Integrated Master in Bioengineering

The effect of pharmaceuticals and personal care products on the behavior of planktonic and sessile *Burkholderia cepacia* from drinking water

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Diana Isabel Ribeiro Madureira | 201104125
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FEUP **FACULDADE DE ENGENHARIA**
UNIVERSIDADE DO PORTO

Supervisor: Manuel Simões (PhD)
Co-supervisors: Lúcia Chaves Simões (PhD)
Inês Gomes (MSc)

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Abstract

Pharmaceuticals and personal care products (PPCPs) are emerging contaminants (ECs), which have been detected in water bodies (surface, ground- and drinking water - DW) at trace concentrations. PPCPs were reported to affect non-target organisms, for example fishes, river biofilms communities or bacteria from the activated sludge in wastewater treatment plants. Putative concerns for human health due to their presence in DW have been only addressed under the perspective of a chemical contamination. However, DW is not a sterile environment and the presence, even at low concentrations, of PPCPs may have repercussions on the behavior, structure and interactions of DW bacterial communities. By affecting bacteria, PPCPs may contribute to the deterioration of DW microbial quality and consequently have an adverse impact on public health.

The main objective of this work was to understand the effect of eight PPCPs – ibuprofen (IBP), diclofenac (DCF), antipyrine (ANTP), carbamazepine (CBZ), tylosin (TY), clofibric acid (CLO), galaxolide (HHCB) and tonalide (AHTN) – and their mixture on *Burkholderia cepacia*, a bacterium isolated from DW. In a first phase, bacterial susceptibility to chlorine, as sodium hypochlorite (NaOCl), and to the antibiotic trimethoprim-sulfamethoxazole (TMP-SMX) was studied in the presence of PPCPs. Motility was also investigated in the presence and absence of the selected PPCPs. The PPCPs were tested on planktonic bacteria at the concentration reported for DW and at a concentration 100 times higher. In a second phase, the effects of PPCPs on the formation and on the susceptibility of 24 h old biofilms (formed in microtiter plate) and of 7 d old biofilms (formed on polyvinyl chloride coupons in a continuously operating bioreactor) to NaOCl were assessed. The PPCPs studied were ANTP, CBZ, HHCB and CLO for the 24 h old biofilm and HHCB and CLO for 7 d old biofilm. The NaOCl treatment was performed using a concentration used in drinking water distribution system (DWDS) (0.5 ppm) and a concentration 10 times higher. The 7 d old biofilms formed in the absence of any PPCPs were also treated in the presence of ANTP, CBZ and CLO.

At 100 times the residual concentration, CBZ, HHCB, ANTP, TY and DCF decreased the susceptibility of *B. cepacia* to NaOCl. HHCB and ANTP also seemed to increase the tolerance of the bacterium to TMP-SMX. In addition, HHCB affected both swimming and swarming motilities of *B. cepacia*; recognized physiological aspects relevant for the early stages of biofilm formation.

On the 24 h old biofilms, the presence of the PPCPs did not affect the formation of biofilm, but those formed in the presence of CBZ, CLO and HHCB were less susceptible to 5 ppm NaOCl. However, the 7 d old biofilms developed under continuous exposure to CLO and HHCB had lower cell density and thickness than those formed in the absence of PPCPs. The tolerance to NaOCl was not affected by the presence of PPCPs.

The presence of PPCPs during the treatment of the 7 d old biofilms (formed without exposure to PPCPs) with 5 ppm NaOCl reduced the antimicrobial action of the disinfectant.

In conclusion, the overall results obtained in this work suggest that PPCPs may affect the behavior of planktonic and sessile *B. cepacia*. In their presence, there was a decrease in the susceptibility to disinfection of planktonic cells and of 24 h old biofilms and a reduction on the biofilm formation ability for longer growth periods (7 days). In addition, PPCPs also seem to affect the microbial tolerance to antibiotics.

Keywords: biofilm, *Burkholderia cepacia*, drinking water, planktonic cells, pharmaceuticals and personal care products.

Resumo

Os fármacos e produtos de cuidado pessoal (PPCPs) são considerados contaminantes emergentes (ECs), uma vez que têm sido encontrados nas águas de superfície, subterrâneas e até na água potável. A sua presença na água pode prejudicar os ecossistemas. Por exemplo, já foi demonstrado que os PPCPs afetam peixes e comunidades de biofilmes fluviais. Para além disso, eles também têm efeito sobre as bactérias presentes nas lamas ativadas, usadas no tratamento de águas residuais. A preocupação com a saúde humana, derivada da presença dos PPCPs na água potável, só foi abordada sob o ponto de vista de uma contaminação química. No entanto, a água potável não é um ambiente estéril e a presença de PPCPs, ainda que a baixas concentrações pode ter repercussões no comportamento, estrutura e interações da comunidade bacteriana, presente na água potável. Se afetarem as bactérias, os PPCPs podem contribuir para a deterioração da qualidade microbiológica da água e, consequentemente ter um impacto negativo na saúde pública.

Este trabalho tem como principal objetivo perceber o efeito de 8 PPCPs (ibuprofeno, diclofenac, antipirina, carbamazepina, tilosina, ácido clofíbrico, galaxolide e tonalide), individualmente e em conjunto, sobre a *Burkholderia cepacia*, uma bactéria isolada da água potável. Na primeira fase deste trabalho, o efeito dos PPCPs foi avaliado através da determinação da suscetibilidade da bactéria *B. cepacia* ao agente antimicrobiano hipoclorito de sódio (NaOCl) e ao antibiótico trimetoprim – sulfametoxazol, na presença e na ausência dos PPCPs. Foi também estudada a ação dos PPCPs na mobilidade da bactéria. A ação dos PPCPs sobre as células planctónicas foi investigada a concentrações encontradas na água potável e a concentrações 100 vezes maiores. Numa segunda fase, o efeito dos PPCPs foi estudado na formação e na suscetibilidade de biofilmes de 24 horas (formados em microplaca) e de 7 dias (formados em cupões de PVC, num biorreator a operar em contínuo). Os PPCPs selecionados foram a antipirina, a carbamazepina, o ácido clofíbrico e o galaxolide para os biofilmes de 24 h, e o ácido clofíbrico e o galaxolide para biofilmes de 7 dias. Os biofilmes foram tratados com NaOCl a uma concentração encontrada nos sistemas de distribuição de água potável (0.5 ppm) e a uma concentração 10 vezes superior. Para além destes ensaios, o biofilme de 7 dias, formado sem nenhum PPCP estar presente, foi tratado na presença e na ausência de antipirina, carbamazepina e ácido clofíbrico.

A carbamazepina, o galaxolide, a antipirina, a tilosina e o diclofenac, a concentrações 100 vezes as detetadas na água potável, diminuíram a suscetibilidade da *B. cepacia* ao tratamento com NaOCl (5 ppm), enquanto o galaxolide e a antipirina aumentaram, moderadamente, a tolerância da bactéria ao antibiótico trimetoprim-sulfametoxazol. O galaxolide aumentou o *swarming* e o *swimming*, que estão associados à fase inicial de formação do biofilme.

A presença de PPCPs não alterou a formação dos biofilmes de 24 h, mas a presença de carbamazepina, ácido clofíbrico e galaxolide tornaram o biofilme menos suscetível ao NaOCl (5 ppm). No entanto, no caso dos biofilmes de 7 dias, formados na presença de ácido clofíbrico e de galaxolide, estes tinham menos densidade celular e menor espessura do que o biofilme formado sem estar exposto a nenhum PPCP.

A presença de PPCPs durante o tratamento dos biofilmes de 7 dias (formados sem exposição aos PPCPs) reduziram o efeito antimicrobiano do desinfetante.

Em suma, os resultados obtidos neste trabalho sugerem que os PPCPs podem afetar o comportamento das células planctónicas e em biofilme. Na presença dos PPCPs, houve uma diminuição na suscetibilidade à desinfeção nas células planctónicas e em biofilmes de 24 h. Verificou-se também uma redução na formação do biofilme para um tempo de exposição mais longo (7 dias). Para além disso, os PPCPs afetaram a tolerância ao antibiótico.

Palavras-chave: água potável, biofilme, células planctónicas, *Burkholderia cepacia*, fármacos e produtos de cuidado pessoal.

Table of contents

List of figures

List of tables

Glossary

AHTN - tonalide

ANTP – antipyrine

ARB - antibiotic resistant bacteria

ARG - antibiotic resistance genes

CAF - caffeine

CBZ - carbamazepine

CFU - colony-forming unit

CLO - clofibric acid

CV - crystal violet

DCF - diclofenac

DMSO - dimethyl sulfoxide

DW - drinking water

DWTP - drinking water treatment plant

DZP - diazepam

EC - emerging contaminant

EPS - extracellular polymeric substances

ERY - erythromycin

GAC - granular activated carbon

HHCB - galaxolide

IBP - ibuprofen

KP - ketoprofen

LC$_{50}$ - lethal concentration 50

MBC - minimum bactericidal concentration

MW - molecular weight

NaOCl - sodium hypochlorite

NF - nanofiltration

NP - naproxen

NSAID - non-steroid anti-inflammatory and analgesic

OD - optical density

OD$_{570}$ - optical density at 570 nm

PAC - powder activated carbon

PPCPs - pharmaceuticals and personal care products

RO - reverse osmosis

SBR - sequencing batch reactor

SMX - sulfamethoxazole

TMP -trimethoprim

TMP-SMX -trimethoprim-sulfamethoxazole

TY - tylosin

UV - ultra-violet

WWTP - wastewater treatment plant

Chapter 1

1. Work outline

1.1. Background and project presentation

Emerging contaminants (ECs) are a group of contaminants, whose presence in surface, ground- and drinking water (DW) has been investigated, particularly, in the last decade. ECs cover different groups of compounds and substances used daily worldwide. Some of these contaminants are currently unregulated or being subjected to regulation processes (Rivera-Utrilla et al., 2013). Pharmaceuticals and personal care products (PPCPs) are one of the most relevant groups of ECs. They include a broad range of products used especially for human health and care and, to a lesser extent, in veterinary care (Daughton and Ternes, 1999). They are used in large quantities and, consequently, they have been detected in trace concentrations in surface and groundwater (Boyd et al., 2003; Ellis, 2006). These water bodies are treated in order to obtain safe DW. In spite of the treatment, some PPCPs, mainly pharmaceuticals, were detected in DW (Mompelat et al., 2009; Stackelberg et al., 2004).

The access to clean DW is essential for human health (Berry et al., 2006). Therefore, DW safety should be ensured by monitoring its physicochemical and microbiological properties. From the point of view of a chemical hazard, the concentrations of pharmaceuticals detected in DW are several orders of magnitude below the minimum therapeutic dose (the lowest concentration to cause the desired effect on the target population), hence the probability of pharmaceuticals cause risks to human health, directly, is very low (WHO, 2011a, 2011b). Nevertheless, it is still necessary to consider the possible risks caused by long-term exposure and by the combined effects of mixtures of pharmaceuticals (WHO, 2011b).

In addition, in DW there are microorganisms such as bacteria, protozoa, algae, fungi and virus (Farkas et al., 2012), which can contribute to the survival and multiplication of potentially pathogenic bacteria (Eichler et al., 2006). When PPCPs are in DW, these microorganisms are exposed to them. However, their effect on these microorganisms has not been assessed yet. On surface water and wastewater, it was observed that PPCPs are likely to affect non-target organisms, sometimes in unexpected ways (Kraigher et al., 2008; Lovett, 2012). Thereby, as microbial safety of DW is essential, there is a need to understand how the microbial community will behave in the presence of PPCPs.

1.2. Objectives

The main objective of this work was to understand how the presence of ECs, particularly, PPCPs, affect the dynamics of planktonic and biofilm bacteria in DW. Therefore, a bacterium, *Burkholderia cepacia*, isolated from a DW distribution system (DWDS), was used.

To understand if the presence of ECs affects the susceptibility of planktonic bacteria to chlorine, as sodium hypochlorite (NaOCl), the minimum bactericidal concentration (MBC) was determined in the presence and in the absence of eight PPCPs: ibuprofen (IBP), antipyrine (ANTP), diclofenac (DCF), carbamazepine (CBZ), clofibric acid (CLO), galaxolide (HHCB), tonalide (AHTN) and tylosin (TY), at the concentrations reported in DWDS and at a concentration 100 times higher. To evaluate if the PPCPs contribute to the development of pathogen tolerance, the susceptibility of *B. cepacia* to antimicrobial agent Trimethoprim-Sulfamethoxazole (TMP-SMX) was also determined in presence and absence of PPCPs. Furthermore, the effect of contaminants on the motility of *B. cepacia* was investigated.

PPCPs were also studied on their influence on biofilm formation and control. Biofilms were formed in polystyrene microtiter plates for 24 h and in a chemostat reactor for 7 days in presence and absence of selected PPCPs. Those biofilms were also treated with NaOCl.

1.3. Thesis organization

This thesis is divided in five chapters. Chapter 1 describes the motivations for the development of this work as well as the main objectives.

Chapter 2 consists of a brief literature review with main focus on the presence of ECs in water, since their entry in environmental waters until the finished DW. This review also provides information on the known effects of antibiotics on WWTPs bacterial communities and of PPCPs on aquatic species. Furthermore, the technological approaches used for the removal of PPCPs are also addressed.

Chapter 3 studies if the presence of selected PPCPs; pharmaceuticals: ANTP, CBZ, IBP, CLO and DCF; musks: HHCB and AHTN; and a veterinary drug TY; affects the susceptibility of planktonic *B. cepacia* to NaOCl, its motility and tolerance to the antimicrobial agent TMP-SMX.

Chapter 4 investigates the effect of the PPCPs ANTP, CBZ, CLO and HHCB on the formation and control of 24 h old biofilms. The biofilms were treated with NaOCl at a residual concentration, found in DWDS (0.5 ppm) and at a concentration 10 times higher, 5 ppm. Steady-state biofilms (7 d old) were formed, with and without CLO and HHCB being present, and were also treated with NaOCl at the same concentrations.

Chapter 5 presents the main conclusions of this work and provides some proposals for future work on this topic.

Chapter 2

2. Literature review

2.1. Drinking water problems

Water is one of the most important resources on Earth and it has a central role in the production of energy and food, in industrial processes and on the quality and health of ecosystems (Shannon et al., 2008; Simões and Simões, 2013). In the case of human beings, the access to safe and reliable drinking water (DW), in sufficient quantity and adequate quality is crucial to ensure a good health (Hunter et al., 2010). Nevertheless, 663 million people still lack access to safe DW (WHO, 2015).

Waterborne diseases, i.e. any illness caused by the consumption of chemically and microbiologically contaminated DW, are responsible for 3.4 million deaths per year (Pandey et al., 2014). In fact, more than half of the deaths are caused by microbial intestinal infections (Cabral, 2010). In developing countries, microbial waterborne diseases are the greatest cause of concern, as they are the cause of 45% of all deaths in these countries (Simões and Simões, 2013). These diseases are due to the consumption of water contaminated with pathogenic organisms, such as bacteria, viruses, protozoa and helminths (WHO, 2011a). The majority of waterborne pathogens are introduced in DW through human and animal feces. In developed countries, there are some reports of outbreaks caused by water microbiological unsafe (Craun et al., 2006).

However, the chemical contamination of DW is also a concern in developed countries. The chemical contaminants can be naturally occurring chemicals, such as arsenic or barium, or produced by anthropogenic activity. The second category includes chemicals resultant from industrial and agricultural activities and from human dwelling (WHO, 2011a). Furthermore, the chemicals used in the DW treatment or that are in contact with DW, in another way, can also contribute to water contamination, when there is formation of by-products. Although microbiological contamination is the largest cause of waterborne diseases worldwide, chemicals in water are also related to health risks, especially when associated with long-term exposure (Thompson et al., 2007). For example, high consumption of arsenic through DW has been connected to skin, bladder and lung cancers, dermal lesions, peripheral neuropathy and peripheral vascular disease (WHO, 2011a). A new class of micropollutants has been detected in DW: the emerging contaminants (ECs).

The ECs are a vast group which comprises pharmaceuticals, personal care and life-style compounds, pesticides, some industrial compounds, drugs of abuse, food additives and engineered nanomaterials (Lapworth et al., 2012; Pal et al., 2010; Petrie et al., 2014; Sorensen et al., 2015). They have not been well studied and they are still unregulated or are being through a regularization process (Rivera-Utrilla et al., 2013). Therefore, there is a lack of information on their occurrence and their behavior in water (surface, drinking and groundwater) (Lapworth et al., 2012). This group comprises newly synthetized compounds and substances that have been in the environment for some time, but only recently, their presence, as well as their importance, has been clarified (Daughton, 2004). Nonetheless, these compounds are produced and used continuously and therefore are released to the environment (Kolpin et al., 2002), contaminating surface and groundwater. In recent years, the number of compounds considered as ECs has increased due to the development of the analytical approaches (Farré et al., 2012; Pal et al., 2010). In surface and groundwater, they were detected in trace concentrations, from ng/L to µg/L (Kasprzyk-Hordern et al., 2009; López-Serna et al., 2013). As they are not removed in conventional treatment plants, some of them have been detected in tap water (Benotti et al., 2009; Stackelberg et al., 2004). Therefore, it is important to study their sources, routes till finished DW and their direct or indirect effects for the ecosystems and the human health.

According to Sorensen et al. (2015), ECs can have adverse ecological effects and can potentially affect human health. In fact, there are some studies regarding the risks of ECs, such as pharmaceuticals, personal care products and pesticides, to human health (Blanset et al., 2007; Gaffney et al., 2015; Herin et al., 2011) and their effects on aquatic species (Beggel et al., 2010; Brodin et al., 2013; Cleuvers, 2003; Luckenbach and Epel, 2005). However, for the majority of ECs, the toxicological data available is not enough to establish regulatory limits (Pal et al., 2014).

Pharmaceuticals and personal care products (PPCPs) are the main focus of this work as they are a diverse group of ECs used on a daily basis, and therefore introduced continuously into the environment.

2.2. Pharmaceuticals and personal care products as environmental contaminants

2.2.1. Pharmaceuticals and personal care products detected in water

PPCPs are ubiquitous at low concentrations, persistent and bioaccumulative in surface water and groundwater (Daughton and Ternes, 1999; Rivera-Utrilla et al., 2013). The pharmaceuticals enter in water through personal hygiene products, pharmaceutical industry waste, hospital waste and therapeutic drugs (Rivera-Utrilla et al., 2013), which means that they are found in the influent of wastewater treatment plants (WWTPs). Table 1 presents the therapeutic groups most frequently detected in water.

Table 1 - Therapeutic groups most commonly detected in water cited from Rivera-Utrilla et al. (2013)

Compound group/ class	Compound
Non-steroid anti-inflammatory drugs (NSAIDs) and analgesics	Paracetamol, acetylsalicylic acid, IBP and DCF
Antidepressants	Benzodiazepines
Anticonvulsants	CBZ
β-blocker	Atenolol, propranolol, and metoprolol
Lipid-lowering drugs	Fibrates
Antiulcer drugs and antihistamines	Ranitidine and famotidine
Antibiotics	Tetracyclines, macrolides, β-lactams, penicillins, quinolones, sulfonamides, fluoroquinolones, chloramphenicol and imidazole derivatives
Others	Cocaine, barbiturates, methadone, amphetamines, opiates, heroin, and other narcotics

Personal care products are also produced and used worldwide in large quantities. According to Ellis (2006), the most relevant groups with environmental concerns are: fragrances such as nitro-, polycyclic and macrocyclic musks and phthalates; sunscreen agents like benzophenone, methylbenzylidene camphor; and insect repellents (N, N-diethyl-meta-toluamide). For example, synthetic musks are used in soaps, shampoos, deodorants, cosmetics, fragrances or detergents (Schnell et al., 2009). In fact, the usage

and production of the polycyclic musks HHCB and AHTN, in USA, is above 450 tons per year (Peck, 2006).

2.2.2. Sources

After being ingested, some pharmaceuticals are not completely metabolized in the human body (Zhang et al., 2008). They are then excreted through feces and urine as they were consumed (non-metabolized parent compound), as a mixture of metabolites or as conjugates (Castiglioni et al., 2006). Their use on humans occurs mainly in hospitals or in private houses (Reddersen et al., 2002). Thereby, they are present in domestic and hospital effluents, discharged in the sewer systems, which will be treated in WWTPs. The incorrect disposal at households of expired pharmaceutical products can also contribute to their spread in water (Reddersen et al., 2002). Lin and Tsai (2009) observed a substantial presence of pharmaceuticals used for human health in the hospital and industrial effluents discharged in surface waters. In addition, spills that may happen during the production also contribute for their presence in the environment (Reddersen et al., 2002). Veterinary pharmaceuticals, for example antibiotics and estrogens enter into the aquatic environment due to agriculture, aquaculture and meat industry (Lin et al., 2008). The application of manure contaminated with veterinary drugs is one of the ways of contaminating groundwater (Sui et al., 2015). As these drugs are introduced into the ground- or surface waters without undergoing treatment in WWTP, it is more complicated to follow their route in the water (Rivera-Utrilla et al., 2013).

As personal care products are used externally they are not metabolized in the human body. Therefore, they are introduced in wastewater without being degraded during showering or bathing (Eggen and Vogelsang, 2015; Pal et al., 2014). They can also enter directly into recreational waters (for example, sunscreen agents or musks) (Daughton and Ternes, 1999).There is also a possibility of groundwater contamination by surface water and vice-versa. For example, when the surface water is used to recharge artificially the aquifer (Sui et al., 2015). Figure 1 displays the main sources of PPCPs in DW.
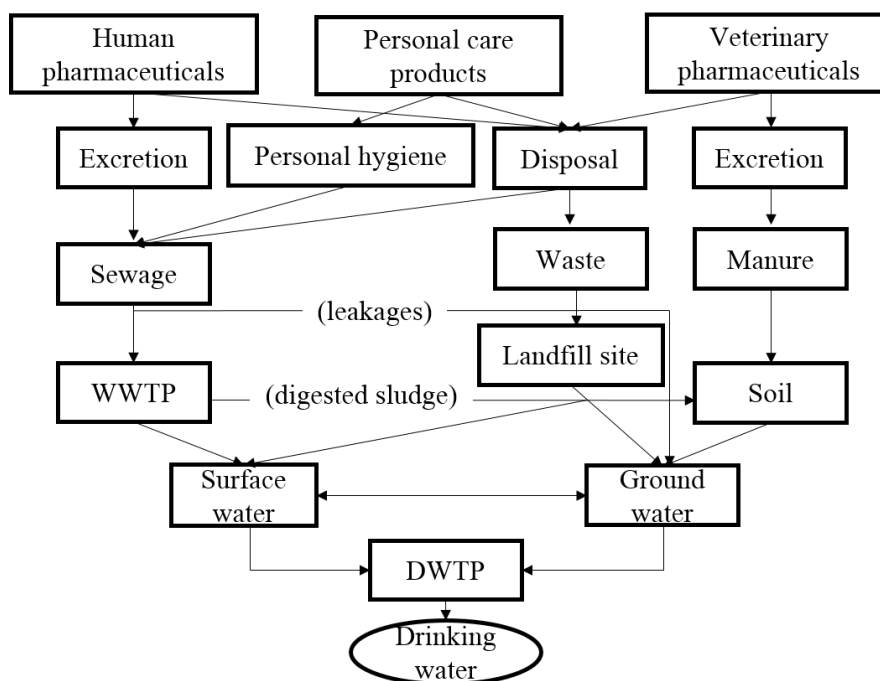
Figure 1- Sources, fate and transport of PPCPs in the environment
(adapted from Ternes 1998).

2.2.3. Behavior in wastewater treatment plants

Some PPCPs are not completely removed in the WWTPs (Boyd et al., 2003). The removal occurs through adsorption (primary treatment) or/and biodegradation (secondary treatment). Carballa et al. (2005) studied the removal of seven PPCPs: the musks HHCB and AHTN, the tranquillizer diazepam (DZP), the antiepileptic CBZ and the anti-inflammatories IBP, naproxen (NP) and DCF by flotation and coagulation-flotation (primary treatment). AHTN and HHCB were the most removed (35-70%) by flotation, followed by DZP and CBZ (40-50%; 20-45%, respectively) (Carballa et al 2005). In the coagulation-flotation assay, the musks and DCF were reduced by 50-70%, but the other compounds had reductions around 25% (DZP and NP) or were not removed (CBZ and IBP) (Carballa et al 2005). The PPCPs which are not removed in primary treatment undergo the biological treatments. The conventional secondary treatment, *i.e.* activated sludge and trickling filters, is not designed to remove these organic compounds since they are present at low concentration and have complex molecular structure (Jones et al., 2005). Kasprzyk-Hordern et al. (2009) concluded that both activated sludge and trickling filters were able to remove most of the personal care products studied (sunscreen agents, preservatives and disinfectants/ antiseptics). However, different elimination percentages were found for pharmaceuticals. A study using activated sludge reported removals of 30% to 75% for the anti-inflammatories IBP and NP and for the antibiotic SMX. This treatment

did not remove x-ray contrast substance (iopromide); so it remained in the water body (Carballa et al 2005). Radjenović et al. (2007) reported that CBZ is only partially removed from wastewater (10-20%) in WWTPs. Joss et al., (2005) also observed similar results in biological treatment: DCF was removed in part (20-40%), CBZ was not removed and great part of IBP was eliminated. The musks HHCB and AHTN achieved removals of 50%, apparently due to sorption into the sludge.

Due to the incomplete elimination of these compounds, the PPCPs along with their metabolites enter the aquatic environment through the treated effluent of WWTPs (Boleda et al., 2011; Boyd et al., 2003; Rivera-Utrilla et al., 2013). Hughes et al. (2013) indicated that 203 pharmaceuticals have been detected in river waters worldwide (41 different countries) and the maximum concentration (6.5 mg/l) was found for the antibiotic ciprofloxacin.

Once the treated effluent is discharged, the PPCPs concentration is diluted in surface water. They can also be adsorbed to sediments, colloids, suspended solids and dissolved organic matter. Furthermore, they can suffer chemical, physicochemical and biotic transformation and photodegradation (Mompelat et al., 2009). Mompelat et al. (2009) reviewed the mechanisms of degradation of pharmaceuticals in the aquatic environment.

2.2.4. Fate of pharmaceuticals and personal care products in water

Wen et al. (2014) studied the presence of five pharmaceuticals (IBP, ketoprofen (KP), NP and DCF (NSAIDs) and CLO (metabolite of a blood lipid regulator drug)) in Huangpu River which is used to provide about 30% of Shanghai's municipal water supply. They detected IBP and DCF in all water samples; KP and IBP were in higher concentration followed by DCF. Wen et al. (2014) also found that the levels of the pharmaceuticals were higher in the dry season due to the higher month rainfall and to the higher temperature in the wet season which favors biodegradation of pharmaceuticals.

The variation of concentration of some pharmaceuticals can also be attributed to the seasonality of some pharmaceutical contaminants. In other words, antihistamines (for allergies) showed a peak in summer months while pharmaceuticals used to treat nasal congestion (pseudoephedrine) and cough (pholcodine) were mainly prescribed during winter (Petrie et al., 2014). Coutu et al. (2013) observed in an experiment performed in a WWTP that the concentration of six antibiotics (TMP, norfloxacin, ciprofloxacin, ofloxacin, clindamycin, and metronidazole) also varied depending on the season. A higher mass flux for ciprofloxacin and norfloxacin was identified during winter and

spring, as they are used to treat seasonal diseases, such as pneumonia. In the same study, an intra-day variation was found and a peak was detected between 7 and 9 am and it is due to the accumulation of administered drugs in urine during sleep (Coutu et al., 2013). It is expected to see inter-day variability for some drugs such as anticancer drugs and x-ray media contrast since they are used especially during the weekdays. The variability between weekdays and weekends is also influenced by the type of location (mostly residential or industrial) (Petrie et al., 2014).

Surface water and groundwater are used as sources of raw water in DW treatment plants (DWTPs) to obtain finished DW for human consumption. Even though there is a decrease of their concentrations from wastewater to the environmental sources of water, the presence of PPCPs in surface water and groundwater is still harmful, as these watersheds are used as main sources for DW supply. Mompelat et al. (2009) reviewed the pharmaceuticals detected on tap water and reported that the NSAIDs, x-ray contrast and anticonvulsants are the groups more often detected.

Table 2 display occurrences of PPCPs in finished DW or in tap water.

Table 2 - Maximal concentration of the pharmaceuticals detected in tap water/finished DW.

Group	Compound	Maximal concentration detected (ng/l)	References
Pharmaceuticals			
Anticonvulsants	CBZ	258; 140; 18; 601; 6.9; 14	Stackelberg et al. (2004)[1]; Stackelberg et al. (2007); Benotti et al. (2009); Kleywegt et al. (2011); Wang et al. (2011)[2]; Gaffney et al. (2015)
	Phenytoin	1.3; 19; 10	Vanderford and Snyder (2006)[3]; Benotti et al., (2009); Huerta-Fontela et al. (2011)
β-blocker	Atenolol	18; 23	Benotti et al., (2009); Huerta-Fontela et al. (2011)
	Sotalol	3	Huerta-Fontela et al. (2011)
Antidepressants and anti-anxiety	DZP	23.5; 0.33	Zuccato et al. (2000)[1]; Benotti et al., (2009)
	Meprobamate	5.9; 42	Vanderford and Snyder (2006)[3]; Benotti et al., (2009)
	Fluoxetine	0.82	Benotti et al., (2009)
Iodinated X-ray contrast media	Diatrizoate	1200	Pérez and Barceló (2007)[3]
	Iopromide	<50	Pérez and Barceló (2007)[3]

Group	Compound	Maximal concentration detected (ng/l)	References
		Pharmaceuticals	
NSAIDS and analgesics	Acetaminophen	9.5	Wang et al. (2011)[2]
	AMDOPH	900	Reddersen et al. (2002)
	DCF	6	Stumpf et al. (1996)[1]
	ANTP	400; 250	Reddersen et al. (2002)[1]; Zühlke et al. (2004)
	DP	1100	Zühlke et al. (2004)
	NP	11	Carmona et al. (2014)
	IBP	3; 25; 39	Stumpf et al. (1996)[1]; Kleywegt et al. (2011); Carmona et al. (2014);
	PDP	240	Zühlke et al. (2004)
	Propyphenazone	120; 80	Reddersen et al. (2002)[1]; Zühlke et al. (2004)
Lipid regulators	Bezafibrate	27	Stumpf et al. (1996)[1]
	CLO	70; 170; 5.3; 19	Stumpf et al. (1996)[1]; Heberer and Stan (1996)[1]; Zuccato et al. (2000)[2]; Carmona et al. (2014)
	Gemfibrozil	2.1; 4	Benotti et al. (2009); Kleywegt et al. (2011)
Diuretic	Hydrochlorothiazide	7	Huerta-Fontela et al. (2011)
Opioidinalgesics	Codeine	30	Stackelberg et al. (2007)
Psychostimulant	Caffeine (CAF)	119; 60; 35.6; 46	Stackelberg et al. (2004); Stackelberg et al. (2007); Wang et al. (2011)[2]; Gaffney et al. (2015)
Veterinary medicine	Ty	1.7; 31	Zuccato et al. (2000)[1]; Kleywegt et al. (2011);
		Personal care products	
Musks	Phantolide	0.06	Wombacher and Hornbuckle (2009)
	HHCB	82; 2.2; 33	Stackelberg et al. (2004); Wombacher and Hornbuckle (2009); Benotti et al. (2009);
	AHTN	490; 68; 0.51	Stackelberg et al. (2004); Stackelberg et al. (2007); Wombacher and Hornbuckle (2009);
Sunscreen agent	Benzophenone	130	Stackelberg et al. (2004)
Antibacterial agent	Triclosan	1.2; 3.5; 59.6	Benotti et al., (2009); Wang et al. (2011)[2]; Padhye et al. (2014)
Insecticide	N,N- diethyl-meta-toluamide	93; 24	Benotti et al. (2009); Padhye et al. (2014)

[1]Cited from Jones et al. (2005); [2]Highest concentration detected during Summer season; [3]Cited from Mompelat et al. (2009);

Boleda et al. (2011) used the following classes of pharmaceuticals: anti-inflammatory, antibiotic, antilipidemic, antacid, x-ray contrast media, barbiturates, antibacterial/antimicrobial and phosphodiesterase V inhibitors to study the effects of DW treatments on pharmaceuticals and drugs of abuse removal. NSAIDs (DCF, acetaminophen, IBP, NP and salicylic acid at concentrations up to 292 ng/L; 260 ng/L; 230 ng/L; 152 ng/L and 31.8 ng/L, respectively) were at higher concentrations in the influent of the DW treatment plant (DWTP). The conventional treatment was able to degrade/transform 33 of the 40 pharmaceuticals and drugs of abuse. Nevertheless, seven of them, iopromide, nicotine, benzoylecgonine, cotinine, CAF, acetaminophen and erythromycin, were detected in the finish water at trace concentrations.

Gaffney et al. (2015) monitored 31 pharmaceuticals in Lisbon's (Portugal) DW supply system. They controlled the water sources (Rivers Tagus and Zêzere and groundwater) and the DW from the exit of the DWTP until the consumer's tap. The levels of pharmaceuticals detected in water sources were under 50 ng/L. The treatment removed eight compounds present in the source waters. Only CAF, CBZ, atenolol, sulfadiazine, sulfapyridine and erythromycin (ERY) were detected and quantified in DW. Three sulfonamides were quantified in 0.5% to 2.4% of the samples. CAF and CBZ were present in 86% and 96% of the DW samples against 84% and 69% of raw water, which means they not react with chlorine (treatment used). Conventional DW treatment processes remove 50% of pharmaceuticals, while the removal percentages are higher when advanced treatment technologies (ozone, nanofiltration, reverse osmosis, advanced oxidation and activated carbon) are used (WHO, 2011a).

The WHO considers that the presence of pharmaceuticals in DW will not cause significant adverse effects on human health, since they are in concentrations orders of magnitude below the lowest therapeutic doses (WHO, 2011a). Nevertheless, as it will be discussed in the next section, the presence of these contaminants along with the presence of other PPCPs can affect, in unexpected ways, non-target organisms and microorganisms (Kraigher et al., 2008; Sui et al., 2015). In other words, although their presence as chemical contaminants is unlikely to damage human health, there is no information on how they affect the bacteria in DW.

2.2.5. Effects of pharmaceuticals and personal care products on organisms

The continuous release of PPCPs to the environment, particularly of pharmaceuticals (pharmaceuticals are produced to cause a biological effect), is likely to cause chronic

toxicity as well as adverse effects on non-target organisms (Farré et al., 2008; Roig et al., 2009; Wen et al., 2014).

Li and Lin (2015) performed a toxicity test on *Cyprinus carpio*, using a mixture of 19 pharmaceuticals detected at high concentrations in the wastewater and in the effluent (10 mg/l each = 190 mg/l). The acute toxicity test showed a lethal concentration 50 (LC_{50}) of 60.68 mg/l of the mixture over 96 hours (3.19 mg/l for each pharmaceutical). Comparison with studies completed individually with these pharmaceuticals showed non-effect at a concentration higher than 10 mg/L and much higher LC_{50}, demonstrating that the mixture has a synergistic effect. As pharmaceuticals are present at low concentrations in water and they must not cause toxic effects when used in treatments, acute toxicity is unlikely to happen in aquatic environment (Cleuvers, 2004; Fent et al., 2006). Cleuvers (2003) also observed that a mixture of CBZ and CLO caused much greater effects than *each* compound on *Daphnia magna* and the mixture of IBP and DCF led to stronger effects on *Daphnia magna* and *Desmodesmus subspicatus*.

An *in vitro* experiment tested the cytotoxicity of 16 PPCPs (11 pharmaceuticals and five synthetic musks) to the rainbow trout liver cell line RTL-W1 (Schnell et al., 2009). This experiment showed that polycyclic musks (HHCB, AHTN, celestolide), followed by anti-depressives (fluoxetine, paroxetine and fluvoxamine) were the most toxic to the cells. For mixtures of PPCPs of the same class, it was found that there was an additive effect. However, when PPCPs of different classes were combined, a synergistic effect was observed (Schnell et al., 2009).

Pomati et al. (2006) used a mixture of 13 pharmaceuticals at low concentrations (ng/L) to assess how they affect the human embryonic cells HEK293 and determined that the presence of the drugs inhibited their growth. They also changed the cells' morphology and decreased the cell-cell contacts.

Some authors studied the influence of PPCPs on the aquatic organisms' behavior. In the mating of *Pimephales promelas* species, the males make the nest, the females bring the eggs and then the males take care of them. Fluoxetine, an antidepressant, was reported to affect the male population of *Pimephales promelas* as the presence of this pharmaceutical compound, at environmentally relevant concentrations, increased the time they spent building the nests and, therefore fishes started to be less social, i.e., they reduced their interactions with the females (Lovett, 2012). Brodin et al. (2013) studied the effect of oxazepam, a commonly prescribed anxiolytic, on the behavior of *Perca fluviatilis*. They found that a low concentration (in environmental range) led to increases in the fishes'

activity, diminished their sociability and increased the food rate as they start to eat first and deplete the food faster.

Luckenbach and Epel (2005) reported that nitro- and polycyclic musks were able to inhibit the activity of multidrug efflux transporters in the gills of the marine mussel *Mytilus californianus*. These transporters are liable for multixenobiotic resistance and when they are inhibited, they cannot prevent the entry of xenobiotics on the mussel. After the exposure to the musks, the activity was only partially recovered after a period of 24 h to 48 h (Luckenbach and Epel, 2005). Therefore, as PPCPs are continually introduced in water, there is a possibility that they can cause almost imperceptible effects on non-target organisms, which can accumulate and give rise to long-term irremediable changes (Daughton and Ternes, 1999).

All these studies show that the presence of PPCPs affect aquatic organisms and cell lines. However, there is almost no information about how they affect bacteria. Kraigher et al. (2008) demonstrated that the presence of five pharmaceuticals, IBP, NP, KP, DCF and CLO, at 50 µg/L, caused a shift in the activated sludge bacterial community structure in a wastewater treatment bioreactor, since their presence led to a reduction of the diversity of bacteria.

Taking into account the effects observed on non-target organisms and on activated sludge bacterial community one can wonder whether the presence of PPCPs, in ng/L range in DW, also affects the DW planktonic bacteria. Some studies with bacteria can be used to suggest potential effects that can be possibly observed on bacteria from DW contaminated with PPCPs. Dantas et al. (2008) found hundreds of soil bacteria able to use at least one of 18 antibiotics (natural, synthetic and semisynthetic) as a sole source of carbon in 11 different soils tested. It was also found that if they could use an antibiotic as sole source of carbon, they were resistant to all the antibiotics of that class (Dantas et al., 2008).

Chlorpromazine, an antipsychotic, and trans-chlorprothixene at one-half of their minimum inhibitory concentration (MIC), reduce the MIC of the antibiotics penicillin, cefuroxime and tobramycin against non-beta-lactamase producing corynebacteria, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. Nevertheless, similar combinations of the non-antibiotics with ampicillin had no effect on the MIC of ampicillin against *Escherichia coli* (Kristiansen et al., 2010). The presence of chlorpromazine and trans-chlorprothixene reversed the resistance of several bacteria to penicillin, cefuroxime and tobramycin. Therefore,

similarly PPCPs in DW might influence the susceptibility of bacteria to antimicrobial agents.

2.2.5.1. Effects of antibiotics on microbial communities

The main focus of this work was the other classes of pharmaceuticals and personal care products, even if antibiotics, another group of pharmaceuticals, are also ECs. They are continually introduced in the environment, which leads to the occurrence and development of antibiotic resistance genes (ARG) and antibiotic resistant bacteria (ARB) (Xu et al., 2015). Therefore, the influence of antibiotics on bacterial communities might clarify the possible interactions between bacteria and PPCPs, since the effects of such interactions are scarcely known.

WWTP have a central role on the proliferation of antibiotic resistance, plus the occurrence of ARGs and ARBs during wastewater treatment has been studied (Hocquet et al., 2016; Mao et al., 2015; Rizzo et al., 2013). The activated sludge is a favorable environment for the development and spreading of resistance due to the high density and diversity of bacteria, which are exposed to antibiotics at sub-inhibitory concentrations (Auerbach et al., 2007). Some authors focused their investigation on how the presence and biodegradation of antibiotics influenced the bacterial communities. Fan and He (2011) observed in an anaerobic sequencing batch reactor (SBR), inoculated with seeding sludge acclimated with 100 µg/L of ERY, that the bacteria were able to degrade the antibiotic when in the presence of exogenous source of carbon and nutrients. This can be justified by the acquisition of ereA, a resistant gene, responsible for ERY tolerance. The degradation of ERY also caused a shift in the microbial community and *Zoogloea*, a producer of extracellular polymeric substances (EPS), became the dominant species (Fan and He, 2011). This suggests that the microbial community started to form biofilm in order to protect the bacteria that did not develop resistance to ERY. Changes in the production of EPS were also observed in anaerobic ammonia-oxidizing (anammox) bacteria when the antibiotics amoxicillin, florfenicol and sulfamethazine, individually, were added to reactors with synthetic wastewater (Zhang et al., 2015). Although there was a decrease in the secretion of EPS and in the specific activity of bacteria, initially, the increase in the concentrations of antibiotics led to the production of a higher quantity of EPS and to a recovery of the specific activity. Aydin et al. (2016) observed a change in the bacterial community relative abundance when ERY and tetracycline were added. Over a year, the concentration of both antibiotic were increased gradually which led to a

reduction of the percentage of Gram-negative *Bacteroidetes*, *Proteobacteria* and *Fusobacterium*, while *Acidobacteria* and *Actinobacteria* relative abundance augmented. Huerta et al. (2013) explored the influence of antibiotic residues and ARGs on bacteria in the water and on the sediments of three water reservoirs. An association was found between ARGs responsible for the resistance to macrolides and the composition of bacterial composition. However, the authors do not exclude the influence of pollution on the community.

2.2.5.2.Biofilms

Some authors studied the accumulation of pharmaceuticals in biofilm and how they affected the biofilm community (Corcoll et al., 2014, 2015; Huerta et al., 2015).

Huerta et al. (2015) detected seven pharmaceuticals (of 44 studied) in the biofilm. There were compounds only detected in the biofilm, whereas there were others present at low concentrations in surface water (for example, sulfamethoxazole (SMX) and CBZ) or high concentrations (IBP) in the surface water, but not in the biofilm. The bioaccumulation of pharmaceuticals in biofilms can lead to adverse effects on organisms that use the biofilm as source of food, for example invertebrates (Corcoll et al., 2015).

Corcoll et al. (2014) studied the effect of two NSAIDs, IBP and DCF on the tolerance of several biofilms of various river sites exposed at different concentrations of these contaminants. It was demonstrated that both algae and microbial communities, in the biofilm, when exposed to the mixture of DCF and IBP at the concentrations found in environment, gained tolerance towards them. The presence of pharmaceuticals in water changed the biofilm structure and, together with other factors, particularly variation of nutrients concentration and the discharge of WWTP effluent led to the natural selection of the species more tolerant to these conditions (Corcoll et al., 2014).

A mixture of nine commonly detected pharmaceuticals (CBZ, SMX, ERY, metoprolol, atenolol, IBP, DCF, gemfibrozil, hydrochlorothiazide) led to changes in the biofilm structure and metabolism (Corcoll et al., 2015). The biofilms were developed in artificial streams, using inoculum from a slightly polluted site (Corcoll et al., 2015). The authors observed a reduction of the diversity of bacteria, but the abundance was maintained. A reduction of algae biomass, diversity and growth rate was also reported. There was an increase in the respiration rate which can possibly be caused by the change in bacterial population numbers or, possibly, it was increased by the presence of the pharmaceuticals.

As 95% of bacteria are attached to surfaces in DWDS and only 5% are found in the bulk phase (Flemming et al., 2002), it is important to understand if PPCPs detected in tap water also has an effect in biofilm bacteria in DW, as it was observed on river biofilms.

2.2.6. Technologies for degradation and removal of pharmaceuticals and personal care products

Sections 2.2.3 and 2.2.4 showed that DWTP and WWTP were not designed for removal of emerging contaminants, since they are a relatively recent problem. Therefore, advanced treatment is required in order to remove PPCPs in WWTP and DWTP. In the last decade, several technologies, such as advanced oxidation processes (ozonation, ultra-violet (UV) and Fenton, among others), activated carbon adsorption and membrane filtration, have been investigated and proved to be effective (De la Cruz et al., 2013; Reungoat et al., 2010; Snyder et al., 2007a).

2.2.6.1.Advanced oxidation processes

The ozonation process can occur directly when the ozone molecule reacts with organic compounds, especially with those with aromatic rings and heteroatoms or unsaturated compounds or, indirectly, due to the action of secondary oxidants resultant from the interaction between the ozone molecule and the water, such as the radical hydroxyl (Von Gunten, 2003; Rosal et al., 2010). Rosal et al. (2010) used ozonation as a tertiary treatment in WWTP and were able to remove the majority of compounds using doses lower than 90 µM. In DW, ozonation is used with various purposes, such as taste and odor control, color removal, disinfection and microcontaminants removal (Camel and Bermond, 1998). A study comparing conventional treatments with ozonation showed that, on the one hand, the conventional treatment did not decrease the concentration of the ECs studied (PPCPs CAF and CBZ, cotinine (metabolite of nicotine) and the pesticide atrazine), but when ozonation was included before filtration the removal efficiencies attained values between 66 and 100% (Hua et al., 2006).

UV is used for disinfection of DW and wastewater. However, the intensity applied for disinfection (30 mJ/cm^2) is unable to remove PPCPs alone, as this energy is not enough to break the compounds with low molecular weight (Snyder et al., 2003). Therefore, UV has been studied combined with ozone and with H_2O_2 (Irmak et al., 2005; Wols et al., 2013). Wols et al. (2013) examined the degradation of 40 pharmaceuticals using UV/ H_2O_2. About 75% of the compounds were removed with high efficiencies when UV and

H_2O_2 were both applied. Nevertheless, the intensity of UV necessary was still between 500 and 1000 mJ/ cm^2.

Fenton is based on the redox reaction between Fe(II) and H_2O_2 with formation of radical hydroxyl and with Fe(II)/Fe(III) acting as catalyst. In photo-Fenton this process is enhanced by UV-Vis irradiation (Méndez-Arriaga et al., 2010). Méndez-Arriaga et al. (2010) studied the degradation of IBP using Fenton and Photo-Fenton processes, achieving removal efficiencies of 25% to 60% and efficiencies of 80% to 100%, respectively. The increase on the efficiency was due to the generation of hydroxyl radicals from both photo-Fenton and from the reaction of UV-Vis/ H_2O_2. To render photo-Fenton a practical application, Klamerth et al. (2010) proposed a photo-Fenton system operating at a pH between 6 and 7 and using low concentrations of Fe and H_2O_2 and achieved high degradation of the ECs in synthetic and real effluents (Klamerth et al., 2010). De la Cruz et al. (2013) also demonstrated that a photo-Fenton system using UV_{254}; H_2O_2 and the iron dissolved in the influents attained degradations over 80% for five indicative substances with low removals in conventional treatment (CBZ, DCF, SMX, benzotriazole and mecocrop), in WWTP. Other advanced oxidation technologies such as ultrasound energy and photocatalysis have also been assessed as possibilities to eliminate PPCPs from water (Belgiorno et al., 2007; Czech and Rubinowska, 2013).

2.2.6.2.Activated carbon adsorption

Granular activated carbon (GAC) and powder activated carbon (PAC) were tested for the removal of PPCPs and endocrine disrupting products in full-scale treatment utilities. Both were efficient at removing the micropollutants. However, the efficiency of PAC depends on the dose used and contact time and the efficiency of GAC is affected by GAC replacement or regeneration (Snyder et al., 2007a).

2.2.6.3.Membrane filtration

Reverse osmosis (RO) is a treatment which separates the pollutants by passing water through a membrane under pressure (Lee et al., 2009), while nanofiltration (NF) membrane is typically operated under lower pressures (Lee et al., 2009). PPCPs can be rejected in NF/RO membrane by three mechanisms: size exclusion or steric hindrance effect, hydrophobic interaction and electrostatic interaction (Ganiyu et al., 2015). For instance, Radjenović et al. (2008) studied the removal of pharmaceuticals from groundwater in a DWTP with two removal lines: RO membranes and NF membrane.

RO/NF achieved high removal rejections for not charged pharmaceuticals with molecular weight (MW) higher than the MW cut-off of the membrane due to size exclusion effect. Very high efficiencies were obtained for the majority of negatively charged compounds and for positively charged pharmaceuticals (> 95% and > 90%, respectively).

Currently, one of the main focuses of research is the combination of different technologies in order to take advantage of a technology already used in WWTP or DWTP, to overcome the drawbacks of one another and, thereby avoiding the presence of PPCPs in tap water (Ganiyu et al., 2015).

Chapter 3

3. Effect of pharmaceuticals and personal care products on *Burkholderia cepacia* planktonic cells

3.1. Introduction

The detection of several PPCPs in finished DW and tap water in the last two decades has motivated the scientific community to investigate the harms of these compounds for human health. For example, Gaffney et al. (2015) and Snyder (2008) performed risk assessment analysis for the compounds detected in finished DW and concluded that PPCPs are several orders bellow the concentrations which would affect human health. Schriks et al. (2010), on the other hand emphasize that PPCPs are not present individually in DW and therefore it is important to consider the effect of mixtures.

In fact, it is necessary to understand how the consumption of PPCPs, through the ingestion of contaminated DW, affects the human being. However, as discussed in section 2.1, the quality of DW not only depends on its chemical safety, but also on its microbiological safety. The presence of bacteria in DW do not represent a problem, unless pathogens are present, or there is an excessive bacterial growth, which can lead to growth of protozoa and invertebrates and affect the microbial quality (Prest et al., 2016). It seems that PPCPs would only affect chemical quality of DW. Nevertheless, as microbial communities live in DWDS, one cannot disregard the possible effect of PPCPs on bacteria behavior. Some questions remain to be answered: Are PPCPs affecting the susceptibility of DW bacteria to disinfection? If they are; what are the consequences for DW safety? And as consumers of DW, what are the dangers to public health? These questions are still unanswered, but effects of PPCPs on other non-target organisms were observed in ecosystems, as demonstrated in section 2.2.5. Furthermore, bacteria and bacterial population has some plasticity, i.e., they are able to adapt to changes in the environment and that can also modulate their possible interaction with PPCPs (Justice et al., 2008).

Most bacteria in DW are in biofilms attached to the pipe surfaces and only 5% are in the bulk phase (Berry et al, 2006). Biofilms are one of the major problems in DWDS, as they can harbor pathogens and deteriorate the quality of DW (Wingender and Flemming, 2011). In biofilm, bacteria have advantages relatively to the planktonic state, such as the

increased resistance to chlorine (Farkas et al., 2012). Consequently, it is important to understand if PPCPs are able to affect biofilms and change their properties.

Another emerging problem for DW industry and for public health is the detection of antibiotics, ARG and ARB in finished DW and in tap water (Bergeron et al., 2015; Xi et al., 2009; Ye et al., 2007). There is a hypothesis that PPCPs can contribute to the development of resistance in bacteria (Daughton and Ternes, 1999). In fact, according to Boxall et al. (2012), this is one of the most important questions regarding PPCPs: are they contributing for the selection of resistant microorganisms? Xi et al. (2009) suggested that the DW treatment could increase the resistance to antibiotics or favor the transference of ARGs. What if PPCPs could also enable the same effect? What are the consequences for the consumers of DW?

Therefore, this chapter aims at answering some of the questions raised, particularly the possible changes in microbial susceptibility to disinfection and in the resistance to antibiotics, as well as the influence of PPCPs on the first stage of biofilm formation. The PPCPs tested were from five different classes: non-steroidal anti-inflammatory drugs (NSAIDs), anticonvulsants, lipid regulators, veterinary drugs and musks.

3.2. Materials and methods

3.2.1. Microorganism and culture conditions

The bacterium used was *B. cepacia*, a strain isolated from DW in Braga (Portugal) (Simões et al., 2007a) and it was stored in a cryovial at -80 ºC, with 30 % (v/v) glycerol. The bacterium was cultured in R2A before the tests.

B. cepacia was grown overnight in batch culture using 25 ml of R2B, a low nutrient medium (0.5 g/L peptone (Oxoid, England), 0.5 g/L glucose (CHEM-LAB, Belgium), 0.1 g/L magnesium sulfate heptahydrate (Merck, Germany), 0.3 g/L sodium pyruvate (Fluka, Germany), 0.5 g/L yeast extract (Merck, Germany), 0.5 g/L casein hydrolysate (Oxoid, England), 0.5 g/L starch soluble (Sigma-Aldrich, Germany) and 0.393 g/L di-potassium phosphate trihydrate (Applichem Panreac, Germany)), at 25 ± 2 ºC (Velp Scientifica FOC 215E, Italy) and under 150 rpm of agitation (IKA KS 130 basic, Germany).

3.2.2. Pharmaceuticals and personal care products

The PPCPs tested were three NSAIDs: ibuprofen (IBP; Alfa Aesar, Germany), diclofenac (DCF; Fluka, Germany) and antipyrine (ANTP; Alfa Aesar, Germany); two musks: galaxolide (HHCB; Sigma-Aldrich, Germany) and tonalide (AHTN; Sigma-Aldrich, Germany), the anticonvulsant carbamazepine (CBZ; Acros Organics, USA), the lipid regulator clofibric acid (CLO; Acros Organics, USA) and the veterinary medicine tylosin (TY; Sigma-Aldrich, Germany). Stock solutions were prepared using dimethyl sulfoxide (DMSO) (Fisher Scientific, UK). The stimulant caffeine (CAF; Fluka, Germany) and the mixture of sulfamethoxazole (SMX; Alfa Aesar, Germany) and trimethoprim (TMP; Alfa Aesar, Germany) were also tested as controls, due to their antibacterial properties (Al-Janabi, 2011; Wüst and Wilkins, 1978). The PPCPs and their residual concentrations found in DW are displayed in Table 3.

The final stock solutions were prepared at concentrations one hundred times higher than the desired final concentration and then were diluted 1:100 when added to the samples (100 µL in 10 mL).

Table 3 – List of PPCPs selected for this study and respective concentrations found in DW.

Class	PPCPs	Concentration found in DW (ng/L)	Reference
NSAIDs	IBP	3	Stumpf et al. (1996)[1]
	DCF	6	Stumpf et al. (1996)[1]
	ANTP	400	Reddersen et al. (2002) [1]
Musks	HHCB	2.2	Wombacher and Hornbuckle (2009)
	AHTN	0.51	Wombacher and Hornbuckle (2009)
Anticonvulsant	CBZ	258	Stackelberg et al., (2004)[1]
Lipid regulator	CLO	170	Heberer and Stan (1996)[1]
Veterinary medicine	TY	1.7	Zuccato et al., (2000)[1]
Stimulant	CAF	119	Stackelberg et al. (2004)
Antimicrobial agent	TMP- SMX	1.7 and 8.2, respectively	Wang et al. (2011)

[1]Cited from Jones et al. (2005).

The ECs were tested at the concentrations detected in DW and at concentrations 100 times higher.

3.2.3. Minimum bactericidal concentration

The bacterial cells were grown as described in section 3.2.1. The culture was harvested by centrifugation (Eppendorf centrifuge 5810R, Germany) at 3777 g for 15 min and then the cells were resuspended in sterile synthetic tap water (STW). STW intended to mimic tap water and was composed of 100 mg/L sodium bicarbonate ($NaHCO_3$; Fischer Scientific, UK), 13.4 mg/L magnesium sulfate heptahydrate ($MgSO_4.7H_2O$; Merck, Germany), 0.700 mg/L diphosphate potassium (K_2HPO_4; Aplichem Panreac,Germany), 0.300 mg/L monopotassium phosphate (KH_2PO_4; CHEM-LAB, Belgium), 0.0100 mg/L ammonium sulfate (($NH_4)_2SO_4$; LabKem, Spain), 0.0100 mg/L sodium chloride (NaCl; Merck, Germany), 0.001 mg/L iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$; VWR PROLABO, USA), 1.00 mg/L sodium nitrate ($NaNO_3$; LabKem, Spain), calcium sulfate ($CaSO_4$; LabKem, Spain) and 1.00 mg/L humic acid (Sigma-Aldrich, Germany) as carbon source (Epa and Homeland, 2011). Subsequently, the optical density (OD) was measured and set to 0.04 at 610 nm.

Afterwards, thirteen aliquots of bacterial suspension were diluted 10 times using synthetic tap water in order to attain a final number of cells between 10^3 and 10^6 cells/mL, which are typical numbers of heterotrophic bacteria found in DW (Prest et al., 2016). Therefore, thirteen different bacterial suspensions were prepared in sterile 50 mL tubes (VWR International, USA): four controls (bacterial suspension only, bacterial suspension with DMSO, CAF and TMP-SMX), eight bacterial suspensions to which one of the eight contaminants was added and a bacterial suspension with the mixture of the eight contaminants.

The determination of the MBC of NaOCl (Acros Organics, USA) for *B. cepacia* was accomplished by the microdilution method according to Andrews (2001). The biocide solutions were prepared using a stock solution of 13% (v/v) NaOCl. The tested concentrations ranged from 0.1 to 10 ppm. In a 96 flat-bottom microtiter plate (Orange scientific, Belgium), for each bacterial suspension (controls and the bacterial suspensions in the presence of the contaminants), 180 μL of bacterial suspension was added to 20 μL of the biocide solution. The microtiter plates were incubated at 25 ± 2 ºC and under agitation (150 rpm) for 24 hours. Afterwards, NaOCl was neutralized according to the procedure described by Knapp et al. (2013) using sodium thiosulfate (Labkem, Spain) at

a final concentration of 5% (BSI, 2009). After 10 min, 10 µL of each well was transferred to R2A plates and incubated at 25 ± 2 ºC for 24 h.

The MBC was determined as the lowest concentration of sodium hypochlorite where the bacteria were unable to grow on the R2A agar plates (Johnson et al., 2002). All tests were performed in triplicates with at least two repeats.

3.2.4. Motility assay

The bacterial cells were grown overnight in R2B, as described in section 3.2.1 and then the OD at 610 nm was adjusted to 0.04. Following this, thirteen bacterial suspensions were prepared according to section 3.2.3. The contaminants were tested at a concentration 100 times higher than the concentration detected in DW.

The bacterial suspensions were incubated for one week at 25 ± 2 ºC and under agitation (150 rpm). Afterwards, the bacterial cells were harvested by centrifugation (3777 g, 15 min) and were resuspended in Luria-Bertani broth (LBB) (Liofilchem, Italy). The OD was adjusted to 0.04 at 610 nm and each bacterial suspension was diluted 10 times using LBB. A volume of 15 µL of the suspensions was applied in the center of plates containing 1% tryptone (Merck, Germany), 0.25% NaCl (Merck, Germany) and 0.3% and 0.7% (w/v) agar (VWR PROLABO chemicals, Belgium) for swimming and swarming motilities, respectively (Butler et al., 2010; Stickland et al., 2010). As the medium porosity is directly related to the concentration of agar, the use of different concentrations of agar allows the characterization of different types of bacterial motility. Plates were incubated at 25 ± 2 ºC and the motility halos were measured after 24, 48 and 72 hours of incubation. The aim was to assess if the motility of *B. cepacia* is affected by the presence of the PPCPs. Two plates were used to evaluate the motility of the bacteria for each condition and the experiment was repeated twice.

3.2.5. Antibiotic susceptibility: disk diffusion

The Kirby-Bauer disk diffusion susceptibility test was performed according to the Performance Standards for Antimicrobial Susceptibility Testing of Clinical and Laboratory Standards Institute (CLSI, 2007).

As in section 3.2.3, thirteen bacterial suspensions were prepared and subjected to the PPCPs at concentration 100 times the concentration detected in DW for one week. Subsequently, the bacteria were centrifuged (3777 g, 15 min), resuspended in Mueller Hinton broth (MHB) (Oxoid, England) and the OD of each suspension was adjusted to

0.135 at 610 nm. Each bacterial suspension was then spread on Mueller Hinton agar (MHA) (Oxoid, England), using a swab dipped in the cell suspension. Sterile disks were placed onto the center of the inoculated plates and impregnated with 10 µL of TMP-SMX solution (final mass in the disk was 1.25/23.75 µg, respectively) (CLSI, 2007). Two plates with a disk with 10 µL of DMSO were also prepared to observe if it inhibited the bacterial growth. The plates were incubated at 37 ± 2 ºC for 24 h and the inhibition zone was determined after incubation. This test assessed if the presence of PPCPs influenced the susceptibility of *B. cepacia* to the antimicrobial agent. The test was performed in duplicate with two repeats.

3.2.6. Statistical analysis

The mean and standard deviation within samples were calculated for all results. Student's *t*-test was used to determine the statistical significance value (significant difference was assumed for *P*<0.05).

3.3. Results and discussion

3.3.1. Minimum bactericidal concentration

B. cepacia is a Gram-negative bacillus found in DWDS (Simões et al., 2007, 2010; Zanetti et al., 2000). This bacterium was already described as a multiresistant opportunistic pathogen, which has been considered harmful for individuals with cystic fibrosis (CF) (Govan et al., 1996). In fact, pulmonary colonization of *B. cepacia* has led to cepacia syndrome, a fatal pneumonia, in 20% of the patients (Hutchison et al., 1998). In this work, *B. cepacia* was used as microorganism model to study the impact of the presence of PPCPs in bacteria found in DWDS. As chlorine is widely used to disinfect DW (WHO, 2003) and it is also the most used strategy to ensure microbial safety in DWDS, it is important to know if the presence of PPCPs in DW will affect the disinfection. In this regard, the MBC of chlorine, as NaOCl, was determined in the presence and in the absence of PPCPs. Table 4 summarizes the range of values and mean values of MBC found for *B. cepacia*, in the presence and absence of contaminants, at the residual concentration detected in DWDS and at a concentration 100 times higher.

Table 4 – Minimum bactericidal concentration of NaOCl for *B. cepacia*, in the absence of contaminants, in the presence of the solvent DMSO and in the presence of each contaminant, at residual concentration and at a concentration 100 times higher, and of the mixture of all contaminants. The results are displayed in a value range and in (mean value ± standard deviation) ppm.

Concentration	Residual concentration		100 × residual concentration	
Control	**MBC (ppm) Value range**	**MBC (ppm)**	**MBC (ppm) Value range**	**MBC (ppm)**
B. cepacia	[0.5;2.5]	1.3±1.0	[0.5;2.5]	1.3±1.0
B. cepacia in presence of DMSO	[2.5;4]	3.5±0.9	[2.5;4]	3.5±0.9
CAF	----	5.0±0.0	---	5.0±0.0
TMP-SMX	---	5.0±0.0	[5;7]	6.0±1.4
***B. cepacia* in the presence of ECs**				
Concentration	**Residual concentration**		**100 × residual concentration**	
Condition	**MBC (ppm) Value range**	**MBC (ppm)**	**MBC (ppm) Value range**	**MBC (ppm)**
IBP	[2.5;4.5]	3.3±1.2	[3;4]	3.5±0.7
DCF	[2.5;4.5]	3.1±0.9	[4;5]	4.5±0.5
ANTP	[2.0;4.5]	3.3±1.0	---	5.0±0.0
CBZ	[2.0;4.5]	3.1±1.0	[4;5]	4.5±0.7
CLO	[2.0;4.5]	2.9±1.2	[3;5]	4.0±1.4
TY	[2.0;4.5]	3.2±1.3	---	4.0±0.0
HHCB	[2.0;4,5]	3.1±1.0	---	4.0±0.0
AHTN	[1.0;4.0]	2.8±1.3	[3;4]	3.5±0.7
Mixture of all contaminants	[2.5-4.5]	3.7±1.0	[3;5]	4.0±1.4

The mean values of MBC were above the residual concentration of chlorine present in DWDS, since free chlorine in DWDS should be at a residual concentration lower than 1 mg/L and closer to 0.5 mg/L to guarantee a concentration at the delivery point of 0.2-0.5 mg/L (WHO, 2011a). In other words, the concentration actually present in DWDS is not adequate to eliminate completely planktonic *B. cepacia*. Virto et al. (2005) determined microbial inactivation for Gram-negative bacteria, *Escherichia coli* and *Yersinia enterocolitica,* in distilled water, describing values in the range of 0.3-0.7 ppm and 0.8-1.2 ppm, respectively, which are similar to the values found for *B. cepacia.* Gomes et al. (2016) investigated the effect of NaOCl on planktonic and sessile bacteria isolated from DW and determined that concentrations of 125 ppm and 175 ppm of NaOCl are necessary

to inhibit *Acinetobacter calcoaceticus* and *Stenotrophomonas maltophilia* growth, respectively. However, the values found by Gomes et al. (2016) are considerably higher than the MBC values found for *B. cepacia* in all the conditions tested. One possible explanation for the variation found, apart from the different susceptibility of bacteria, is the medium growth used during the determination of the minimum inhibitory concentration (Gomes et al., 2016). In this case, STW was used to mimic DW composition, which is similar to distilled water used by Virto et al. (2005), while Gomes et al. (2016) used a richer medium, composed by glucose, peptone and yeast extract. In fact, Virto et al. (2005) observed an increase in inhibitory concentrations when a medium growth was used instead of distilled water, as the organic matter present in the medium will interact with chlorine, leading to a higher demand of NaOCl.

At the concentrations reported in DW, the results suggest that the presence of PPCPs did not affect the susceptibility of planktonic bacteria to NaOCl, except for the controls CAF and TMP-SMX (Table 4). In contrast, with concentrations 100 times higher, a decrease on the susceptibility ($P<0.05$) was achieved when the bacteria underwent the effect of DCF, ANTP, CBZ, HHCB and TY. Although, CAF was used as a control due to its antimicrobial properties, at the concentration found in DW, the value of MBC in its presence is similar to those obtained in the presence of ANTP, CBZ and DCF ($P>0.05$) (Al-Janabi, 2011). The increase on MBC could happen due to the effect of these PPCPs on the resistance of *B. cepacia* or due to the reaction between the contaminants and NaOCl. There are some works which tested the efficiency of chlorine to degrade some microcontaminants (Deborde and von Gunten, 2008; Gibs et al., 2007). Simazaki et al. (2008) observed a decrease of 70% of DCF concentration (after 24 h), when water was treated with NaOCl, at laboratorial scale. ANTP, in a kinetic study, reacted with chlorine, being reduced by more than 99% after 30 minutes (Rodil et al, 2012). Chamberlain and Adams (2006) also concluded that TY can be significantly removed during chlorination. Concerning CBZ, it was shown using a DWTP effluent with free chlorine at 1.2 mg/L that CBZ is persistent and does not react with chlorine (Gibs et al., 2007). In addition, Soufan et al. (2013), in an assay to study the kinetics of the reaction of CBZ with chlorine confirmed that at conditions similar to the DW (pH 7 and 1 mg/L free chlorine), a low reactivity of chlorine with CBZ is likely to happen. The removal of HHCB with chlorine was tested at a full-scale DW facility and it was achieved a low removal value (<20%) (Snyder et al., 2007b). Therefore, the hypothesis of an increase in MBC due to the reaction between PPCPs and chlorine may be valid for ANTP, DCF and TY, but not for CBZ and

HHCB. Regarding the inexistence of studies about the effect of PPCPs on bacteria tolerance to chlorine and the obtained results, it is not possible to affirm the cause for the decrease of susceptibility.

When the results obtained for *B. cepacia* on the presence of PPCPs are compared with the control using the solvent DMSO, it is observed that there is no difference between the solvent and any of PPCPs ($P>0.05$), which suggests that the effects observed may be caused, in part, by the solvent. In order to clarify if these results are affected by DMSO, future work is required with an alternative solvent.

3.3.2. Motility assay

Motility is an important factor affecting biofilm formation and pathogenesis of microorganisms (O'Toole and Kolter, 1998). Henrichsen (1972) described several types of motilities, being among them swimming and swarming, which are flagellar-mediated motilities. Swimming is performed by individual cells, in liquid environments, while swarming is a coordinated movement of a group of bacteria on solid surfaces. The second implies that cells differentiate in swarming phenotype, characterized by a greater number of flagella and by a longer cellular shape (Calvio et al., 2005; Harshey, 2003).

Flagellar-mediated motility is important for transport and to establish the first contact with abiotic surfaces, as it helps overcoming repulsive forces between the surface and the bacteria (O'Toole and Kolter, 1998; Sauer et al., 2002). Therefore, it contributes for the reversible attachment stage in biofilm formation.

In this work, swimming and swarming motility of *B. cepacia* were evaluated after the bacteria have been exposed to PPCPs for one week at concentrations 100 times higher than the residual concentrations (Table 5). Swimming and swarming motility have been previously reported for *B. cepacia* (Huber et al., 2001; Simões et al., 2007; Zlosnik et al., 2014). For all controls and for all PPCPs, there is a significant increase in motility halos ($P<0.05$) over the time. For swimming, on the first 24 h, the presence of PPCPs did not affect the motility of *B. cepacia* ($P>0.05$). However, after 48 h HHCB, DCF and the control CAF led to a significant increase on swimming, when compared to *B. cepacia* not exposed to PPCPs. After 72 h, besides these PPCPs, the presence of ANTP also enabled an increase in the swimming halo ($P<0.05$). The exposure to DCF, CAF, ANTP and HHCB increased by 15%, 13%, 10% and 9% the swimming motility halo of *B. cepacia*, respectively.

Concerning swarming, after 24 h smaller halos were observed for IBP, CBZ, CLO, TY, for the controls CAF and TMP-SMX and for the mixture of PPCPs than for bacterium not exposed to PPCPs ($P<0.05$). Nevertheless, after 48 h the presence of PPCPs did not alter the swarming ability except for the HHCB which caused a significant increase and for the antimicrobial agent TMP-SMX which reduced motility ($P<0.05$). The effect of HHCB was also verified after 72 h. This musk enhanced swarming motility by 23%. Swimming and swarming are important in the beginning of biofilm formation (O'Toole and Kolter, 1998; Shrout et al., 2006). Therefore, these results suggest that the presence of HHCB can improve biofilm formation by *B. cepacia*.

Besides the contribution of swarming for the transition between planktonic and sessile states, it was also reported that this type of motility can increase bacterial resistance to antibiotics (Butler et al., 2010; Lai et al., 2009).

Table 5 - Motility (swimming and swarming) of *B. cepacia* when in presence and in the absence of emerging contaminants. The results are shown in (mean value ± standard deviation) mm. The values do not include the diameter of the spot produced by the 15 µL of bacterial suspension on the agar plate.

Control	*B. cepacia*		DMSO		CAF		TMP-SMX	
Time (h)	Swimming (mm)	Swarming (mm)	Swimming (mm)	Swarming (mm)	Swimming (mm)	Swarming (mm)	Swimming (mm)	Swarming (mm)
24	14.9±5.4	8.4±0.9	14.4±5.7	7.9±0.9	15.8±2.7	5.9±0.6	13.9±1.7	5.9±0.3
48	32.3±4.0	22.5±3.1	32.8±3.1	23.5±1.3	37.4±4.3	18.7±3.5	34.0±5.1	15.8±4.1
72	47.8±5.0	29.9±4.1	49.0±2.9	29.8±1.8	53.9±2.1	25.8±0.6	52.0±8.3	26.4±1.0

PPCPs	IBP		DCF		ANTP		CBZ	
Time (h)	Swimming (mm)	Swarming (mm)	Swimming (mm)	Swarming (mm)	Swimming (mm)	Swarming (mm)	Swimming (mm)	Swarming (mm)
24	14.8±6.6	6.7±0.8	15.2±3.0	8.3±1.1	14.9±5.8	7.4±1.0	12.9±4.6	5.8±0.3
48	31.9±4.4	24.9±6.5	35.9±1.6	24.4±0.5	35.1±3.1	24.9±2.4	32.5±1.5	25.9±0.3
72	49.0±2.4	31.7±5.8	54.9±5.2	32.2±0.4	52.5±1.9	32.7±1.2	51.0±4.4	32.4±1.0

PPCPs	CLO		TY		HHCB		AHTN	
Time (h)	Swimming (mm)	Swarming (mm)	Swimming (mm)	Swarming (mm)	Swimming (mm)	Swarming (mm)	Swimming (mm)	Swarming (mm)
24	15.2±6.2	6.7±0.8	15.0±5.8	6.4±1.0	15.5±5.5	7.4±0.5	14.5±6.0	7.2±1.3
48	33.1±3.8	25.3±5.1	34.5±3.1	26.4±1.5	35.0±2.7	27.4±1.0	32.6±4.8	25.8±2.3
72	54.3±6.3	32.9±4.6	52.0±5.1	33.8±2.5	52.3±1.6	36.9±1.7	48.5±5.4	32.7±1.5

PPCPs	Mixture of all PPCPs	
Time (h)	Swimming (mm)	Swarming (mm)
24	12.5±4.3	6.7±1.5
48	33.1±5.6	23.9±3.9
72	50.0±4.9	30.2±4.2

3.3.3. Susceptibility of *B. cepacia* to antimicrobial agent trimethoprim-sulfamethoxazole

The detection of ARB and ARG in source water and DW is a public health concern and it is a problem for DW industry (Bergeron et al., 2015; Xi et al., 2009). Besides the possible adverse effects of PPCPs to human health and their ecological effects, some authors also suggest that they may have an active role on the development of bacteria resistance to antibiotics (Daughton and Ternes, 1999; Rosal et al., 2010). Therefore, in this work, the susceptibility of *B. cepacia* to TMP-SMX was investigated in the presence and in the absence of PPCPs at a concentration 100 times higher than the concentrations detected in DW.

Table 6 shows the inhibition halos measured for *B. cepacia* alone and when incubated in the presence of PPCPs for 7 days.

Table 6 - Inhibition halos of *B. cepacia* in the absence and presence of PPCPs, which were tested at concentrations 100 times the concentration detected in DW. The inhibition halos are presented in (mean value ± standard deviation) mm. These values include the diameter of the disk (6 mm).

Control	Inhibition halo (mm)
B. cepacia	28.6±1.1
DMSO	27.9±3.0
CAF	26.0±1.4
TMP- SMX	26.4±0.1
B. cepacia in the presence of ECs	
Condition	**Inhibition halo (mm)**
IBP	28.9±1.7
DCF	27.1±1.1
ANTP	26.0±1.4
CBZ	27.7±3.7
CLO	27.4±3.3
TY	26.9±1.5
HHCB	25.6±1.3
AHTN	27.6±1.3
Mixture of all contaminants	26.8±2.6

According to CLSI (2007), *B. cepacia* is considered resistant to TMP-SMX if the inhibition zone diameter is ≤ 10 mm, intermediate if the diameter is between 11 and 15 mm and it is susceptible if the diameter ≥ 16 mm. Therefore, it was observed that *B. cepacia* was always susceptible to TMP-SMX even when it was exposed to PPCPs. For the majority of the PPCPs no significant difference was observed, when compared with the bacteria not exposed to compounds ($P>0.05$). However, ANTP, HHCB and the controls TMP-SMX and CAF reduced significantly the inhibition halo of *B. cepacia* ($P<0.05$). This is the first evidence that PPCPs may affect, even if moderately, the susceptibility of bacteria to antibiotics. The antimicrobial agents TMP and SMX (used as a control in this work) are also PPCPs and were detected in finished DW at concentrations in the range of ng/L. The antibiotics at high sub-inhibitory concentrations in the environment can lead to proliferation of resistance and selection of ARB (Bernier et al., 2013; Martinez, 2009). Gullberg et al. (2011) showed that even at low concentrations antibiotics can exert selective pressure. For example, the minimal selective concentration for ciprofloxacin was 100 ng/L (Gullberg et al., 2011). Therefore, the presence of PPCPs in DW associated with the presence of antibiotics may affect bacteria tolerance to antimicrobial compounds, which may endanger human health.

3.4.   Conclusions

The presence of PPCPs at residual concentrations did not change the susceptibility of *B. cepacia* to NaOCl. When the concentrations were increased 100-fold, the MBC in the presence of DCF, ANTP, CBZ, HHCB and TY was significantly higher. However, a rise in the MBC of DMSO was also determined, and therefore it is not clear if the decreases observed in susceptibility were caused by the presence of the mentioned PPCPs or if they were due to DMSO.

Some of the PPCPs showed significant effects on motility of the studied bacteria and on its susceptibility to antimicrobial agent TMP-SMX. HHCB, DCF and ANTP enabled swimming motility after 72 h. In addition, HHCB was also able to cause an increase in swarming motility. HHCB and ANTP were responsible for a decrease in susceptibility of *B. cepacia* to the tested mixture of antibiotics.

Chapter 4

4. Effect of selected pharmaceuticals and personal care products on *Burkholderia cepacia* biofilms formation and susceptibility to sodium hypochlorite

4.1. Introduction

Biofilm is a microbial community, adhered at a solid-liquid interface and surrounded by a matrix of extracellular polymeric substances (EPS) (Sauer et al., 2007). Briefly, biofilm formation comprises the pre-conditioning of the adhesion surface, transport of bacteria from the liquid phase to the surface, reversible and irreversible adhesion of bacteria, production of cell-cell signaling molecules and EPS, growth, maturation and detachment (Simões et al., 2010). It is known that the sessile mode of life is preferred by bacteria in aquatic environments, including DWDS, in detriment to living as planktonic cells (Melo and Bott, 1997). In fact, living in a biofilm gives some advantages to bacteria. It protects them from extreme environmental conditions and the EPS matrix can accumulate and concentrate nutrients (Flemming and Wingender, 2010). In addition, there are enzymes in EPS matrix which are able to degrade more complex nutrient sources and thereby, enable the sessile bacteria to use them (Flemming and Wingender, 2010). The presence of biofilms in DWDS can affect the aesthetic and the quality of water, by deteriorating the taste, color and odor of water. The detachment of patches of biofilm can also increase the numbers of bacteria in the bulk phase (Wingender and Flemming, 2011). Furthermore, biofilms can harbor pathogens (September et al., 2007).

The biofilm growth can be affected by nutrient availability, temperature, hydraulic conditions, pipe material and by the presence of residual disinfectant (Manuel et al., 2007; Norton and LeChevallier, 2000; Silhan et al., 2006). However, residual chlorine has little effect on biofilm bacteria (Simões and Simões, 2013).

In the last twenty years several studies reported the presence of PPCPs in DW (Carmona et al., 2014; Gaffney et al., 2015; Reddersen et al., 2002). In the environment they were reported to affect the abundance (Lawrence et al., 2005) and variety (Corcoll et al., 2015) of bacteria in river biofilms. Therefore, as biofilm can affect greatly the microbial quality of DW, it is of utmost importance to know if PPCPs can play an active role in the

formation of DW biofilms. Furthermore, it is essential to investigate if they can influence the susceptibility of biofilms to the treatment with chlorine, since chlorine is the most used disinfectant to ensure the microbiological quality of DW (WHO, 2003, 2011a).

4.2. Material and methods

4.2.1. Microorganism and culture conditions

The microorganism and culture conditions used for biofilm formation are described in section 3.2.1.

4.2.2. Biofilm formation and treatment in microtiter plates

The method of development of biofilms was adapted from Stepanović et al. (2000). The bacterium was grown and prepared according to sections 3.2.1 and 3.2.3. As described previously in section 3.2.3, different bacterial suspensions were prepared. The OD was adjusted to 0.04 and then diluted 10 times with R2B. The contaminants tested were: CBZ (258 ng/L), CLO (170 ng/L), ANTP (400 ng/L) and HHCB (2.2 ng/L). ANTP and HHCB were selected based on the results of Chapter 3. They seemed to decrease the susceptibility to NaOCl and to increase the tolerance to TMP-SMX and also affected *B. cepacia* motility. Other two compounds were selected: CLO and CBZ. CBZ is one of the most frequently detected PPCPs in water bodies (Zhang et al., 2008) as it is very recalcitrant. CLO is also relatively persistent in environment (Corcoran et al., 2015). Both pharmaceuticals have several occurrences in DW (Carmona et al., 2014; Stackelberg et al., 2004, 2007; Stumpf et al., 1996)

At least 24 wells were filled with 200 µL of each bacterial suspension. The bacterial suspension without any contaminant, the cells in presence of DMSO and in the presence of the antibiotics TMP-SMX, at concentrations found in DW (1.7 and 8.2 ng/L, respectively), were used as controls. Six negative control wells were filled with 200 µL of R2B broth. The microtiter plates were then incubated for 24 h, at 25 ± 2 °C and under agitation (150 rpm) to allow biofilm formation.

After 24 hours, the growth medium was carefully removed and each well was washed with 200 µL of STW. For each bacterial suspension, eight wells were filled with 200 µL of STW and used as positive control; eight wells were treated with 0.5 ppm NaOCl and the biofilm from the remaining wells was treated using 5 ppm NaOCl. The microtiter

plates were then incubated at the conditions previously mentioned (25 ± 2 ºC and under 150 rpm of agitation) for one hour.

4.2.2.1. Biofilm mass quantification by crystal violet

The mass quantification by crystal violet (CV) was performed for six wells of each condition and the method was adapted from Simões et al (2007). Briefly, each well was washed with STW and then, the biofilm was fixed with 250 µL (per well) of 96% ethanol, for 15 min. The microplates were emptied and left to dry. After this, 200 µL of 1% (v/v) CV (Merck, Germany) was used to stain the adhered cells for 5 min. The dye was removed and each well was washed with 200 µL of STW. Finally, 200 µL of 33% (v/v) glacial acetic acid (Fisher Scientific, UK) was added to solubilize the CV bounded to the fixed cells. The optical density was measured at 570 nm, using a microplate reader (BioTek Instruments, Inc, USA) (Stepanović et al., 2000). The experiments were performed in six replicates and with two repeats.

Biofilm removal was calculated using Equation 1:

$$\%BR = \frac{OD_{570}\text{ of biofilms not exposed to NaOCl} - OD_{570}\text{ of biofilms treated with NaOCl}}{OD_{570}\text{ of biofilms not exposed to NaOCl}}$$ (Eq. 1)

4.2.2.2. Assessment of the culturability on R2A

For each different bacterial suspension and for each condition (0 ppm, 0.5 ppm and 5 ppm of NaOCl), two wells were used to quantify colony-forming units (CFU). After the incubation period, a neutralization step was performed using sodium thiosulfate at a final concentration of 5%, as Knapp et al. (2013) described. Following this, the biofilm from each well was scraped for one minute using 200 µL of saline water (0.85%) and reserved in a microcentrifuge tube (Borges et al., 2014). This step was repeated three times. The biofilm suspension was then serial diluted to 10^{-5} and two 10 µL aliquots of each dilution were transferred to a R2A plate. The plates were incubated at 25 ± 2 ºC for 48 h and then the colonies were enumerated for plates where the number of colonies was between 10 and 300. The assessment of culturability was carried out in duplicate with two repeats.

4.2.2.3. Biofilm formation in microtiter plates using pharmaceuticals and personal care products at higher concentrations

An assay using all PPCPs at concentrations 100000 times higher was also performed according to section 4.2.2. The biofilm was treated with 5 ppm and 100 ppm NaOCl and

the culturability on R2A was assessed, as described in section 4.2.2.2. The tests were conducted in duplicate with two repeats.

4.2.3. Biofilm development in a continuous bioreactor

4.2.3.1.Preparation of the polyvinyl chloride coupons

Polyvinyl chloride (PVC) coupons (2.0 cm × 2.0 cm and 0.1 cm of thickness) were used for the biofilm formation in the bioreactor. They were washed using a commercial detergent, rinsed with tap water and then they were immersed in 70% ethanol for 30 min. Afterwards, they were rinsed with sterile distilled water and left to dry. Finally, they were exposed to UV light (40 W) for 1 h and 30 min, before being placed in the bioreactor.

4.2.3.2.Biofilm formation on polyvinyl chloride coupons in a continuous bioreactor

A continuous well-stirred 2 L glass bioreactor was used to develop the biofilm. The bioreactor was aerated, agitated with a magnetic stirrer (Age Magnetic Stirrer, Velp Scientifica, Italy) and continuously fed with sterile diluted R2B medium (0.05 g/L peptone, 0.05 g/L glucose, 0.01 g/L magnesium sulfate heptahydrate, 0.03 g/L sodium pyruvate, 0.05 g/L yeast extract, 0.05 g/L casein hydrolysate, 0.05 g/L starch soluble and 0.0393 g/L di-potassium phosphate trihydrate), at a constant rate (230 mL/h), which allowed the biofilm activity to prevail over planktonic growth (Ferreira et al., 2013). The reactor was inoculated on first day, by adding 250 mL of bacterial suspension, grown as described in section 3.2.1 to 2 L of STW. The feeding process started approximately 2 h after the inoculation.

The biofilm was formed on 22 PVC coupons, placed in the bioreactor, as shown in Figure 2. The reactor was operated for 7 days to allow the biofilm to reach the steady-state.
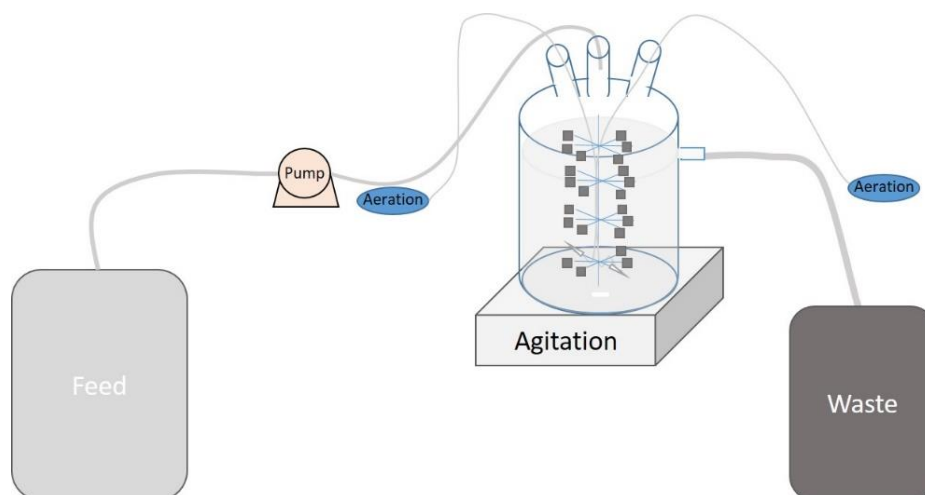
Figure 2 – Representation of the bioreactor setup

The biofilm on PVC coupons was also developed in the presence of two PPCPs, CLO and HHCB, individually, at the concentrations detected in DW. In these experiments, two stock solutions were prepared: one to add to the reactor, before initiating the feeding process, and one added to the feed. The stock solutions were prepared according to section 3.2.2. However, in the preparation of the stock solution for feed, only 20 mL de DMSO was added and sterile distilled water was used to make up to 400 mL (40 L of feed were prepared).

4.2.3.3.Biofilm treatment

After 7 days, the coupons were carefully transferred for 100 mL beakers (two coupons per condition) and exposed to different treatments: 0.5 ppm NaOCl and 5 ppm NaOCl. A control with only STW was carried out. The beakers were placed on a multi-place magnetic stirrer (Ika-werke RO 15, Germany) and exposed to NaOCl for 1 h. Afterwards, sodium thiosulfate was added to neutralize the biocide action (BSI, 2009). The biofilm formed without being subjected to any PPCP was also treated in the presence of the PPCPs ANTP, CLO and CBZ (0.5 and 5 ppm of NaOCl), of the solvent DMSO (5 ppm NaOCl) and with the mixture of SMX and TMP (0.5 and 5 ppm of NaOCl).

4.2.3.4.Assessment of the viability using a staining technique

The Live/Dead® BacLight ^{TM} kit (Invitrogen, USA) was used to evaluate the viability of *B. cepacia* and consists of two nucleic acid-binding stains: SYTO9^{TM} (green fluorescing) which is able to enter all cells and allows the assessment of total counts of bacteria and propidium iodide (PI) that only enters damaged cells with compromised cytoplasmic

membranes (Berney et al., 2007). The mixture of the two stains produces red fluorescence in damaged cells (Berney et al., 2007).

After NaOCl neutralization, each coupon was transferred to a 50 mL tube (VWR International, USA) with 15 mL of saline solution (0.85%) and the biofilm was suspended in the saline solution using a vortex (VWR V3, USA) for 2 min. The staining procedure was adapted from Borges et al. (2013). Briefly, 500 µL of each sample was stained with 250 µL of diluted reagent A (SYTO9^{TM}) and 50 µL of diluted reagent B (PI) and filtered through a Nucleopore® (Whatman, UK) black polycarbonate membrane (pore size 0.22 µm). Prior filtration, the dyes were left to react for 7 minutes in the dark at room temperature. Following this, the membrane was mounted on BacLight® mounting oil according to the instructions provided by the manufacturer.

The stained bacteria were observed using a Leica DM LB2 microscope, equipped with fluorescence illumination. A $100 \times$ oil immersion fluorescence objective, the microscope camera (Leica DFC 300 FX, Germany) and LAS V4.2 software were used to obtain 10 micrographs per coupon. The optical filter combination for optimal viewing of stained coupons consisted of a 480 to 500 nm excitation filter combined with a 485 nm emission filter (Chroma 61000-V2 DAPI/FITC/TRITC). The red and green cells were measured using the image processing software ImageJ v1.50 (NIH, USA), according to Gomes et al. (2016). The results were presented as the mean percentage value of cells stained with PI.

4.2.3.5. Assessment of the culturability on R2A

As mentioned previously in section 4.2.3.4, the coupons were transferred to 50 mL tubes with 15 mL of saline solution (0.85%). The tubes were vortexed for 2 min to suspend the biofilm in the saline solution. Subsequently, the samples were diluted to the appropriate cell density in saline solution and two drops of 10 µL of each dilution (for each coupon) were spread on R2A agar plates. The colony enumeration was accomplished after 48 h incubation at 25 ºC. Final results are displayed as log of CFU/ cm^2 of the adhesion surface.

4.2.3.6. Biofilm topography and thickness

Two coupons were placed in a 100 mL beaker with STW and then were used to determine the biofilm topography using the microscope Nikon Eclipse LV 100 (software, NIS-Elements AR 4.13.05) fitted with fluorescence illumination and a microscope camera (Nikon Digital Sight DS-Ri1, Japan). Prior to observation, each coupon was stained with

400 µL of 4', 6-diamidino-2-phenylindole (DAPI; Merck, Germany) at 0.5 µg/mL (Simões et al., 2007b). Subsequently, the stained coupons were incubated for 7 minutes in dark at room temperature.

The optical filter combination for optimal viewing of stained coupons consisted of a 359 nm excitation filter combined with a 461 nm emission filter. The software NIS Elements AR was used for determine a 3D structure of the biofilm. 3D topography was performed using a joystick (Prior Scientific Ltd, UK). Thereby, for at least five fields of view for each coupon, the base and the top of the biofilm were defined and five horizontal plans were taken between the base and the top of the biofilm. The 3D structure was then created. Afterwards, three vertical random planes, for each field of view, were selected and 1279 points of thickness were determined by the software for each plane. The biofilm thickness was determined as the mean value of all values measured for the five fields of views.

4.2.4. Statistical analysis

The mean and standard deviation within samples were calculated for all results. Student's *t*-test was used to determine the statistical significance value (significant difference was assumed for $P<0.05$).

4.3. Results and discussion

As PPCPs are contaminants of emerging concern, the potential adverse effects resultant from their interaction with DW biofilms has not been studied to this date. Therefore, four PPCPs were selected to test the effect of PPCPs on the formation and susceptibility of biofilms of *B. cepacia* isolated from DW. Regarding the results of Chapter 3, HHCB and ANTP were chosen as they seem to affect the susceptibility to antibiotics and to disinfectant NaOCl as well as the motility of *B. cepacia*. Besides these compounds, other two from other classes were also selected: CBZ and CLO. CLO is the active agent of lipid regulator fibrate and it has been detected in wastewater, surface, groundwater and DW (Heberer et al., 1998; López-Serna et al., 2013; Ternes, 1998). It is considered as relatively persistent in the environment, with a lifespan of 21 years (Corcoran et al., 2015). In DW, CLO was detected at concentrations in the order of tens and hundreds ng/L (Carmona et al., 2014; Heberer and Stan, 1996; Stumpf et al., 1996). CBZ is an anticonvulsant and it is one of the pharmaceuticals more often detected in water (Zhang et al., 2008). It is probably one of the most recalcitrant. CBZ is very poorly removed in

WWTP and resistant to biodegradation (Radjenović et al., 2007; Tixier et al., 2003). In addition, it has also been detected in DW by several authors (Benotti et al., 2009; Kleywegt et al., 2011; Stackelberg et al., 2004, 2007).

4.3.1. Biofilms developed in microtiter plates for 24 h

The microtiter plates were used to allow the formation of biofilm in the presence and absence of PPCPs, at the concentrations detected in DW, for 24 h. The biofilms were then treated with NaOCl, at a concentration used to guarantee the biological stability in DWDS (0.5 ppm) and at a concentration 10 times higher (WHO, 2011a). Figure 3 displays the effect of NaOCl on the culturability of *B. cepacia*, in the presence and in the absence of PPCPs.
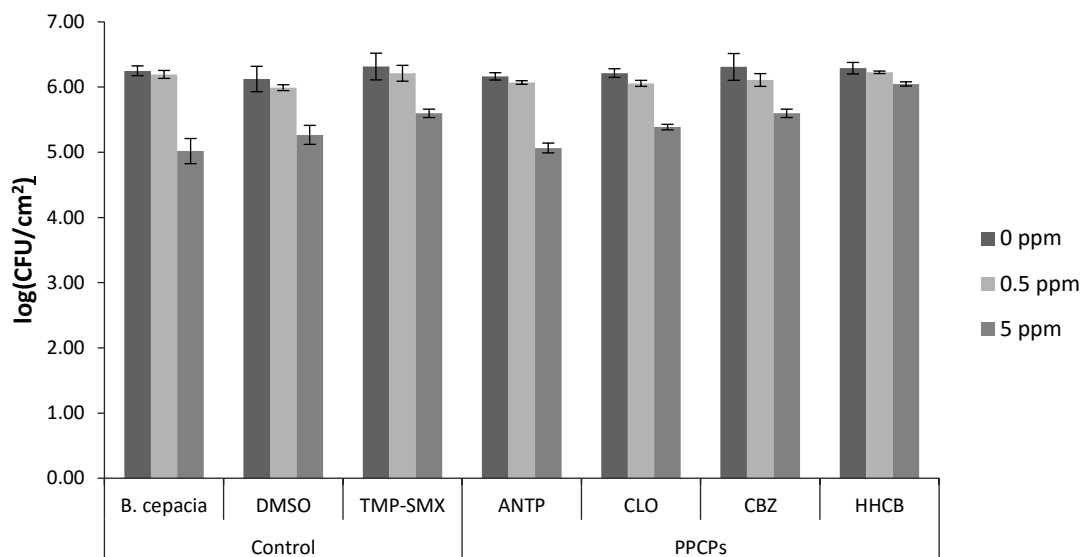
Figure 3 – Effect of NaOCl (■ - 0 ppm; ▪ - 0.5 ppm; ▪ – 5 ppm) on 24 h old *B. cepacia* biofilms, formed in microtiter plates. *B. cepacia* formed biofilm without any compounds, in the presence of the controls DMSO and TMP-SMX and in the presence of ANTP, CLO, CBZ and HHCB. The results are displayed in mean logarithm of CFU/cm^2 ± standard deviation.

B. cepacia formed significantly less biofilm in the presence of the solvent DMSO ($P<0.05$). Although HHCB increased swarming and swimming motilities (section 3.3.2), that did not correspond to higher biofilm formation ($P>0.05$). The other PPCPs also did not alter biofilm formation ($P>0.05$).

For each condition, the treatment with NaOCl at 0.5 ppm did not reduce significantly, the number of CFU ($P>0.05$), except for CBZ and CLO ($P<0.05$). The reductions obtained were: 0.06; 0.13; 0.10; 0.09; 0.20; 0.16 and 0.06 log (CFU/cm^2) for *B. cepacia* not exposed to PPCP and for *B. cepacia* biofilms formed in the presence of DMSO, TMP-SMX, ANTP, CLO, CBZ and HHCB, respectively. 0.5 ppm of NaOCl is a concentration characteristically used to prevent bacterial growth in DWDS (Prest et al., 2016; WHO,

2011a). Despite that, the low reductions achieved with 0.5 NaOCl are not completely unexpected, since the mean MBC values, determined in section 3.3.1, were always higher than 0.5 ppm and biofilm bacteria has increased resistance to disinfection (Araújo et al., 2011).

When the concentration of NaOCl was increased 10 times, there was a significant reduction in the number of CFUs for all cases. When cells were exposed to CLO, CBZ and HHCB, the log (CFU/cm^2) reduction achieved was significantly lower than the reduction obtained for *B. cepacia* (0.83; 0.71 and 0.23 log (CFU/cm^2), respectively, against 1.23 log (CFU/cm^2). The biofilms formed in the presence of ANTP and the biofilms developed in the absence of PPCPs had similar susceptibilities to NaOCl at 5 ppm. Based on these results, one can infer that the selected PPCPs did not affect biofilm formation, but three of the PPCPs (CLO, CBZ and HHCB) could reduce its susceptibility to the treatment with NaOCl (treatment with 5 ppm NaOCl).

In order to assess biofilm removal due to exposure to NaOCl at 0.5 and 5 ppm, a staining procedure with CV was conducted in the 96-well microplates (Figure 4).
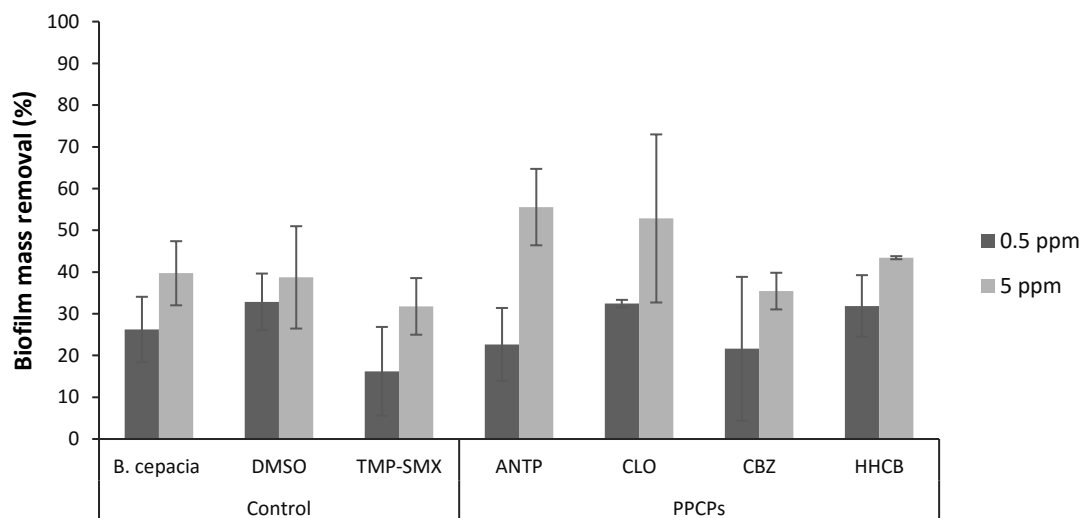
Figure 4 - Percentage of biofilm mass reduction of *B. cepacia* biofilms treated with 0.5 (■) and 5 ppm (■) NaOCl. *B. cepacia* formed biofilm without any compounds (only cells, control), in the presence of the solvent DMSO (control) and the antimicrobial agent TMP-SMX (control) and in the presence of ANTP, CLO, CBZ and HHCB. The results are displayed as % (mean biofilm mass reduction ± standard deviation).

When the biofilm was treated with NaOCl at 0.5 ppm, the presence of CLO caused a higher removal of the biofilm (32% against 26%, when *B. cepacia* was not exposed to any compound) (P<0.05). TMP-SMX (16%) was responsible for a lower reduction (*P*<0.05). In the treatment with 5 ppm NaOCl, only ANTP (56%) affected the removal of biofilm, while those formed in the presence of other PPCPs had similar removals to *B. cepacia* without undergo the effect of any compound (*P*>0.05).

The removal of the biofilm was determined using CV, a basic dye, which binds to the negatively charge surface molecules (on both live and dead cells) and to the polysaccharides of the EPS matrix (Li et al., 2003). Removal and CFU reduction are different actions. Nevertheless, taking into account the removals attained with 0.5 ppm, it would be expected to observe higher reductions.

4.3.2. Biofilm formed on coupons in a bioreactor for 7 days

A chemostat was used to develop *B. cepacia* biofilms for 7 days, on PVC coupons. For example, Ferreira et al. (2013) used a similar setup to develop *Pseudomonas fluorescens* biofilms. PVC was the support material chosen for biofilm formation as it is frequently used in DWDS (Simões et al., 2007a).

4.3.2.1. Biofilm formed without the influence of pharmaceuticals and personal care products

The 7 d old biofilm formed on coupons was treated for one hour with 0.5 and 5 ppm NaOCl. This treatment was also applied in the presence of TMP-SMX, ANTP, CBZ and CLO. The effect of NaOCl on *B. cepacia* culturability is displayed in Figure 5.
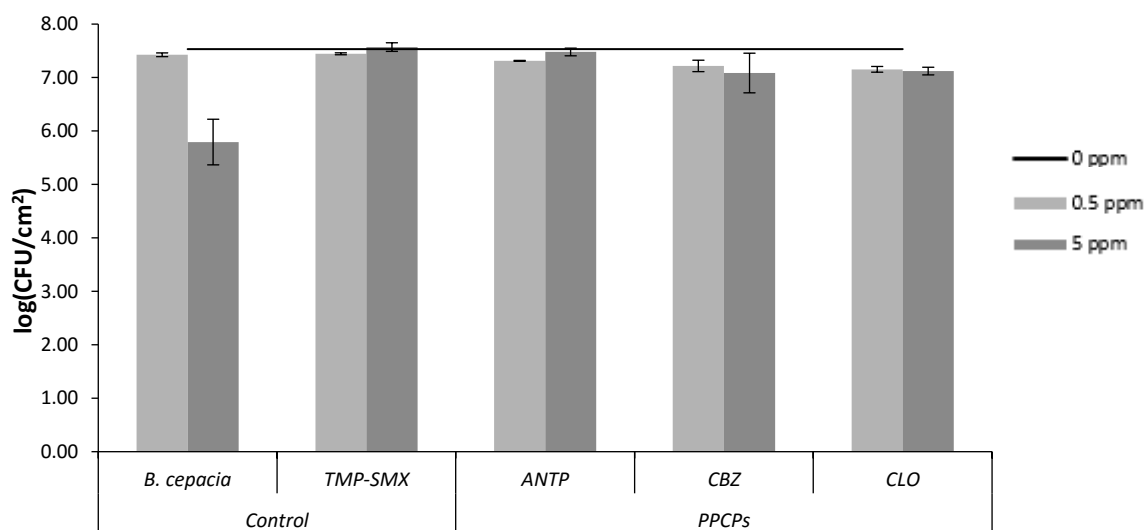
Figure 5 -Effect of NaOCl (■ - 0 ppm; ■ - 0.5 ppm; ■ - 5 ppm) on culturable *B. cepacia* biofilm cells. The biofilm was treated in the presence and in the absence of the control TMP-SMX and the PPCPs ANTP, CBZ and CLO. The results are displayed in mean logarithm of $CFU/cm^2 \pm$ standard deviation.

The number of CFU on the coupons, which did not undergo any treatment, was 7.53 log (CFU/cm^2). When comparing the reduction on the number of CFU caused by the treatment with NaOCl, there is a significant reduction for both 0.5 (0.11 log (CFU/cm^2) reduction) and 5 ppm (1.74 log (CFU/cm^2) reduction). However, when the coupons were also exposed to PPCPs, during the treatment, there was a change in the reduction pattern

with 5 ppm NaOCl. When ANTP, CBZ or the antibiotic SMX-TMP were present, there was no considerable reduction in the CFU numbers ($P>0.05$), except for CLO ($P<0.05$). Even though, the reduction achieved in the presence of CLO (0.41 log (CFU/cm^2) reduction) was much smaller than the reduction observed when *B. cepacia* was only exposed to 5 ppm NaOCl, without any PPCP.

The effect of PPCPs on the treatment of the biofilm with 5 ppm NaOCl was also assessed through the integrity of cytoplasmatic membrane. The percentage of cells stained with PI, i.e., with damaged cytoplasmatic membrane, is presented in Figure 6.
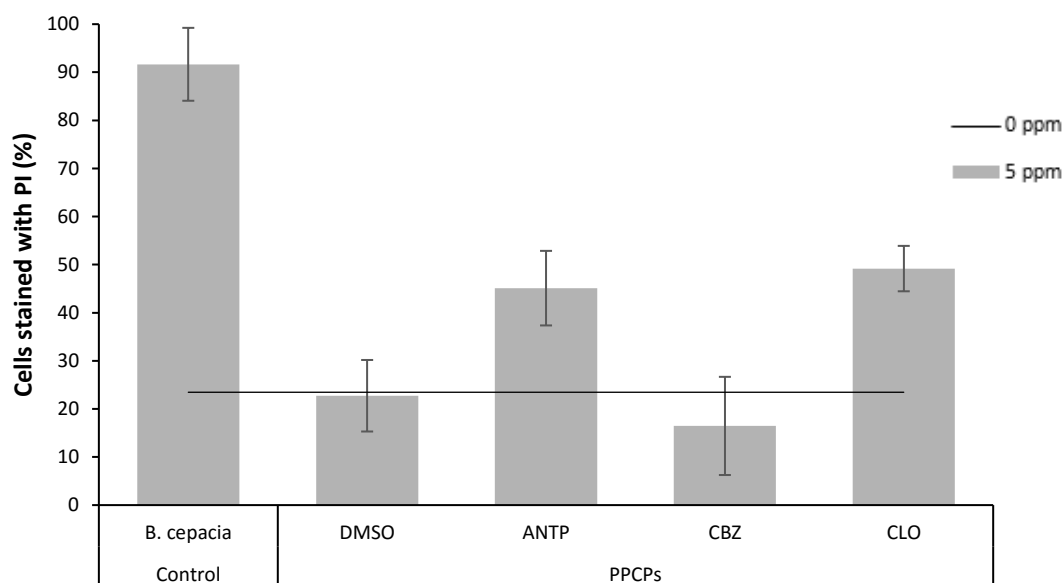
Figure 6 – Effect of ANTP, CBZ, CLO and of DMSO on the treatment of *B. cepacia* biofilm with NaOCl
(■ – 0 ppm; ▪ - 5 ppm). The results are shown as % (mean cells stained with PI ± standard deviation).

The results show that the treatment with NaOCl compromised the integrity of the cell membrane, when *B. cepacia* was not subjected to PPCPs during the treatment. This was expected as this disinfectant when in water forms NaOH and HOCl, which can dissociate in H^+ and OCl^-. HOCl and OCl^- can inhibit enzymes, damage the cell membrane and possibly affect the membrane transport capacity (Fukuzaki, 2006). Therefore, in the presence of NaOCl, approximately 92% of the cells had a damaged cytoplasmatic membrane. However, when PPCPs were present, the percentage of damaged cells was much lower (45% for ANTP; 16% for CBZ and 49% for CLO) ($P<0.05$). These results are consistent with the CFU numbers. The differences already existent between the biofilm on the different coupons can justify the results to some extent. However, it seems that PPCPs also have an important role on the lack of efficacy of NaOCl at killing the biofilm bacteria. The assessment of membrane integrity, coupled with the assessment of

culturability, hints at the possibility of a reaction between NaOCl and the PPCPs. Nevertheless, as discussed in section 3.3.1, the reaction between CBZ and chlorine is negligible (Soufan et al., 2013) which raises once again the question about the possible effect of DMSO on the results.

In this regarding, the effect of the solvent DMSO on the treatment with 5 ppm NaOCl was also assessed through the cells culturability and the cells viability. In the presence of DMSO, the log reduction caused by the disinfectant was 0.19 log. In addition, NaOCl only damaged the cytoplasmatic membrane of 23% of the cells which is similar to the percentage of damaged cells of the untreated biofilm ($P>0.05$). Furthermore, in viability assay, the cells organization in the sample with DMSO was very similar to the control. For example, after the action of NaOCl the cells were mainly isolated from one another, while in control and in the sample treated with NaOCl in the presence of DMSO, the cells were very close (Figure 7). Therefore, chlorine apparently reacted with DMSO and did not affect cells viability and culturability.
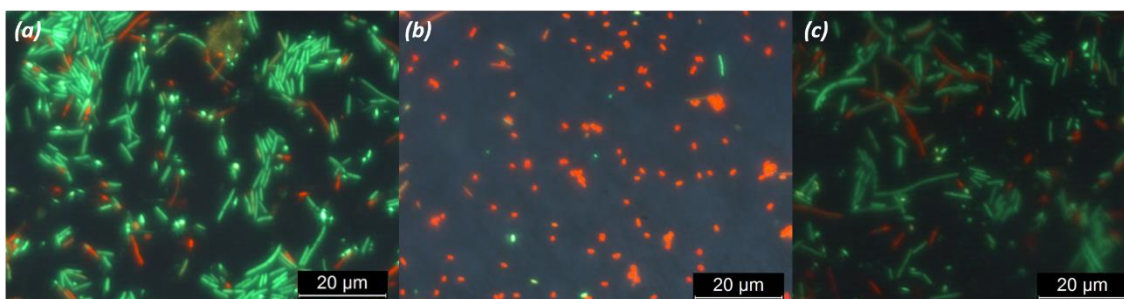
Figure 7 – Epifluorescence photomicrographs of *B. cepacia* biofilm cells without any treatment **(a)**; after being treated with 5 ppm NaOCl **(b)** and after applying the treatment with NaOCl in the presence of DMSO **(c)**. Viable cells are green and damaged cells are red (magnification, 1000 ×; bar = 20 µm).

4.3.2.2. Comparison between biofilm formed in the presence and in the absence of galaxolide and clofibric acid

The effect of PPCPs was also tested on biofilm formation on PVC coupons in the bioreactor. For 7 days, the cells were exposed continuously to HHCB and CLO, individually. After this period, the biofilm on the coupons was treated using 0.5 and 5 ppm NaOCl (Figure 8).
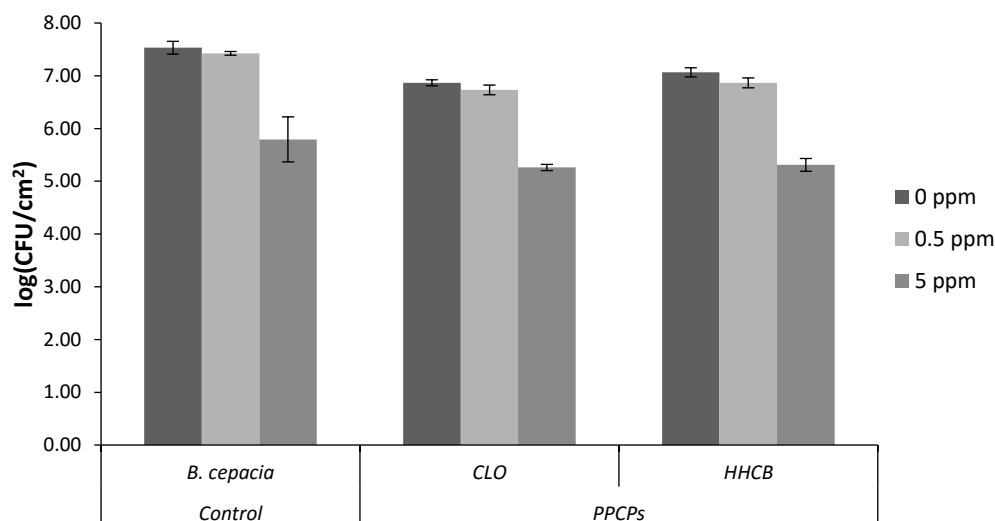
Figure 8 - CFU of 7 d old biofilm adhered on PVC coupons for *B. cepacia* without being exposed to any PPCP and when it underwent the effect of CLO and HHCB, after treatment with NaOCl (■ - 0 ppm; ■ - 0.5 ppm; ■ - 5 ppm). The results are displayed in mean logarithm of CFU/cm^2 ± standard deviation.

The cell density of *B. cepacia* biofilm was significantly lower when the bacterium was exposed to PPCPs (7.53 log (CFU/cm^2)): 6.87 and 7.06 log (CFU/cm^2) for *B. cepacia* biofilm formed when CLO and HHCB were present, respectively. When the biofilms were treated with NaOCl (0.5 ppm and 5 ppm), the reductions attained in the presence of PPCPs were similar to the reduction achieved when *B. cepacia* was not exposed to PPCPs (*P*>0.05). Therefore, these results do not corroborate the findings of the 24 h old biofilm formed in the microtiter plate, since in that assay CLO e HHCB did not affect the formation of the biofilm, but decreased the susceptibility of the biofilm cells to the treatment with NaOCl. However, the biofilm formed in the microtiter plate was in the early stages of development, while the biofilm formed in the bioreactor has already achieved the steady-state. Based on these results, one does not know what phase of the biofilm formation was affected by PPCPs. Besides that, in the bioreactor, the conditions were quite different: the medium was diluted, the exposure period was longer and the PPCP, at the residual concentration, was continuously fed to the reactor. Therefore, these parameters could also affect the interaction between the compound and *B. cepacia*.

The assessment of the membrane integrity was also performed for *B. cepacia* in the presence and in the absence of CLO (Figure 9). The assessment of the membrane integrity was not performed for HHCB due to the lack of resources.
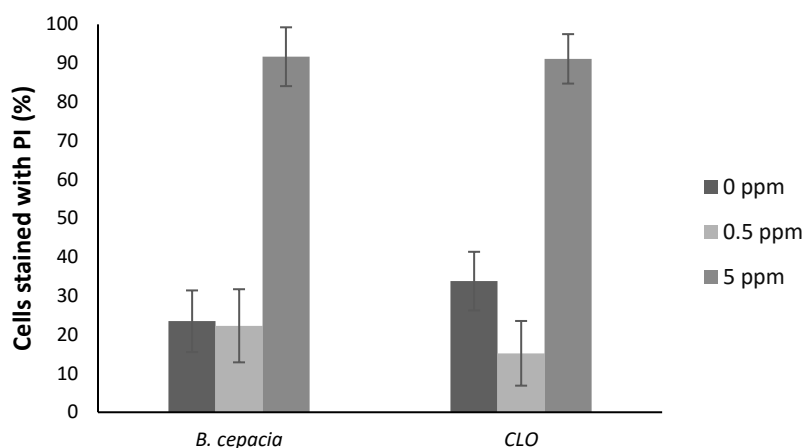
Figure 9 - Effect of NaOCl (■ - 0 ppm; ▪ - 0.5 ppm; ■ - 5 ppm) on a 7 d old biofilm formed in the presence and in the absence of CLO. The results are shown as % (mean cells stained with PI ± standard deviation).

In the biofilm, without the influence of any PPCP, the percentage of cells stained with PI was similar for the control and for the treatment with 0.5 ppm NaOCl (23% and 22%, respectively) ($P>0.05$). When the biofilm was formed in the presence of CLO, the fraction of stained cells was significantly lower for the biofilm treated with 0.5 ppm than for the non-treated biofilm ($P<0.05$). However, this difference is likely to be due to the variances between the biofilm cell numbers already existent before the treatment. The treatment with 5 ppm NaOCl rendered approximately the same percentage of cells with damaged cytoplasmatic membrane for control and for the biofilm formed in the presence of CLO (93% and 92%, respectively).

For a better characterization of the differences between the biofilm formed on the coupons, in the presence and absence of CLO and HHCB, the thickness of the biofilm was determined through the biofilm topography (Figure 10). The values obtained are summarized in the Table 7.
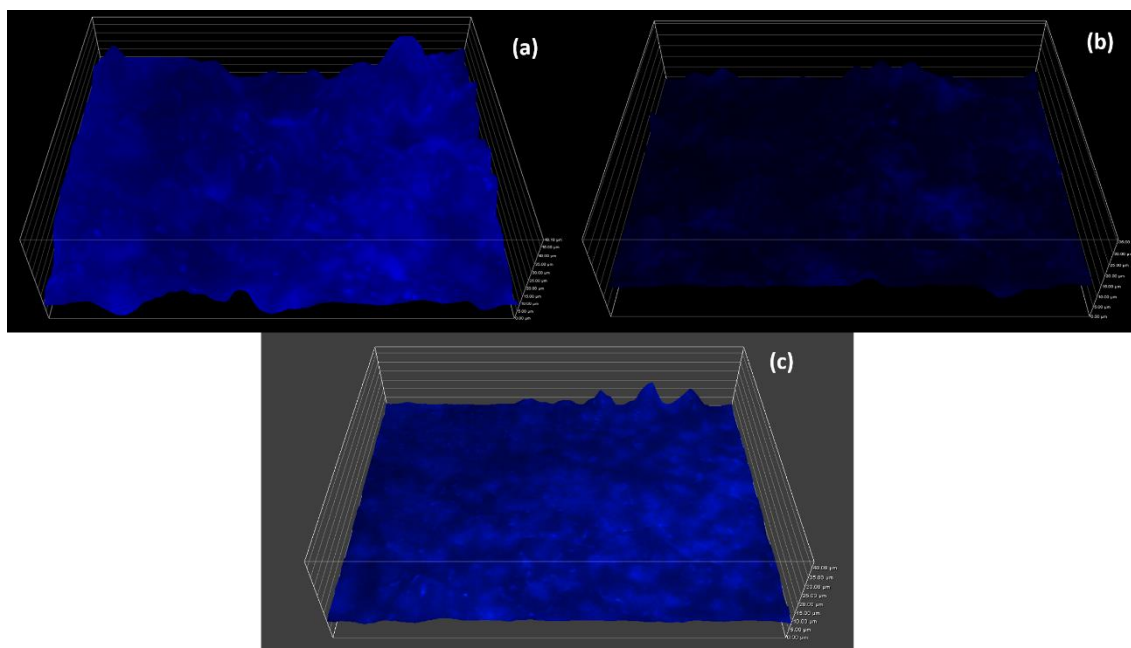
Figure 10 – 3D structure of the *B. cepacia* biofilms formed for 7 d on PVC coupons, in the absence **(a)** and in the presence of CLO **(b)** and HHCB **(c)** (magnification, 500 ×). The vertical axis represents the thickness of the biofilm. It starts in 0 µm (base of the biofilm) and the unit is 5 µm.

Table 7 – Thickness of the biofilm formed by *B. cepacia* in the presence and in the absence of CLO and HHCB. The values are displayed in (mean biofilm thickness ± standard deviation) µm.

Biofilm thickness (µm)		
Control	CLO	HHCB
15.5±6.5	6.9±2.6	9.3±2.7

The presence of CLO and HHCB, individually, in the feed of the bioreactor, affected the biofilm formation and development, since when *B. cepacia* was exposed to the PPCPs, the biofilm was thinner ($P<0.05$). The thickness of the biofilm formed with CLO present was also significantly lower ($P<0.05$) than the thickness of the biofilm formed in the presence of HHCB. It was observed that higher CFU numbers corresponded to a higher thickness.

Even though the presence of PPCPs have not been studied in DW biofilms yet, some authors investigated their presence and effects on fluvial biofilms (Corcoll et al., 2014, 2015; Huerta et al., 2015; Lawrence et al., 2005). These biofilms are constituted by bacteria, cyanobacteria, algae, protozoa and fungi (Corcoll et al., 2014) and, therefore they are very different from the studied biofilm. In spite of the differences, they still can give some insights on the behavior of bacteria in the presence of PPCPs.

Lawrence et al. (2005) developed a model riverine biofilm in an annular bioreactor in the presence of IBP, CBZ, CAF and furosemide, at 10 µg/L, individually. The presence of the compounds affected the abundance of biomass and the biofilm thickness. While CAF and furosemide increased the biomass, CBZ has decreased it. Regarding the thickness, CAF increased it, significantly. Even though these biofilms and *B. cepacia* biofilm are not comparable, this study supports that the presence of PPCPs during the biofilm formation process can affect bacteria and biofilm characteristics.

4.3.3. Biofilm formed on microtiter plate in presence of PPCPs at concentrations 100000 times the residual concentrations

Besides the tests using PPCPs at residual concentrations, another test with all the PPCPs at a concentration 100000 times the residual was carried out. The objective of this assay was to assess if macro-concentrations would produce amplified effects in the formation of biofilms and treatment with NaOCl. The results are shown in Figure 11.
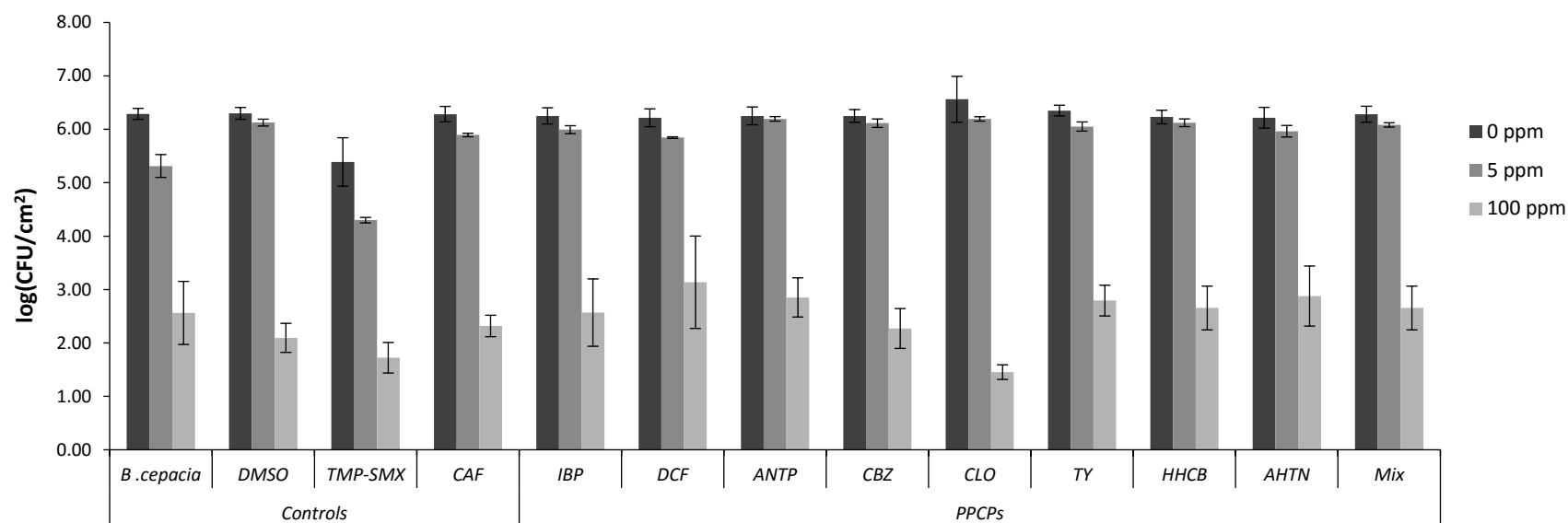
Figure 11 - Biofilm formation and treatment with NaOCl (■ - 0 ppm; ▨ - 0.5 ppm; ▨ - 5 ppm), in the presence and in the absence of PPCPs, at a concentration 100000 times the concentration reported in DW. The results are displayed as mean log (CFU/cm^2) ± standard deviation.

In the presence of CLO, the biofilm cell density was 0.27 log (CFU/cm^2) ($P<0.05$) higher than *B. cepacia* biofilm without PPCPs. The other PPCPs and control CAF did not alter significantly the biofilm formation ($P>0.05$). When control TMP-SMX was present, the number of cells was lower than for *B. cepacia* biofilm (without PPCPs) (0.9 log (CFU/cm^2) difference) ($P<0.05$), which can be justified by the antimicrobial action of this mixture. In the other assays, as the concentrations used were in the ng/L range it was not observed an inhibitory behavior. Nevertheless, in this case, the concentrations tested were already in the order of hundreds of µg/L causing a decrease in cells culturability. In the literature, a study demonstrated that the lowest concentration of a mixture of TMP and SMX that inhibited *E. coli* growth was 0.03 µg/mL of TMP and 0.075 µg/mL of SMX (Woodbine, 1977). Therefore the concentration of TMP-SMX (0.17 µg/mL TMP and 0.82 µg/mL SMX) tested was above these values, which explains its inhibitory behavior on *B. cepacia*.

The treatment of the biofilms with 5 ppm NaOCl reduced by approximately 1 log (CFU/cm^2) the number of cells in the biofilm formed by *B. cepacia* without any PPCPs and on the biofilm formed under the influence of TMP-SMX ($P<0.05$). In the biofilms developed in the presence of the controls DMSO and CAF and in the presence of the PPCPs and of the mixture, the log (CFU/cm^2) reduction was significantly lower and varied between 0.06 and 0.39, with ANTP, CBZ and HHCB, achieving the lowest reductions ($P<0.05$). CBZ, HHCB and CLO had a similar behavior at residual concentrations and at this concentration of NaOCl. In both cases, they reduced the susceptibility of sessile *B. cepacia* to the treatment, with lower CFU reduction for the macro-concentrations.

The log (CFU/cm^2) reduction achieved with 100 ppm was identical for the majority of the cases, except for the biofilm formed in the presence of controls DMSO and CAF and PPCPs CBZ and CLO which suffered higher reductions ($P<0.05$). These results are quite unexpected, since biofilms treated with 5 ppm NaOCl were more tolerant to the treatment when in the presence of the PPCPs.

When chlorine reacts with organic compounds it can produce by-products (Canosa et al., 2006). This can also happen for PPCPs (Quintana et al., 2010). As PPCPs and NaOCl are at high concentrations, by-products may be formed at higher concentrations. If those by-products are toxic for the cells, they might explain the changes observed in susceptibility for the treatment with 100 ppm NaOCl. In this study, CBZ and CLO were the PPCPs responsible for higher log (CFU/cm^2) reduction in the treatment with 100 ppm NaOCl. Regarding their chlorination, CLO achieved a degradation lower than 20% and negligible degradation was reported for CBZ for low concentrations of chlorine (1 mg/L) (Simazaki et al., 2008; Soufan et al., 2013). In other words, the presence of by-products would not be plausible explanation. Nevertheless, a higher degradation of CBZ and CLO is expected in the treatment with 100 ppm NaOCl. For example, 58% of CBZ was removed in a treatment with chloride dioxide at 13.5 mg/L which shows that a higher concentration of disinfectant can improve the degradation (Kosjek et al., 2009). Therefore, the possible production of by-products potentially toxic for cells cannot be ruled out. However, further analysis would be necessary to conclude if this hypothesis is in fact responsible for these results.

4.4. Conclusions

The presence of PPCPs at residual concentrations did not improve 24 h biofilm formation. The treatment with 0.5 ppm only reduced significantly the biofilm formed in the presence of CBZ and CLO. However, when treated with 5 ppm NaOCl, the biofilms formed in the presence of CBZ, CLO and HHCB were less susceptible to the treatment.

The 7 d old biofilm was formed in the presence of CLO and HHCB and also in the absence of any PPCPs. The cell density and the biofilm thickness were lower for the biofilm formed during exposition to each PPCP. The susceptibility of *B. cepacia* biofilm to the treatment with 0.5 and 5 ppm was not affected by the PPCPs.

When the control biofilm (without exposure to PPCPs) was treated with 5 ppm NaOCl, in the presence of CBZ and ANTP, there was no significant reduction of CFU. Furthermore, the percentage of cells with damaged membrane was much lower in the presence of CBZ, ANTP and CLO. However, this was also verified in the presence of the solvent DMSO, which suggests it can be responsible for the lack of efficacy of NaOCl.

Another assay, using the eight PPCPs at concentrations 100000 times higher than the concentrations reported in DW, was performed to assess the effect of macro-concentrations on bacteria. In the presence of CLO, the biofilm had a higher cell density. The treatment with 5 ppm NaOCl produced lower log CFU reductions for biofilms formed in the presence of PPCPs. Nevertheless, when a higher concentration of NaOCl was tested (100 ppm) the biofilm formed in the presence of the majority of PPCPs and the control (*B. cepacia* only) had a similar susceptibility to NaOCl. The biofilms formed in the presence of CBZ and CLO experienced higher log (CFU/cm^2) reductions of CFU.

Chapter 5

5. Concluding remarks and future perspectives

5.1. General conclusions

The continuous usage of PPCPs and their consequent introduction into the environment has led to their appearance in DW, as they are not completely removed by conventional treatments. Their presence as chemical contaminants and their possible risks to human health have been investigated. However, to this date there is no information on how they can affect the microbiological quality of DW. Therefore, this work sought to assess if PPCPs can have an effect on a DW bacterium behavior and on its susceptibility to disinfection.

The main conclusions that can be drawn from this work are the following:

PPCPs, at concentrations 100 times the concentrations detected in DW, can affect the susceptibility of planktonic *B. cepacia* to the treatment with NaOCl and to the antimicrobial agent TMP-SMX. ANTP and HHCB increased the tolerance to TMP-SMX, while ANTP, HHCB, DCF, CBZ and TY decreased the susceptibility of the bacterium to NaOCl. Nevertheless, the solvent DMSO also decreased the susceptibility of *B. cepacia* to NaOCl, which suggests that it can be responsible to some extent for the results observed.

ANTP, HHCB and DCF improved the swimming motility, while only HHCB enabled the swarming motility.

HHCB, ANTP, CBZ and CLO, at residual concentrations, were tested on the formation of 24 h old biofilms and treatment with NaOCl. They did not influence the development of the biofilms. However, HHCB, CBZ and CLO increased the tolerance of *B. cepacia* biofilm to the treatment with 5 ppm NaOCl. *B. cepacia* also formed 7 d old biofilm in the presence of CLO and HHCB. Both PPCPs led to a lower formation of biofilm and also affected its thickness as *B. cepacia* formed thinner biofilms when in the presence of PPCPs. Nevertheless, no changes were observed in the susceptibility to the treatment with NaOCl. When 7 d old biofilm formed without any PPCPs was treated with 5 ppm NaOCl in the presence of ANTP, CLO and CBZ the cell density was similar to the biofilm without exposure to any treatment. This effect was also observed in the presence of

DMSO. In other words, it seems that DMSO when added during the treatment diminished the effect of NaOCl on *B. cepacia*.

Macro-concentrations of all PPCPs were also tested in 24 h old biofilms and affected the biofilm treatment with 5 ppm NaOCl, by decreasing the biofilm susceptibility. Only CLO increased biofilm formation. In addition, a higher concentration of NaOCl (100 ppm) was also used to treat the biofilm and, in the presence of CLO and CBZ, the biofilm was more susceptible.

Based on these conclusions, the presence of PPCPs seems to affect the susceptibility of both planktonic and sessile *B. cepacia*. In the biofilms, the conditions and the period of the biofilm formation (24 h old and 7 d old) seems to play an important role on how PPCPs influence the bacterium. Different concentrations of NaOCl may also dictate how the compounds will interact with the bacterium. This work is the first evidence of the effects of PPCPs on a DW bacteria. However, further analysis would be necessary to validate the effect of these PPCPs in *B. cepacia*.

5.2. Future work

As it was referred, some of the results seemed to be affected by the choice of the solvent. Therefore, in the future, it would be important to carry out the experimental assays, using a different solvent in order to ensure that the differences obtained between the control and the contaminants are due to their effect alone. During this work, it was performed a preliminary assay using three different solvents, besides the DMSO: acetone (propanone), ethanol and propylene glycol (1,2-propanediol). The results are showed in the Table 8.

Table 8 – MBC of NaOCl for *B. cepacia* and for *B. cepacia* in the presence of the solvents DMSO, acetone, ethanol and propylene glycol.

Condition	MBC (ppm)
Control (*B. cepacia*)	3
DMSO	5
Acetone	3
Ethanol	2
Propylene glycol	2

These results suggest that acetone could be used as solvent without modifying the susceptibility of *B. cepacia*. However, further experiments would be required to choose the most appropriate solvent.

In this work, different ranges of concentrations (concentration reported in DW and concentrations 100- and 100000-fold) were tested, but the exposure time was always short (24 h, generally, and, in the case of the bioreactor, 7 d). In this regard, it would be interesting to determine the effect of the variable time. In other words, longer experiments, for example for one month, could be conducted to observe if the presence of PPCPs affect the susceptibility and the behavior of bacteria over time. Furthermore, it would also be important the optimization of the experimental conditions in order to find the best way to mimic, in a relatively short time, the effects that in the nature can take many years to happen and to be noticed.

Since PPCPs are not detected individually in water it would be interesting to test the effect of groups of compounds, from the same class or from different classes to assess their putative additive or synergistic effects against the bacteria.

A quantification of the PPCPs present in those biofilms formed in the bioreactor should be done in order to observe if the compound is or not at the same concentration found in the water phase.

Finally, the effect of PPCPs should be determined for other DW bacteria, as they may affect different bacteria in different ways.

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