Are emerging contaminants affecting drinking water microbial biofilms?

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Abstract The presence of biofilms in drinking water (DW) distribution systems is unavoidable as well as the presence of trace levels of different emerging contaminants (ECs). ECs constitute a potential risk for the "One Health" trilogy. In particular, the presence of antibiotics in the environment has been associated with antibiotic-resistance spread worldwide. However, the information about the pressure caused by non-antibiotic and non-pharmaceutical ECs remains scarce. This study aims to highlight the possible impact of different pharmaceutical and even some non-pharmaceutical ECs on the behavior of bacteria isolated from DW. Few recent works reported the impact of a continuous exposure to specific ECs on DW microbiota; however, this topic still remains unexplored by the scientific community. In order to provide more realistic knowledge about the continuous exposure of DW biofilms to ECs, *Acinetobacter calcoaceticus* isolated from DW, *was* used to form biofilms. These biofilms were exposed to several pharmaceutical (ibuprofen, ciprofloxacin, carbamazepine) and non-pharmaceutical (caffeine) ECs. The results demonstrated that the presence of these ECs may affect DW bacteria behavior, namely the ability to form biofilms and the tolerance to antibiotics. Additionally, this work demonstrates the possible impact of ECs on DW bacteria behavior and highlights that the impact of ECs on DW microbial community is dependent on the bacterial diversity, environmental conditions and also the time of exposure.

Keywords: antimicrobial tolerance, biofilms, exposure conditions, micropollutants

1. Introduction

The presence of emerging contaminants (ECs) in aquatic environments is unavoidable, constituting a worldwide concern. Although ECs are only detected at trace levels in aquatic environments, their presence may cause alarming effects on the biota. Several works reported alterations in fish behavior due to the continuous exposure to ECs, mainly pharmaceuticals (Jacquin *et al.* 2020). Moreover, microorganisms may be affected by ECs (Gomes *et al.* 2020). Several studies reported that antimicrobial agents are associated with the dissemination of antibiotic resistance in fluvial and marine environments as well as in wastewater treatment plants (WWTPs) (Gomes *et al.* 2020). For example, Qiu *et al.* (2019) found a positive correlation between the presence of sulfamethoxazole in water and sediments from rivers and the detection of *blaD* gene and *Fusobacteria*, suggesting that antibiotics may be positively linked to the expression of antibiotic resistance genes (ARG) in certain fluvial bacteria. Furthermore, Wang *et al.* (2017) found that the presence of polycyclic aromatic hydrocarbons in coastal waters is responsible for an increase in the dissemination of ARGs. Despite all this information about the effects of ECs in fluvial and marine sources or in WWTPs, the information available in the literature regarding the impact of ECs in drinking water (DW) microbiome is still very limited. It is known that biofilm formation along DW distribution systems (DWDS) and plumbing systems is unavoidable. In fact, 95% of the bacteria found in DWDS are attached on the pipe walls as biofilms (Flemming *et al.* 2002). Therefore, these biofilms are continuously exposed to trace concentrations of ECS that are able to reach DW. Several works described the detection of ECs in DW worldwide, as recently reviewed by Gomes *et al.* (2020). However, only few works described the impact of the presence of ECs in the DW microbiome.

To the best of our knowledge only four works reported the impact of ECs in DW bacteria (Gomes *et al.* 2018b, Gomes *et al.* 2019b, Gomes *et al.* 2019c, Wang *et al.* 2019). Three of the referred works (Gomes *et al.* 2018b, Gomes *et al.* 2019b, Gomes *et al.* 2019c) were developed by the team of the main authors of the present study. Therefore, this study aims to demonstrate the impact of ECS in DW microbial community, by gather compiling all the information obtained in previous works regarding ECS impact on DW isolated bacteria (*Burkholderia cepacia* and *Stenotrophomonas maltophilia*) with new results regarding ECs` impact on *A. calcoaceticus* isolated from DW.

2. Methods: How the impact of ECs in DW bacteria has been determined?

2.1. Biofilms and bacteria

Most of the work developed was based on the use of bacteria isolated from DWDS (*Burkholderia cepacia* and *Stenotrophomonas maltophilia*) (Gomes *et al.* 2018b, Gomes *et al.* 2019a, Gomes *et al.* 2019b). *S. maltophilia* was used to form single species biofilms in polyvinyl chloride (PVC) coupons in microtiter plates, for 26 d (Gomes *et al.* 2018b) and 12 weeks (Gomes *et al.* 2019b).

In the current study, *A. calcoaceticus* isolated from a DWDS was used to form 7 days-old biofilms in PVC coupons. *A. calcoaceticus* biofilms were exposed to selected ECs for 7 days. Then, the impact of ECs on bacteria behavior (biofilm formation ability and antibiotic susceptibility) was assessed

2.2. *Emerging contaminants*

Pharmaceutical and personal care products (PPCPs) were selected as ECs, since those are often detected in water sources. Among PPCPs, ECs were selected according the most worrying classes attending their recalcitrance in the environment (carbamazepine - CBZ), impact on antimicrobial resistance (antibiotics) and frequency of detection (anti-inflammatory drugs – IBP and caffeine).

Since ECs in the environment are not found isolated, the impact of the mixture of all the selected ECs on DW bacteria behavior was also evaluated. The solvent used to prepare the ECs solutions was CIP and CAF solutions were prepared in synthetic tap water (STW accordingly Gomes *et al.* (2018a)). CBZ and IBP were prepared in dimethyl sulfoxide (DMSO) at a maximum of 0.005 % (v/v). Therefore, negative controls are presented as CONT (exposure to STW instead of ECs) and as DMSO (exposure to DMSO instead of ECs).

Table 1. *ECs tested in the present works*

ECs	Conc. tested (ng/L)*
CAF	158.7
CBZ	586
CIP	679.7
IBP	223.6

* maximum concentration detected in DW according to Gomes *et al.* (2020)

Previous works have also given special emphasis to the impact of non-antibiotic and even non-pharmaceutical ECs (Gomes *et al.* 2018b, Gomes *et al.* 2019a, Gomes *et al.* 2019b). On the other hand, the work developed by Wang *et al.* (2019) only studied the impact of sulfadiazine (SULF) and CIP at 2000 ng/L on mixed species biofilms formed directly by DW.

2.3. *Assessing the impact of ECs on DW bacteria*

In the current work the impact of selected ECs was assessed on biofilm formation ability and on the bacterial susceptibility to antibiotics.

However, it is important to highlight that to understand the impact of the exposure to ECs, several other tests such as the susceptibility to DW disinfectants and the biofilm tolerance to chlorine disinfection as well as the impact of ECs on bacterial virulence have been performed. Table 2 summarizes the tests that have been performed in current and previous works.

Table 2. *Summary on the parameters evaluated after bacteria/biofilm exposure to selected ECs.*

Bacterial/biofilm properties tested	References
Minimum bactericidal concentration of chlorine	(Gomes *et al.* 2018b, Gomes *et al.* 2019c)
Antibiotics susceptibility	(Gomes *et al.* 2018b, Gomes *et al.* 2019c)
Motility	(Gomes *et al.* 2019c)
Ability to form biofilms	(Gomes *et al.* 2018b, Gomes *et al.* 2019b, Gomes *et al.* 2019c)
Biofilm tolerance to chlorine	(Gomes *et al.* 2018b, Gomes *et al.* 2019b, Gomes *et al.* 2019c)
Siderophores production	(Gomes *et al.* 2019b)
Adhesion and internalization to human cells (Infection)	(Gomes *et al.* 2019b)
EPS production*	(Wang *et al.* 2019)
Bacterial diversity	(Wang *et al.* 2019)
Enzymatic activity	(Wang *et al.* 2019)

* ongoing work

Furthermore, the conditions of exposure to ECs varies from work to work. For example, planktonic studies were performed by exposing bacteria to ECs for 1 d to 7 d(Gomes *et al.* 2019a). However, the exposure of biofilms to ECs took place for longer periods (from 1 week to 12 weeks) (Gomes *et al.* 2018b, Gomes *et al.* 2019a, Gomes *et al.* 2019b)or even for 8 months (Wang *et al.* 2019).

3. Results

3.1. Impact of ECs in DW bacteria – What is known so far?

The impact of ECs on DW bacteria and biofilms is still unexplored. The main results obtained in previous works demonstrated that the ECs' impact may vary between bacteria, ECs class, concentration and time of exposure.

Figure 1 summarizes the main results obtained in previous works attending the impact of ECs on *S. maltophilia* and *B. cepacia* (opportunistic pathogens isolated from a DWDS and often found in tap water). These studies demonstrated that some ECs have not a significant impact on DW biofilms and bacteria. For example, CBZ increased the susceptibility of *B. cepacia* biofilms to chlorine, similarly to CA for *S. maltophilia*

biofilms. However other ECs had a more concerning impact on the bacteria behavior. For example, the exposure to MIX was related to an increase of 32% in the ability of *S. maltophilia* to form biofilms. On the other hand, the exposure to CBZ increased the susceptibility of *B. cepacia* biofilms to the treatment with NaOCl (increasing CFU reduction in 0.64 log CFU/mL) - Figure 1. Therefore, these two works highlight that the impact of specific ECs may be different for different bacteria.

Moreover, it is possible to verify that an increase in the exposure time completely alter the impact of CA in *S. maltophilia*. For 26 d of exposure to CA at 170 and 170.000 ng/L, no significant changes in the behavior of *S. maltophilia* were detected (Gomes *et al.* 2018b). However, an increase in the exposure time up to 12 weeks resulted in significant changes in *S. maltophilia* behavior (Gomes *et al.* 2019b). Therefore, these works revealed that the exposure time is an important factor to consider for the analysis of ECs impact on DW bacteria. Since in real environmental conditions the accumulation of ECs may occur and the exposure time exceeds the 12 weeks, the observed effects may actually be higher.

So, these studies reinforce the research needs on the impact of ECs in DW bacteria and their biofilms.

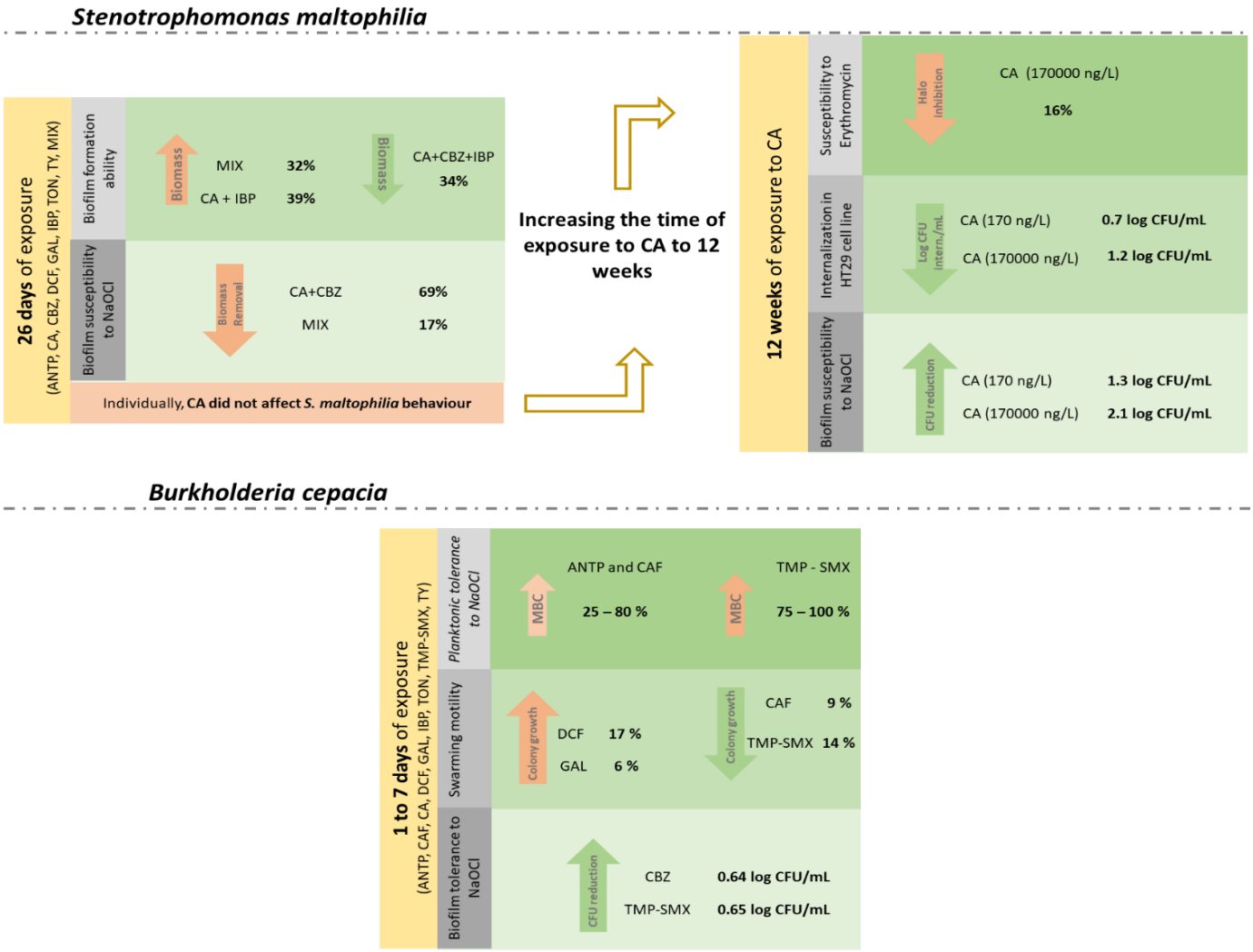

Figure 1. Compilation of the main effects of ECs in DW bacteria based on previous studies (Gomes *et al.* 2018b, Gomes *et al.* 2019a, Gomes *et al.* 2019b).

3.2. Impact of ECs in DW bacteria – Most recent achievements

The impact of CAF, CBZ, CIP, IBP (ECs representative of the most commonly detected classes on DW (Gomes *et al.* 2020)) on *A. calcoaceticus* isolated from DW, is currently being studied. *A. calcoacaeticus* is a bridging bacterium in DW biofilms, being essential to the adhesion of other DW bacteria on DW biofilms (Simões *et al.* 2008). Therefore, it is important to understand if the presence of ECs may also compromise the behavior of this bacterium in DW biofilms. The presented results were obtained by exposing *A. calcoaceticus* to selected ECs for 7 days, and afterwards evaluating the impact on biofilm formation ability and on antibiotics tolerance (in three independent assays). It was observed that the previous exposure to CIP, IBP and the mixture of all the

four selected ECs (MIX) reduced the ability of *A. calcoaceticus* to form biofilms (Figure 2).

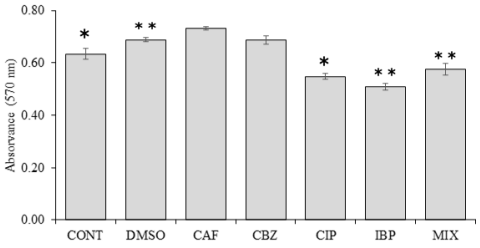

Figure 2. Effect of selected ECs on the ability of *A. calcoaceticus* to form biofilms.
* results with statistically significant differences ($P < 0.05$) – comparison to CONT; ** results with statistically significant differences ($P < 0.05$) – comparison to DMSO.

The impact of ECs exposure on *A. calcoaceticus* susceptibility to antibiotics is also being analyzed. The results from Table 3 demonstrate that the exposure to CIP and CAF increased bacteria susceptibility to CIP and levofloxacin (LEV), respectively.

The results are pointing to non-concerning alterations on *A. calacoaceticus* behavior caused by a previous exposure to the selected ECs.

Table 3. *Effect of selected ECs on A. calcoaceticus susceptibility to antibiotics (CIP and LEV)*

	Inhibition halo (cm)	
	CIP	**LEV**
CONT	2.66 ± 0.02*	2.55 ± 0.01*
DMSO	2.76 ± 0.10	2.78 ± 0.13
CAF	2.82 ± 0.13*	2.78 ± 0.12*
CBZ	2.76 ± 0.06	2.67 ± 0.17
IBP	2.90 ± 0.06	2.59 ± 0.01
CIP	2.85 ± 0.14	2.70 ± 0.09
MIX	2.70 ± 0.02	2.63 ± 0.01

* results with statistically significant differences ($P < 0.05$) – comparison to CONT.

4. Conclusions

The understanding of ECs impact in DW biofilms and bacteria is essential to understand whose ECs should be critically controlled along DWDS, attending their impact on bacterial virulence and DW disinfection efficacy. Therefore, a better understanding of this topic may be of utmost importance to allow DWTPs to focus only on concerning ECs, attending their impact on the DW bacterial community, and consequently DW quality and safety. The variability of results obtained as well as the differences in experimental design are hindering the possibility to understand the real impact of ECs in the DW microbiome. In addition, the impact of ECs in a DWDS microbiome will certainly depend on their concentration, bacterial species present, time of exposure and process conditions.

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