical exposure on the structure and metabolism of stream biofilms. Sci. Total Environ. 503, 159–170.

Cunningham, V.L., Binks, S.P., Olson, M.J., 2009. Human health risk assessment from the presence of human pharmaceuticals in the aquatic environment. Regul. Toxicol. Pharmacol. 53, 39–45.

Cycoń, M., Borymski, S., Żołnierczyk, B., Piotrowska-Seget, Z., 2016. Variable effects of non-steroidal anti-inflammatory drugs (NSAIDs) on selected biochemical processes mediated by soil microorganisms. Front. Microbiol. 7, 1969.

DeLorenzo, M.E., Fleming, J., 2008. Individual and mixture effects of selected pharmaceuticals and personal care products on the marine phytoplankton species *Dunaliella tertiolecta*. Arch. Environ. Contam. Toxicol. 54, 203–210.

Duedahl-Olesen, L., Cederberg, T., Pedersen, K.H., Hojgard, A., 2005. Synthetic musk fragrances in trout from *Danish* fish farms and human milk. Chemosphere 61, 422–431.

EPA, 2011. Development and Testing of Methods to Decontaminate a Building's Plumbing System Impacted by a Water Contamination Event: Decontamination of *Bacillus* Spores. United States Environmental Protection Agency, USA.

Fatta-Kassinos, D., Meric, S., Nikolaou, A., 2011. Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research. Anal. Bioanal. Chem. 399, 251–275.

Fisher, M.M., Eugster, E.A., 2014. What is in our environment that effects puberty? Reprod. Toxicol. 44, 7–14.

Flemming, H.-C., Percival, S.L., Walker, J.T., 2002. Contamination potential of biofilms in water distribution systems. Water Sci. Technol. Water Supply 2, 271–280.

Gavrilescu, M., Demnerova, K., Aamand, J., Agathos, S., Fava, F., 2015. Emerging pollutants in the environment: present and future challenges in biomonitoring, ecological risks and bioremediation. New Biotechnol. 32, 147–156.

Golan-Rozen, N., Chefetz, B., Ben-Ari, J., Geva, J., Hadar, Y., 2011. Transformation of the recalcitrant pharmaceutical compound carbamazepine by *Pleurotus ostreatus*: role of cytochrome P450 monooxygenase and manganese peroxidase. Environ. Sci. Technol. 45, 6800–6805.

Gomes, I.B., Simões, M., Simões, L.C., 2016. The effects of sodium hypochlorite against selected drinking water-isolated bacteria in planktonic and sessile states. Sci. Total Environ. 565, 40–48.

Gomes, I.B., Lemos, M., Mathieu, L., Simões, M., Simões, L.C., 2018. The action of chemical and mechanical stresses on single and dual species biofilm removal of drinking water bacteria. Sci. Total Environ. 631–632, 987–993.

Guslandi, M., 2012. Non-steroidal anti-inflammatory drugs-induced small intestinal injury and probiotic agents. World J Gastroenterol: WJG 18, 4241–4242.

Guyot, A., Turton, J.F., Garner, D., 2013. Outbreak of *Stenotrophomonas maltophilia* on an intensive care unit. J. Hosp. Infect. 85, 303–307.

Henderson-Begg, S.K., Livermore, D.M., Hall, L.M., 2006. Effect of subinhibitory concentrations of antibiotics on mutation frequency in *Streptococcus pneumoniae*. J. Antimicrob. Chemother. 57, 849–854.

Hong, P.-Y., Al-Jassim, N., Ansari, M., Mackie, R., 2013. Environmental and public health implications of water reuse: antibiotics, antibiotic resistant bacteria, and antibiotic resistance genes. Antibiotics 2, 367.

Jones, O.A., Lester, J.N., Voulvoulis, N., 2005. Pharmaceuticals: a threat to drinking water? Trends Biotechnol. 23, 163–167.

Jutkina, J., Rutgersson, C., Flach, C.F., Larsson, D.G., 2016. An assay for determining minimal concentrations of antibiotics that drive horizontal transfer of resistance. Sci. Total Environ. 548-549, 131–138.

Khan, S., Beattie, T.K., Knapp, C.W., 2016. Relationship between antibiotic- and disinfectant-resistance profiles in bacteria harvested from tap water. Chemosphere 152, 132–141.

Lei, M., Zhang, L., Lei, J., Zong, L., Li, J., Wu, Z., et al., 2015. Overview of emerging contaminants and associated human health effects. Biomed. Res. Int. 2015, 12.

López, E., Blázquez, J., 2009. Effect of subinhibitory concentrations of antibiotics on intrachromosomal homologous recombination in *Escherichia coli*. Antimicrob. Agents Chemother. 53, 3411–3415.

Lucero, G.-M.A., Marcela, G.-M., Sandra, G.-M., Manuel, G.-O.L., Celene, R.-E., 2015. Naproxen-enriched artificial sediment induces oxidative stress and genotoxicity in *Hyalella azteca*. Water Air Soil Pollut. 226, 195.

Luis, L.G., Barreto, A., Trindade, T., Soares, A.M., Oliveira, M., 2016. Effects of emerging contaminants on neurotransmission and biotransformation in marine organisms - an *in vitro* approach. Mar. Pollut. Bull. 106, 236–244.

Lundstrom, S.V., Ostman, M., Bengtsson-Palme, J., Rutgersson, C., Thoudal, M., Sircar, T., et al., 2016. Minimal selective concentrations of tetracycline in complex aquatic bacterial biofilms. Sci. Total Environ. 553, 587–595.

Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I., Zhang, J., et al., 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Sci. Total Environ. 473, 619–641.

Martinez, J.L., 2009. Environmental pollution by antibiotics and by antibiotic resistance determinants. Environ. Pollut. 157, 2893–2902.

Osorio, V., Proia, L., Ricart, M., Perez, S., Ginebreda, A., Cortina, J.L., et al., 2014. Hydrological variation modulates pharmaceutical levels and biofilm responses in a Mediterranean river. Sci. Total Environ. 472, 1052–1061.

Pal, A., Gin, K.Y.-H., Lin, A.Y.-C., Reinhard, M., 2010. Impacts of emerging organic contaminants on freshwater resources: review of recent occurrences, sources, fate and effects. Sci. Total Environ. 408, 6062–6069.

Petrie, B., Barden, R., Kasprzyk-Hordern, B., 2015. A review on emerging contaminants in wastewaters and the environment: current knowledge, understudied areas and recommendations for future monitoring. Water Res. 72, 3–27.

Prest, E.I., Hammes, F., van Loosdrecht, M.C.M., Vrouwenvelder, J.S., 2016. Biological stability of drinking water: controlling factors, methods, and challenges. Front. Microbiol. 7, 45.

Proia, L., Morin, S., Peipoch, M., Romani, A.M., Sabater, S., 2011. Resistance and recovery of river biofilms receiving short pulses of triclosan and diuron. Sci. Total Environ. 409, 3129–3137.

Proia, L., Osorio, V., Soley, S., Köck-Schulmeyer, M., Pérez, S., Barceló, D., et al., 2013. Effects of pesticides and pharmaceuticals on biofilms in a highly impacted river. Environ. Pollut. 178, 220–228.

Reddersen, K., Heberer, T., Dünnbier, U., 2002. Identification and significance of phenazone drugs and their metabolites in ground- and drinking water. Chemosphere 49, 539–544.

Ricart, M., Guasch, H., Alberch, M., Barcelo, D., Bonnineau, C., Geiszinger, A., et al., 2010. Triclosan persistence through wastewater treatment plants and its potential toxic effects on river biofilms. Aquat. Toxicol. 100, 346–353.

Richardson, S.D., Kimura, S.Y., 2017. Emerging environmental contaminants: challenges facing our next generation and potential engineering solutions. Environmental Technology and Innovation 8, 40–56.

Rogers, M.A.M., Aronoff, D.M., 2016. The influence of non-steroidal anti-inflammatory drugs on the gut microbiome. Clin. Microbiol. Infect. 22, 178.e1–178.e9.

Sarria, M.P., Santos, M.M., Reis-Henriques, M.A., Vieira, N.M., Monteiro, N.M., 2011. The unpredictable effects of mixtures of androgenic and estrogenic chemicals on fish early life. Environ. Int. 37, 418–424.

Schnell, S., Bols, N.C., Barata, C., Porte, C., 2009. Single and combined toxicity of pharmaceuticals and personal care products (PPCPs) on the rainbow trout liver cell line RTL-W1. Aquat. Toxicol. 93, 244–252.

Schriks, M., Heringa, M.B., van der Kooi, M.M.E., de Voogt, P., van Wezel, A.P., 2010. Toxicological relevance of emerging contaminants for drinking water quality. Water Res. 44, 461–476.

Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H., Negele, R.D., 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac. Aquat. Toxicol. 68, 141–150.
Schwartz, T., Kohnen, W., Jansen, B., Obst, U., 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. FEMS Microbiol. Ecol. 43, 325–335.
SDWC SDWC, BTEHH, BoTaEHH, NRC NRC, 1982. Drinking Water and Health. Vol 4. National Academic Press, Washington DC.
Shaw, L., Phung, C., Grace, M., 2015. Pharmaceuticals and personal care products alter growth and function in lentic biofilms. Environ. Chem. 12, 301–306.
Simões, L., Simões, M., 2013. Biofilms in drinking water: problems and solutions. RSC Adv. 3, 2520–2533.
Simões, L.C., Simões, M., Oliveira, R., Vieira, M.J., 2007a. Potential of the adhesion of bacteria isolated from drinking water to materials. J. Basic Microbiol. 47, 174–183.
Simões, L.C., Simões, M., Vieira, M.J., 2007b. Biofilm interactions between distinct bacterial genera isolated from drinking water. Appl. Environ. Microbiol. 73, 6192–6200.
Snyder, S.A., Westerhoff, P., Yoon, Y., Sedlak, D.L., 2003. Pharmaceuticals, personal care products, and endocrine disruptors in water: implications for the water industry. Environ. Eng. Sci. 20, 449–469.
de Solla, S.R., Gilroy, È.A.M., Klinck, J.S., King, L.E., Mcinnis, R., Struger, J., et al., 2016. Bioaccumulation of pharmaceuticals and personal care products in the unionid mussel *Lasmigona costata* in a river receiving wastewater effluent. Chemosphere 146, 486–496.
Stepanović, S., Vuković, D., Dakić, I., Savić, B., Švabić-Vlahović, M., 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J. Microbiol. Methods 40, 175–179.
Subirats, J., Triado-Margarit, X., Mandaric, L., Acuna, V., Balcazar, J.L., Sabater, S., et al., 2017. Wastewater pollution differently affects the antibiotic resistance gene pool and biofilm bacterial communities across streambed compartments. Mol. Ecol. 26, 5567–5581.
Subirats, J., Timoner, X., Sanchez-Melsio, A., Balcazar, J.L., Acuna, V., Sabater, S., et al., 2018. Emerging contaminants and nutrients synergistically affect the spread of class 1 integron-integrase (intl1) and sul1 genes within stable streambed bacterial communities. Water Res. 138, 77–85.
Sui, Q., Cao, X., Lu, S., Zhao, W., Qiu, Z., Yu, G., 2015. Occurrence, sources and fate of pharmaceuticals and personal care products in the groundwater: a review. Emerging Contaminants 1, 14–24.
Sun, W., Liu, W., Cui, L., Zhang, M., Wang, B., 2013. Characterization and identification of a chlorine-resistant bacterium, *Sphingomonas TS001*, from a model drinking water distribution system. Sci. Total Environ. 458-460, 169–175.
Vajda, A.M., Barber, L.B., Gray, J.L., Lopez, E.M., Bolden, A.M., Schoenfuss, H.L., et al., 2011. Demasculinization of male fish by wastewater treatment plant effluent. Aquat. Toxicol. 103, 213–221.
Vincenti, S., Quaranta, G., De Meo, C., Bruno, S., Ficarra, M.G., Carovillano, S., et al., 2014. Non-fermentative gram-negative bacteria in hospital tap water and water used for haemodialysis and bronchoscope flushing: prevalence and distribution of antibiotic resistant strains. Sci. Total Environ. 499, 47–54.
Vulliet, E., Cren-Olive, C., 2011. Screening of pharmaceuticals and hormones at the regional scale, in surface and groundwaters intended to human consumption. Environ. Pollut. 159, 2929–2934.
Waiser, M.J., Swerhone, G.D., Roy, J., Tumber, V., Lawrence, J.R., 2016. Effects of erythromycin, trimethoprim and clindamycin on attached microbial communities from an effluent dominated prairie stream. Ecotoxicol. Environ. Saf. 132, 31–39.
Weng, S., Sun, P., Ben, W., Huang, C.-H., Lee, L.T., Blatchley Iii, E.R., 2014. The presence of pharmaceuticals and personal care products in swimming pools. Environmental Science & Technology Letters 1, 495–498.
Westerhoff, P., Yoon, Y., Snyder, S., Wert, E., 2005. Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes. Environ. Sci. Technol. 39, 6649–6663.
WHO, 2011. In: Press, W. (Ed.), Pharmaceuticals in drinking water: public health and environment water, sanitation, hygiene and health. World Health Organization, Geneva.
Wilkinson, J.L., Hooda, P.S., Barker, J., Barton, S., Swinden, J., 2016. Ecotoxic pharmaceuticals, personal care products, and other emerging contaminants: a review of environmental, receptor-mediated, developmental, and epigenetic toxicity with discussion of proposed toxicity to humans. Crit. Rev. Environ. Sci. Technol. 46, 336–381.
Wombacher, W.D., Hornbuckle, K.C., 2009. Synthetic musk fragrances in a conventional drinking water treatment plant with lime softening. J. Environ. Eng. 135, 1192.
Xi, C., Zhang, Y., Marrs, C.F., Ye, W., Simon, C., Foxman, B., et al., 2009. Prevalence of antibiotic resistance in drinking water treatment and distribution systems. Appl. Environ. Microbiol. 75, 5714–5718.
Xu, L., Ouyang, W., Qian, Y., Su, C., Su, J., Chen, H., 2016. High-throughput profiling of antibiotic resistance genes in drinking water treatment plants and distribution systems. Environ. Pollut. 213, 119–126.
Zhang, Q.Q., Zhao, J.L., Ying, G.G., Liu, Y.S., Pan, C.G., 2014. Emission estimation and multimedia fate modeling of seven steroids at the river basin scale in China. Environ. Sci. Technol. 48, 7982–7992.