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UNIVERSIDADE DO PORTO

Understanding the effects of copper surfaces and emerging contaminants on planktonic and biofilm behaviour of drinking water bacteria

Dissertation for PhD degree in Chemical and Biological Engineering

Supervisor: Professor Manuel Simões

Co-Supervisor: Dr. Lúcia Simões

Inês Bezerra Gomes

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"Os rios não bebem sua própria água; as árvores não comem seus próprios frutos. O sol não brilha para si mesmo; e as flores não espalham sua fragrância para si. Viver para os outros é uma regra da natureza. (...) A vida é boa quando se está feliz; mas a vida é muito melhor quando os outros estão felizes por sua causa".

Papa Francisco

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Abstract

The access to safe drinking water (DW) is a right for each human being. Unfortunately, there are several problems that may impair DW quality, even in the most developed countries. One of the main concerns in recent years is the presence of emerging contaminants (ECs) in water sources. The lack of efficient technologies to remove ECs from residual waters contributes for their appearance in drinking water distribution systems (DWDS). Low attention has been given to the consequences from the exposure to ECs by the DW microbiome. Biofilm development in DWDS is unavoidable, despite separation and disinfection processes that may be applied before and during the water distribution. Therefore, sessile microorganisms on DWDS pipes are continuously exposed to trace concentrations of ECs.

The aim of this work was to evaluate the consequences from the exposure to different ECs on the behaviour of a bacterial strain, *Stenotrophomonas maltophilia*, isolated from a DWDS. Eight ECs (antypiryne (ANTP), clofibric acid (CA), carbamazepine (CBZ) diclofenac (DCF), galaxolide (GAL), ibuprofen (IBP), tonalide (TON) and tylosin (TY)) were tested at two different concentrations: [DW] – an environmental concentration detected in DW and 100 × [DW]. The presence of some ECs (CA, CA + CBZ, CA + IBP, CA + CBZ + IBP) in DWDS was found to hinder biofilm disinfection with chlorine. CA was the single compound present in all the combinations that altered *S. maltophilia* biofilm behaviour. Therefore, this compound was studied with higher detail and *S. maltophilia* was exposed to CA for a longer period (12 weeks), allowing to find out that CA increased *S. maltophilia* tolerance to erythromycin.

The presence of biofilms in DWDS may also constitute a concern for consumers and distribution companies, affecting the water microbiological quality and may lead to a number of unwanted effects on the organoleptic properties of the distributed water. Of additional concern is the presence of pathogens in biofilms, which may constitute a public health risk when these microorganisms are released from the biofilm. Therefore, this work also aimed to understand the effects from the use of copper materials in DW biofilms. The antimicrobial properties of copper have been studied over the years. However, copper is expensive causing a reduction on its use in DWDS. The use of copper alloys instead of elemental copper materials would counterbalance the current economic limitations. Therefore, in this work six materials with different copper content (0, 57, 79, 83, 96 and 100% of copper content) were used to form single and dual species biofilms with *Acinetobacter calcoaceticus* or/and *S. maltophilia*, both isolated from DW.

In the first part of the work, all the alloys were evaluated in terms of biofilm control (inactivation and removal), regrowth, copper leaching and corrosion. All the copper materials had important impact on biofilm viability and culturability - even those with lower copper content. Nevertheless,

96% copper alloy was the one with most promising results in terms of preventing biofilm regrowth. All the alloys were resistant to corrosion under both chlorinated and unchlorinated conditions. Nevertheless, 83% copper alloy is not suitable for application in chlorinated DWDS as the copper concentration detected in the bulk water was above the maximum value recommended by the Environment Protection Agency (EPA) and the World Health Organization (WHO). All the other alloys suffered lower leaching in the presence and absence of chlorine and the maximum recommended values were not exceeded. No copper was quantified in the 57% copper alloy leachates. *S. maltophilia* was more tolerant to copper than *A. calcoaceticus* in single and dual species biofilms. Therefore, the effects of copper on *S. maltophilia* biofilm control were further investigated.

Supplementary studies were performed to understand how copper materials may influence the efficacy of conventional treatments. A rotating cylinder reactor (RCR) was used to form biofilms for seven days under conditions that simulate real DWDS. The use of this reactor allowed the application of a chemical treatment (10 mg/l of free chlorine for 10 minutes), a mechanical treatment (flushing with 1.5 m/s of fluid flow for 30 s) and the combination of both. Biofilms formed on the copper materials produced lower amounts of extracellular proteins than those formed on stainless steel (0% of copper content). The use of copper materials may be important to overcome chlorine decay along the system, as biofilms formed on 96 and 100% copper materials had lower culturability than those formed on stainless steel (0% of copper) treated with chlorine. The copper materials also reduced the viability of *S. maltophilia* released to the water during chlorine and mechanical treatments.

The use of copper materials in DWDS may play an important role in the control of biofilm development, in the reduction of chlorine usage and reducing the consumers' contact with viable pathogens. Nevertheless, it is also of utmost importance to understand if copper leachates are toxic for the consumers. Therefore, *in vitro* assays using human colon rectal adenocarcinoma cells (HT29) were performed in order to assess potential cytotoxic effects from the ingestion of DW with copper. Leachates formed by the contact with chlorinated water were more toxic for HT29 cells. Most of the leachates also caused DNA damage in HT29 cells. The exception was the alloy with 57% copper content, which demonstrated reduced cytotoxicity and genotoxicity. Therefore, the 57% copper alloy seems to be adequate for application in DWDS based on complementary biofilm control effects and reduced cytotoxicity and genotoxicity.

In conclusion, this work reinforces the importance of studying the effects of non-antibiotic contaminants on the behaviour of environmental microorganisms, particularly their role as drivers affecting resistance evolution and selection. The use of copper alloys in DW biofilm control is promising and can complement disinfection by chlorination.

Resumo

Todo o ser humano tem direito ao acesso a água potável (AP). Infelizmente, existem diversos problemas que podem prejudicar a qualidade da AP mesmo nos países mais desenvolvidos. Nos últimos anos, uma das principais preocupações ambientais tem sido a presença de contaminantes emergentes (CEs) nos recursos de água. Estes contaminantes são frequentemente detetados em AP devido à falta de tecnologias eficientes para a sua remoção completa nas estações de tratamento. No entanto, a comunidade científica não tem prestado muita atenção às possíveis consequências da exposição a CEs no microbioma naturalmente presente em sistemas de distribuição de AP. A formação de biofilmes ao longo do sistema de distribuição de AP é impossível de ser evitada, ainda que diferentes processos de separação e desinfeção sejam aplicados antes e durante o transporte de água. Desta forma, os microrganismos que colonizam as tubagens de transporte de AP estão continuamente expostos a concentrações residuais de CEs.

Um dos principais objetivos deste trabalho foi avaliar as consequências da exposição a diferentes CEs numa estirpe de *Stenotrophomonas maltophilia* isolada de AP. Oito CEs (antipirina (ANT), ácido clofibrico (AC), carbamazepina (CBZ), diclofenac (DCF), galaxolide (GAL), ibuprofeno (IBP), tonalide (TON) e tilosina (TI)) foram selecionados e testado em duas concentrações distintas: a concentração ambiental detetada em AP e uma concentração 100 vezes superior. Os resultados obtidos demonstraram que a presença de alguns CEs (AC, AC + CBZ, AC + IBP, AC + CBZ + IBP) nos sistemas de distribuição de AP pode dificultar a desinfeção dos biofilmes com cloro. O AC é o único composto presente em todas as combinações que promoveram alterações nos biofilmes de *S. maltophilia* e por esse motivo foi estudado com maior detalhe. *S. maltophilia* foi exposta ao AC por um período mais longo (12 semanas) o que permitiu observar que a presneça de AC aumentou a tolerância da *S. maltophilia* à eritromicina.

A presença de biofilmes nos sistemas de distribuição de AP também representa uma preocupação quer para consumidores quer para as empresas de distribuição de AP, afetando a qualidade microbiológica da água e causando inúmeros efeitos indesejáveis nas caraterísticas organolética da água. Mais preocupante é a presença de organismos patogénicos em biofilmes, que podem constituir um risco para a saúde pública se ocorrer a sua libertação para a água transportada. Portanto, este trabalho tem como objetivo adicional a compreensão dos efeitos do uso de materiais de cobre no controlo de biofilmes de AP. As propriedades antimicrobianas do cobre tem sido muito estudadas ao longo dos anos. No entanto, o cobre é um metal caro pelo que o seu uso no transporte de AP tem diminuído. O uso de ligas de cobre pode ser importante para contrabalançar as atuais limitações económicas associadas a este material. Por isso, neste trabalho foram selecionados seis materiais com diferentes teores de cobre (0, 57, 79,83, 96 e 100 % de cobre)

para formar biofilmes simples e duplos (contendo *Acinetobacter calcoaceticus* e/ou *S. maltophilia*, ambas isoladas de AP).

Numa primeira fase, as ligas de cobre foram avaliadas de acordo com a sua capacidade de controlar biofilmes (inativação e remoção) e o seu recrescimento, a lixiviação de cobre e a taxa de corrosão. Todos os materiais de cobre mostraram uma importante ação no controlo da viabilidade e culturabilidade dos biofilmes, mesmo as ligas que continham menor teor de cobre. Contudo, a liga com 96% de cobre foi a liga mais promissora no que respeita à prevenção do recrescimento do biofilme. Todos materiais se mostraram resistentes à corrosão na presença e na ausência de cloro. Porém a liga que contém 83% de cobre não pode ser aplicada em sistemas de distribuição de água clorados, pois os lixiviados resultantes desta exposição continham uma concentração de cobre superior ao valor máximo recomendado pelas entidades reguladoras, Agência de Proteção do Ambiente (EPA) e a Organização Mundial de Saúde (OMS). Todos os restantes materiais sofreram menor lixiviação, na presença e ausência de cloro, e os valores máximos recomendados não foram excedidos. É de salientar que nos lixiviados da liga com 57% de cobre, não foi possível detetar iões de cobre. *S. maltophilia* mostrou-se mais tolerante à presença de materiais de cobre do que a *A. calcoaceticus*, quer em biofilmes simples quer em biofilmes duplos. Por este motivo, os efeitos do cobre no controlo de biofilmes de *S. maltophilia* foram mais aprofundados posteriormente.

Estudos adicionais foram realizados, por forma a perceber o efeito dos materiais de cobre na eficácia de tratamentos de AP convencionais. Utilizou-se um reator de cilindros rotativos (RCR) para formar biofilmes durante sete dias em condições que simulam um sistema de distribuição de AP. O uso deste reator permitiu a aplicação de um tratamento químico (10 mg/L de cloro livre durante 10 minutos), um tratamento mecânico (*flushing* com uma velocidade do fluido de 1.5 m/s durante 30 s) e a combinação de ambos os tratamentos.

Os biofilmes formados em materiais de cobre produziram menor quantidade de proteinas extracelulares do que os biofilmes formados em aço inoxidável (0% de teor de cobre). A utilização de materiais de cobre pode ser importante para superar o decaimento de cloro que normalmente ocorre ao longo do sistema de distribuição, uma vez que os resultados demonstraram que biofilmes formados em materiais com 96 e 100% de teor de cobre tinham menor quantidade de células cultiváveis do que os biofilmes formados em aço inoxidável (0% de cobre) tratados com cloro. Os materais de cobre também reduziram a viabilidade da *S maltophilia* libertada para a água transportada durante a aplicação dos tratamentos com cloro e mecânico.

Em síntese, o uso de superfícies de cobre em sistemas de distribuição de água assume uma ação importante no controlo de biofilmes, na redução da utilização de cloro e na diminuição da exposição dos consumidores a microrganismos patogénicos. No entanto, também é importante

avaliar se os lixiviados produzidos têm algum efeito tóxico para o consumidor. Por esse motivo, células humanas de adenocarcinoma colorretal (HT29) foram utilizadas num ensaio *in vitro*, avaliando os possíveis efeitos da ingestão de AP proveniente de sistemas de distribuição contendo materiais de cobre para as células dos consumidores. Verificou-se que os lixiviados produzidos na presença de cloro foram mais tóxicos para as células HT29. A maioria dos lixiviados demonstrou causar dano genético em células HT29, com a exceção dos lixiviados da liga com 57% de teor de cobre que demonstrou efeitos citotóxicos e genotóxicos reduzidos. Desta forma, a liga com 57% de teor de cobre parece poder ser aplicada em sistemas de distribuição de água, melhorando o controlo da formação de biofilmes sem aparente citotoxicidade e genotoxicidade.

Em conclusão, este trabalho reforça a importância do estudo dos efeitos de contaminantes não-antibióticos no comportamento dos microrganismos ambientais, particularmente o seu papel como fatores que afetam a evolução e seleção de resistência antimicrobiana. O impacto das ligas de cobre no controlo de biofilmes de AP também demonstrou ser relevante no controlo de biofilmes, particularmente como complemento da desinfeção com cloro.

Table of contents

List of figures

List of tables

List of abbreviations

AMO – Amoxicilin

ANTP – Antypiryne

AOPs – Advanced oxidation process

ARB – Antibiotic resistant bacteria

ARG – Antibiotic resistance gene

ASP – Activated sludge process

CA – Clofibric acid

CAS – Chrome azurol A

CBZ – Carbamazepine

CFU – Colony-forming units

CIP – Ciprofloxacin

CLSI – Clinical & Laboratorial Standards Institute

COD – Chemical oxygen demand

CR – Corrosion rate

DBPs – Disinfection by-products

DCF – Diclofenac

DDT – Di-chlorodiphenyltrichloroethane

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DPD – N,N - diethyl-p-phenylenediamine

DW – Drinking water

DWDS – Drinking water distribution system

DWTP – Drinking water treatment plant

EC – Emerging contaminants

EC/EF – Electrocoagulation/electrofiltration

EDCs – Endocrine disruptor compounds

EDTA – Ethylenediamine tetraacetic acid

EPA – Environmental Protection Agency

EPS – Extracellular polymeric substances

ERY – Erythromycin

FBS – Fetal bovine serum

GAC – Granular activated carbon

GAL – Galaxolide

H_2DCFDA – Dichloro-dihydro-fluorescein diacetate

HDPE – High density polyethylene

HGT – Horizontal gene transfer

I3S – Institute for research and Inovation in Health Sciences

IBP – Ibuprofen

KAN – Kanamycin

LBB- Luria – Bertani broth

LEV – Levofloxacin

LMP – Low melting point

MBC – Minimum bactericidal concentration

MBR – Membrane bioreactor

MF – Microfiltration

MHA – Mueller Hinton agar

MIC – Minimum inhibitory concentration

MIX – Mixture of contaminants

MTT – 3 - (4,5 - dimethylthiazol -2-y)-2,5-diphenyltetrazolium bromide

NF – Nanofiltration

NMP – Normal melting point

NP – Nanoparticle

NSAID – Non-steroidal anti-inflammatory drug

OXA – Oxacillin

PAE – Phthalate esters

PBS – Phosphate buffered saline

PBT – Persistence, bioaccumulation and toxicity

PCA – Plate count agar

PE – Polyethylene

PI – Propidium iodide

POE – Point-of-entry

POU – Point-of-use

PPCP – Pharmaceutical and personal care products

PVC – Polyvinyl chloride

R2A – Reasoner's 2A (médium)

RCR – Rotating cylinder reactor

RO – Reverse osmosis

ROS – Reactive oxigen species

SPE – Spectinomycin

SS – Stainless steel

STW – Synthetic tap water

SWCNT – Single walled carbon nanotubes

TBBPA – Tetrabromobisphenol A

TCCACM – Tubular nanofiber/carbon/alumina composite membrane

TE – Tris-EDTA buffer

TET – Tetracycline

TMP-SMX – Trimethoprim- sulfamethoxazole

TON – Tonalide

TVS – Total volatile solids

TY – Tylosin

UF – Ultrafiltration

UK – United Kingdom

USA – United States of America

UV – Ultra-violet

WHO – World Health Organization

WW – Wastewater

WWTP – Waste water treatment plant

[DW] – Concentration detected in DW

$(NH_4)_2SO_4$ – Ammonium sulfate

$CaSO_4$ – Calcium sulfate

CeO_2 – Cerium oxide

ClO_2 – Chlorine dioxide

CO_2 – Carbon dioxide

$CuCl_2$ – Cupric chloride

CuO – Copper oxide

Fe^{3+} – Ferric ion

$FeSO_4.7H_2O$ – Ferrous sulfate heptahydrated

H_2O_2 – Hydrogen peroxide

HCl – Hydrochloric acid

HOCl – Hypochlorous acid

K_2HPO_4 – Potassium phosphate dibasic

KCl – Potassium chloride

KH_2PO_4 – Monopotassium phosphate

$MgSO_4.7H_2O$ – Magnesium sulfate heptahydrated

$Na_2HPO_4.H_2O$ – Dissodium phosphate

$Na_3PO_4.H_2O$ – Trisodium phosphate hydrated

NaCl – Sodium chloride

$NaHCO_3$ – Sodium bicarbonte

$NaNO_3$ – Sodium nitrate

NaOCl – Sodium hypochlorite

NaOH – Sodium hidroxide

ZnO – Zinc oxide

Chapter 1

Work outline

This chapter summarizes the relevance and motivation of the work, the main objectives and a description of the thesis organization.

1.1 Relevance and Motivation

The presence of biofilms in drinking water distribution systems (DWDS) constitutes one of the currently recognized hazards affecting the microbiological quality of water and may lead to a number of unwanted effects on its organoleptic properties. Importantly, biofilms may constitute a persistent reservoir of pathogens, which can cause several waterborne diseases. The risk for human health can be higher if those microorganisms become resistant to antimicrobials. The continuous exposure to disinfectants and antibiotics stimulate bacterial adaptation to antimicrobials. Nevertheless, the existing information about effects of pharmaceuticals and other emerging contaminants (ECs) presented in drinking water (DW) on microbial behaviour and antimicrobial resistance is limited. Despite this lack of information, the presence of ECs in DW and its consequences for human health deserved the attention of the World Health Organization (WHO) that published a report entitled "Pharmaceuticals in drinking water" (WHO 2012).

It is of utmost importance to have efficient disinfection plans to control microorganisms in the bulk phase and their biofilms in order to provide high quality DW. Antimicrobial products, particularly chlorine, have been the main weapons used for DW disinfection and biofilm control in DWDS. Although this strategy is widespread, there are no standardized disinfection strategies with reliable efficacy in the control of biofilms (Simões and Simões 2013). Therefore, there is a global concern on developing alternative strategies to control the presence of biofilms in DWDS and minimize the environmental and public health impacts resulting from the ineffectiveness of traditional techniques.

Several strategies can be used to attempt biofilm prevention and control in DWDS. For instance, preventive strategies have attempted to identify materials that do not promote or can even suppress biofilm formation (Rogers *et al*. 1994). Furthermore, the type of material can also affect biofilm disinfection efficiency and some surfaces have been described as antimicrobial, such as copper and silver (Grass *et al*. 2011, Roe *et al*. 2008). Copper pipes have been used to construct DWDS and home plumbing systems. Some problems can be pointed to this material, as copper leaching to the bulk water and high acquisition costs (WHO 2003b). Nevertheless, other alternatives to apply copper in DWDS pipes should be evaluated, particularly the use of stable copper alloys.

The use of physical strategies, particularly pipe flushing, is cheap, chemical-free and can remove biofilms through the application of high water flows (Mathieu *et al*. 2014, Poulin *et al*. 2010). This strategy can help to improve DW biofilm control by acting synergistically with chemical agents (Simões *et al*. 2009). However, the combined use of different strategies is not yet conveniently explored for DW disinfection and the consequences from biofilm release into the transported water had not been conveniently studied.

It seems essential to develop new strategies to control biofilms in DWDS, based on the use of materials that may retard biofilm formation or in the combination of chemical and physical treatments. Biofilm control is of utmost importance in order to reduce biofilm development and the consequences from microbial exposure to ECs, improving the quality of delivered DW and reducing the risks for consumers.

1.2 Objectives

The main goals of this project were to evaluate the consequences from the exposure of DW bacteria to ECs and the study of different copper materials as a strategy to prevent and control biofilm development in DWDS. A system mimicking DWDS and microorganisms isolated from DWDS were used in order to obtain results that express what happens in DWDS.

The specific objectives are listed below:

- Evaluation of the effects of the continuous exposure to ECs on bacterial resistance to chlorine and antibiotics, providing information on the role of DWDS as a pool of resistant and virulent microorganisms.

- Evaluation of the effects of different pipe materials on biofilm prevention, exploiting the antimicrobial activity of copper and copper alloys as well as the possibility to apply this material in DWDS.

- Study on the antimicrobial mode of action of the copper materials.

- Evaluation of biofilm regrowth potential using materials with different copper content.

- Study of biofilm formation in a rotating cylinder reactor (RCR) mimicking real conditions found in a DWDS and further evaluation of copper materials under such conditions.

- Evaluation of combined strategies to control biofilms on copper materials, studying their influence on microbial release and possible consequences in DW quality and public health.

- Study of the potential risks from copper use in DWDS for consumers and evaluation of cytotoxic and genotoxic effects of leachates resulting from copper exposure to chlorinated and unchlorinated water.

1.3 Synopsis

This thesis is divided in nine main chapters that are summarized below.

Chapter 1 presents the main objectives of this work as well as the motivation for the research goals.

Chapter 2 consists in the literature review that focus on the presence of ECS in water and the known consequences for the microbiomes exposed. The literature review also emphasis strategies employed to avoid the presence of ECs in DWDS and important strategies to improve the control of biofilms along DWDS. These combined strategies may be crucial on the limitation of antimicrobial resistance spread, limiting the prolonged exposure of DW microorganisms to ECs.

Chapter 3 describes in detail the methodology used in the experimental work. This chapter is divided in 8 sub-chapters. The first four are related to general methodology, such as bacterial growth, biofilm substrates and description of selected ECs. The last four are entitled according to the chapters where the respective results are presented.

The main results and respective discussion are presented in chapters 4 to 8. Chapter 4 refers to the evaluation of the effects from the exposure to 8 selected ECs (antypiryne, clofibric acid, carbamazepine, diclofenac, galaxolide, ibuprofen, tonalide and tylosin) on the behaviour of *Stenotrophomonas maltophilia* isolated from a DWDS. This chapter presents the results concerning *S. maltophilia* tolerance to chlorine, levofloxacin and

trimethoprim-sulfamethoxazole and the biofilm formation ability after *S. maltophilia* exposure to different combinations of ECs.

Clofibric acid (CA) was present in all the combinations causing changes in bacterial behaviour (Chapter 4), particularly biofilm formation ability and tolerance to chlorine disinfection. Therefore, the effects of CA on *S. maltophilia* virulence and tolerance to antimicrobials were studied in more detail and are described in Chapter 5.

Six different materials with distinct copper contents (0, 57, 79, 83, 96 and 100%) were studied on single and dual species biofilm growth by *Acinetobacter calcoaceticus* and *S. maltophilia*. The results from biofilm regrowth, and material corrosion rate and copper leaching are further presented in Chapter 6.

Materials with 0, 57, 96 and 100% of copper content were tested for biofilm formation in a RCR under conditions mimicking real DWDS. Chapter 7 presents the results on the comparison of these materials in terms of biofilm control and their performance in disinfection situations (chlorine treatment, flushing treatment and the combination of both). The results from their role on the viability of bacteria released to the bulk phase after each treatment are also presented.

The impacts from the use of copper materials in chlorinated and unchlorinated systems on human cells and bacterial virulence are described in Chapter 8, where the cytotoxic and genotoxic effects of copper leachates in HT29 human colorectal adenocarcinoma are presented.

At last are presented the main conclusions of the work developed and identified future research needs that will improve the knowledge about biofilm control using copper materials and the effects of ECs on the spread of antimicrobial resistance.

Chapter 2

Literature review

This chapter comprises a literature review that collects evidences on the role of ECs' presence in water bodies, with main emphasis in DW. The work compiles the effects of exposure to ECs in microorganisms naturally present in water bodies, including DWDS and reports the possible public health consequences, in terms of tolerance to antimicrobials and the dissemination of antibiotic resistant bacteria and genes. Strategies to reduced DW microbiome exposure to ECs are also reviewed.

2.1 Introduction

Emerging contaminants (ECs) or contaminants of emerging concern are chemicals or materials found in the environment at trace concentrations ($ng.L^{-1}$ or $\mu g.L^{-1}$) with potential, perceived or real risk to the environment, human and animal health. ECs can also be considered as compounds for which published health standards are lacking. For many of these contaminants the threat for the environment and humans is still not fully understood (EPA 2008, Pereira *et al*. 2015). Among huge varieties of ECs, several classes are highlighted: pharmaceuticals and personal care products (PPCPs), nanomaterials, fire retardants, pesticides, plasticizers, surfactants (Pereira *et al*. 2015). More recently, some contaminants resulting from the use of several chemicals, as the disinfection by-products (DBPs), and antibiotic resistant bacteria (ARB) and genes (ARG) have also been described as ECs by Berendonk *et al*. (2015) and Richardson and Ternes (2018). The worldwide use of these contaminants routinely is a result of the economic growth and of the increase of life quality. It is expected that contamination by ECs will increase - not only the typical concentration detected but also the number of ecosystems contaminated - due to the increase of global human population mainly in high-density areas. In developed countries, the use of these contaminants is increasing and its reduction is an almost impossible task as they are absolutely indispensable for health and general life quality (Richmond *et al*. 2017). Despite the increased use of ECs and the consequent higher entrance of contamination in the environment, it should not be forgotten the impact of bioaccumulation and biomagnification (Zenker *et al*. 2014). These are two important concepts that amplify the presence and the exposure to several contaminants that are able to accumulate in some organisms or tissues and that are propagated through the food chain. The concern about the presence of new contaminants in the environment emerged

with the chlorinated hydrocarbons, mainly with the pesticide di-chlorodiphenyltrichloroethane (DDT) in 1940s-1950s (Pereira *et al*. 2015). DDT was the first worrying example of a contaminant with huge impact in the environment and human health due to bioaccumulation and biomagnification in the environment (Pereira *et al*. 2015).

ECs reach different environments through different routes (Houtman 2010, Pal *et al*. 2014), as represented in Figure 2-1. Wastewater treatment plants (WWTPs) are the main point of ECs entrance into the environment, mainly in surface waters (Pal *et al*. 2014). Pharmaceuticals, are not completely metabolized by consumers and are excreted in the urine and feces by humans and animals (Jjemba 2006). Several personal care products and detergents, can also directly reach sewer when applied in shower, sinks, etc. Therefore, ECs used by population are collected in sewer lines and reach WWTPs that are not designed to remove these specific contaminants (Pal *et al*. 2014). Many other contaminants are disposed in the garbage and consequently disposed in landfills, allowing soil and groundwater contamination due to landfill leaching (Ramakrishnan *et al*. 2015). An important source of contaminants is intensive agriculture and livestock (Snow *et al*. 2017). The application of manure in land fields for crops cultivation is the main contamination source of veterinary pharmaceuticals (Lamastra *et al*. 2016). The direct application of pesticides in soil will also affect groundwater quality. The removal of ECs during water treatment in WWTPs and in drinking water treatment plants (DWTPs) is not effective (Gabarrón *et al*. 2016). Therefore, these contaminants have also been detected in DW (Benson *et al*. 2017, Kot-Wasik *et al*. 2016, Peng *et al*. 2019, Tabe *et al*. 2016).

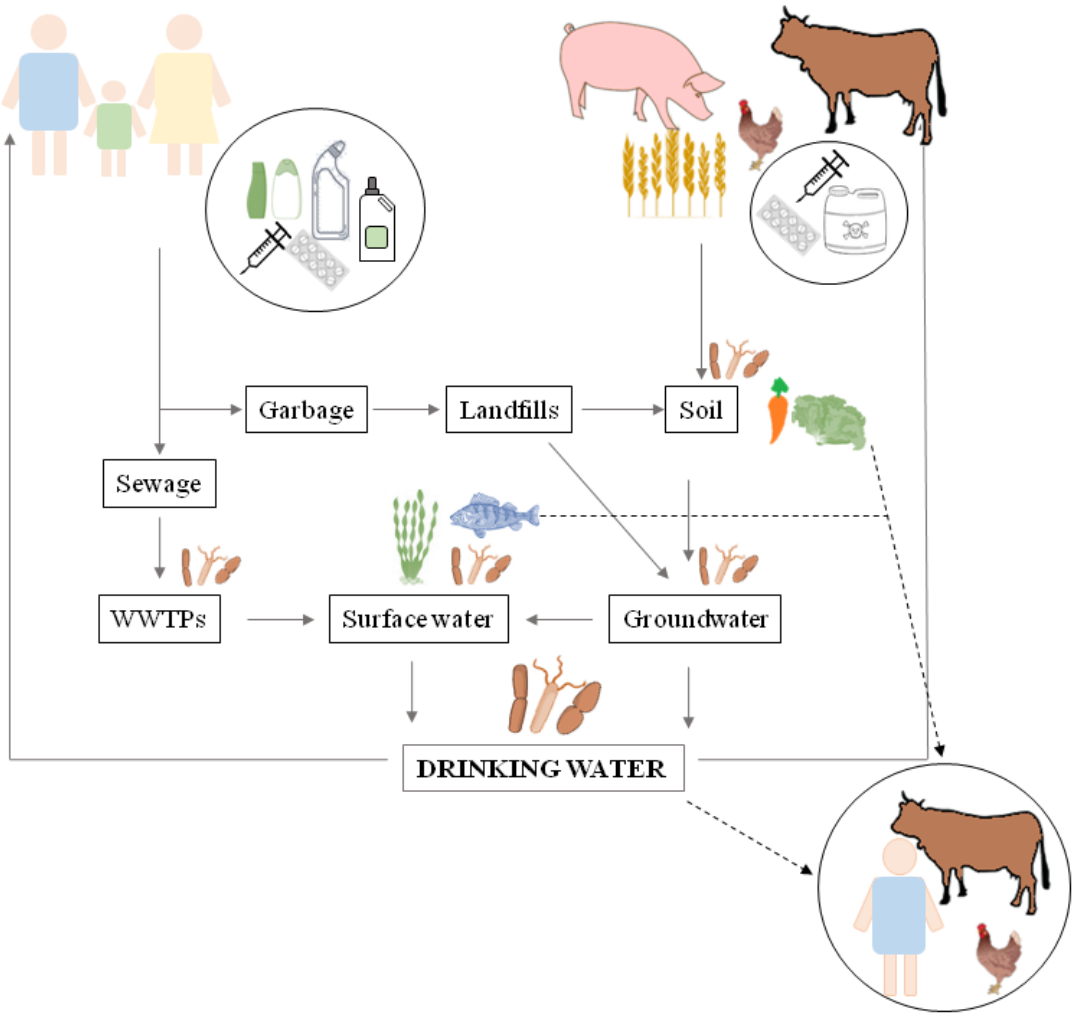

Figure 2-1. Emerging contaminants routes into the environmental ecosystems and exposed biota.

The development of analytical techniques allowed the detection of trace concentrations of many ECs in different environments: surface water, groundwater, DW, swimming pool, soils, sediments, irrigation water (del Carmen Salvatierra-Stamp *et al*. 2015, Glassmeyer *et al*. 2017, Lempart *et al*. 2018). Therefore, several organisms from different environments (such as algae, microorganisms, aquatic animals, vegetables, humans) are continuously exposed to ECs. The effects from such exposure are still not completely understood. The main concern regarding pharmaceuticals and in particular antibiotics is the widespread of antibiotic resistance (Lempart *et al*. 2018, Ye *et al*. 2017). The

contamination of DW is a worrying situation for the consumer as it is a direct route to the human body.

This literature review aims to collect evidences on the role of ECs` presence in water bodies, with main emphasis in DW. The work compiles the effects of exposure to ECs in microorganisms naturally present in water bodies, including DWDS and reports the possible consequences for public health, in terms of tolerance to antimicrobials and the dissemination of ARGs and ARBs (antibiotic resistant bacteria). Two strategies to reduced DW microbiome exposure to ECs are also reviewed: (1) removal and/or reduction of ECs in water bodies/DW; (2) microbial load reduction in DWDS.

2.2 Presence of ECs in drinking water

ECs from all the previously listed categories (PPCPs, nanomaterials, fire retardants, pesticides, plasticizers, surfactants, DBPs, ARB and ARGs) have been detected in DW. Table 2-1 summarizes recent works where the presence of ECs was described in different countries. ECs reach DW due to the inability of DWTPs to completely remove these contaminants. Also, conventional WWTPs are not prepared to remove ECs from received water causing the dissemination of untreated contaminants to surface water or soils (through the reuse of activated sludge as fertilizer) (Petrović *et al*. 2003). Nevertheless, there are several works demonstrating that WWTPs may have important impact on the reduction of specific ECs concentration, by biodegradation using activated sludge, adsorption to the activated sludge, by reaction with disinfectants as chlorine or ozone, or through ultra-violet (UV) treatment (Petrović *et al*. 2003). Taking into account that DW is often produced from surface water and groundwater, the efficacy of ECs removal through DWTPs is also important. Some conventional treatments in DWTPs, such as filtration, oxidation and adsorption, may remove some of these contaminants. Several

studies (Gabarrón *et al*. 2016, Rigobello *et al*. 2013) demonstrated that oxidative processes particularly the use of chlorine are important strategies to reduce ECs levels in DWTPs. The use of granular filters, such as sand, is also a common strategy applied in DWTPs with low ability to remove ECs (Gabarrón *et al*. 2016, Pojana *et al*. 2011). The use of granular activated carbon (GAC) is also a conventional strategy used in DWTPs with important results in the reduction of ECs content (Gabarrón *et al*. 2016, Pojana *et al*. 2011, Rigobello *et al*. 2013). Despite all these barriers presented along conventional DWTPs, the removal of ECs is still inefficient and their presence in final DW remains unavoidable (Tröger *et al*. 2018). The number of works studying the presence of ECs in DW has been increasing and the main works published in the last 10 years are listed in Table 2-1, where it is possible to search for ECs detected in DW and their concentration in DW all over the world.

All the barriers existent in DWTPs and along DWDS also aim to reduce the microbial load in transported DW. However, DW is not sterile and the development of biofilms along DWDS pipes is unavoidable. For this reason, there are several organisms continuously exposed to ECs in DWDS. Bacteria are the microorganisms more abundant in DW biofilms, but other organisms and biological structures are also found in DW biofilms: viruses, protozoa, fungi and algae (Simões and Simões 2013). The presence of ECs may have consequences on the behaviour of DW organisms, particularly on community diversity and function and on the spread of antibiotic resistance. The information regarding the consequences of ECs on DW microbiome is scarce. Therefore, research on this topic is emerging in order to understand the consequences of ECs exposure on DW microbiome and to develop strategies to reduce microbial exposure to ECs and potential negative consequences for the consumers.

Table 2-1. Maximum concentrations (ng/L) of ECs detected in DW worldwide in the last 10 years.

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Algaecide	Irgarol	7.2	DW from treatment plant in Rio Grande do Sul, Brazil	(Caldas *et al*. 2013)
Amphetamine	Mephedrone	0.77 – 2.81	DWTPs in the East Anglia region and tap water in Cambridge, UK	(Peng *et al*. 2019)
	Methamphetamine	2.21	DWTPs in the East Anglia region and tap water in Cambridge, UK	(Peng *et al*. 2019)
	Methylone	1.37	DWTPs in the East Anglia region and tap water in Cambridge, UK	(Peng *et al*. 2019)
Analgesic	Acetaminophen	124	Finished DW from a WTP in Windsor, Ontario, Canada	(Tabe *et al*. 2016)
	Paracetamol	44	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
Anesthesia	Ketamine	0.14 – 1.12	DWTPs in the East Anglia region and tap water in Cambridge, UK	(Peng *et al*. 2019)
Antiarrhythmics	Verapamil	26.7	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)
Antibiotics	Amprolium	179	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Antibiotics (cont.)	Chloramphenicol	2	Tap water from Alzira, Burjassot, Picassent, Valencia and Aldaia, Spain	(Carmona *et al*. 2014)
		0.9	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
	Chlorotetracycline	16 – 65	Finished DW from a WTP in Windsor, Ontario, Canada	(Tabe *et al*. 2016)
	Ciprofloxacin	9.5 – 476	Finished DW from a WTP in Windsor, Ontario, Canada	(Tabe *et al*. 2016)
		8.2 – 679.7	Tap water in Macao and Guangzhou, respectively	(Yiruhan *et al*. 2010)
		10	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)
	Clorsulon	4	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)
	Danofloxacin	23	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)
	Doxycycline	59	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)
	Enrofloxacin	110 – 128	Finished DW from a WTP in Windsor, Ontario, Canada	(Tabe *et al*. 2016)
		5.2 – 8.3	Tap water in Macao and Guangzhou, respectively	(Yiruhan *et al*. 2010)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Antibiotcs (cont.)	Erythromycin	6.0	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
		2 - 6	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)
	Lomefloxacin	37.1 – 179.0	Tap water in Macao and Guangzhou, respectively	(Yiruhan *et al*. 2010)
	Marbofloxacin	8 – 23	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)
	Meclocycline	101	Finished DW from a WTP in Windsor, Ontario, Canada	(Tabe *et al*. 2016)
	Norfloxacin	17.1 - 82.7	Tap water in Macao and Guangzhou, respectively	(Yiruhan *et al*. 2010)
		37 – 80	Finished DW from a WTP in Windsor, Ontario, Canada	(Tabe *et al*. 2016)
	Oxolinic acid	5 – 13	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)
	Roxithromycin	11 - 41	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)
	Sulfadiazine	2 - 30	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Antibiotics (cont.)	Sulfadimethoxine	75	Finished DW from a WTP in Windsor, Ontario, Canada	(Tabe *et al*. 2016)
		39	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)
	Sulfamethoxazole	8.2	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
		8.2	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)
		0. 14 -0.50	Pipeline and tap water from DWDS in Southern China	(Qiao *et al*. 2011)
		1.3	Treated DW from 5 DWTPs in Southern Ontario, Canada.	(Metcalfe *et al*. 2014)
		2	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)
	Sulfaquinoxaline	6	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)
	Tetracycline	19 – 58	Finished DW from a WTP in Windsor, Ontario, Canada	(Tabe *et al*. 2016)
		61	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)
	Trimethoprim	3 - 14	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Antibiotics (cont.)	Tylosin	3	Treated water from DWTPs in Paris Basin	(Guillon *et al*. 2015)
Anticoagulant	Warfarin	0.39 – 3.89	Tap water from Northwest Portugal	(Barbosa *et al*. 2016)
Antidepressant	Bupropion	3.34	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
		10.9	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)
	Citalopram	2.26 – 2.80	DWTPs in the East Anglia region and tap water in Cambridge, UK	(Peng *et al*. 2019)
	Fluoxetine	0.27	DWTPs in the East Anglia region and tap water in Cambridge, UK	(Peng *et al*. 2019)
Anti-diabetic	Metformin	8.0	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
Antihelminthic drug	Carbazochrome	0.68 – 0.89	Pipeline and tap water from DWDS in Southern China	(Qiao *et al*. 2011)
	Mebendazole	18.5	DW from treatment plant in Rio Grande do Sul, Brazil	(Caldas *et al*. 2013)
Antihypertensive	Metaprolol	18.4	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
		18.4	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Antihypertensive (cont.)	Propranolol	2.5	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)
	Ramipril	2.8	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
Anti-ischemic agent	Trimetazidine	4.2	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
Antioxidant and deicing agent	Benzotriazole methyl 1H	247	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
Antipsychotic drug	Sulpiride	0.11 – 0.17	Pipeline and tap water from DWDS in Southern China	(Qiao *et al*. 2011)
Antipyretic	Aminopyrine	0.13	Finished water from DWTPs in Beijing, China	(Cai *et al*. 2015)
	Antipyrine	0.15 – 0.22	Finished water from DWTPs in Beijing, China	(Cai *et al*. 2015)
	Isopropylantipyrine	0.18	Finished water from DWTPs in Beijing, China	(Cai *et al*. 2015)
Antiretroviral	Lamivudin	27.7	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
ARGs	ermB. tetM, tetO, tetQ, tetW, cfr, cmlA, fexA, fexB, floR, oqxB	1.18×10^7 (min: tetO, tetQ; max: 16S) [*1]	Tap water sourced from the East river (South China)	(Su *et al*. 2018)
	sul2, ermB, tetM, tetO, tetQ, tetW, cfr, cmla,feA, fexB, floR, oqxB, qepA	1.74×10^7 (min: tetO, tetQ; max: 16S) [*1]	Tap water sourced from the West river (South China)	(Su *et al*. 2018)
	sul1, ermB,tetO, tetQ, tetW, cfr, cmlA, fexA, fexB, flor, oqxB, qnrS	1.89×10^8 (min: tetO, tetQ; max: cmlA)[*1]	Tap water sourced from the North river (South China)	(Su *et al*. 2018)
Artificial sweetener	Acesulfame	680	Tap water from Tianjin, China	(Gan *et al*. 2013)
	Cyclamate	< 36	Tap water from Tianjin, China	(Gan *et al*. 2013)
	Saccharin	100	Tap water from Tianjin, China	(Gan *et al*. 2013)

[1] * total concentration of ARGs in copies/L

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Artificial sweetener (cont.)	Sucralose	120	Tap water from Tianjin, China	(Gan *et al*. 2013)
		48 - 2400	Finished water from DWTPs in USA	(Mawhinney *et al*. 2011)
		3.7 – 253.7	Treated DW from 5 DWTPs in Southern Ontario, Canada.	(Metcalfe *et al*. 2014)
Beta blocker	Atenolol	0.12 - .18	Pipeline and tap water from DWDS in Southern China	(Qiao *et al*. 2011)
		1.0	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
Bipolar disorder treatment	Lithium	42700	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
		42700	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)
Cocaine and metabolites	Benzoylecgonine	10 – 652	DW from Guarapiranga dam	(Campestrini and Jardim 2017)
		0.24 – 0.61	DW wells in Milan, Italy	(Riva *et al*. 2018)
	Cocaine	0.06 – 4.44	DW wells in Milan, Italy	(Riva *et al*. 2018)
		6 - 22	DW from Guarapiranga dam	(Campestrini and Jardim 2017)
		0.19 – 0.84	DWTPs in the East Anglia region and tap water in Cambridge, UK	(Peng *et al*. 2019)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Disinfection by-products	Bromoform	32 - 4060	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
	Chlorite	< 2.5	Finished DW from a WTP in East Ireland	(Pender *et al*. 2015)
		< 2.5	Finished DW from a WTP in South-east of Ireland	(Pender *et al*. 2015)
	Halo-acetic acid	9200	Finished DW from a WTP in South-east of Ireland	(Pender *et al*. 2015)
		4400	Finished DW from a WTP in East Ireland	(Pender *et al*. 2015)
	Trihalomethanes	21070	Finished DW from a WTP in South-east of Ireland	(Pender *et al*. 2015)
		49430	Finished DW from a WTP in East Ireland	(Pender *et al*. 2015)
	2- chlorophenylacetonitrile	5.0 – 155	Finished water from nine DWTPs	(Zhang *et al*. 2019)
	2,3-dichlorophenylacetonitrile	1.3 – 148	Finished water from nine DWTPs	(Zhang *et al*. 2019)
	2,5-dichlorophenylacetonitrile	0.8 – 16	Finished water from nine DWTPs	(Zhang *et al*. 2019)
	2,6-dichlorophenylacetonitrile	0.5 – 33	Finished water from nine DWTPs	(Zhang *et al*. 2019)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Disinfection by-products (cont.)	3- chlorophenylacetonitrile	0.6 – 69	Finished water from nine DWTPs	(Zhang *et al*. 2019)
	3,4-dichlorophenylacetonitrile	1.6 - 12	Finished water from nine DWTPs	(Zhang *et al*. 2019)
	4- chlorophenylacetonitrile	1.8 – 22	Finished water from nine DWTPs	(Zhang *et al*. 2019)
Diuretic drug	Hydrochlorothiazide	2.9	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
EDCs (synthetic and natural hormones)	Estradiol	11.60	DW from DWTPs in Abrera, Baix Llobregat, Spain	(Kuster *et al*. 2008)
	Estrone	1 – 1.5	Treated DW from 5 DWTPs in Southern Ontario, Canada.	(Metcalfe *et al*. 2014)
	Levonorgestrel	46.4	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
	Nonyl phenol	< 1000	Finished DW from a WTP in South-east of Ireland	(Pender *et al*. 2015)
		< 1000	Finished DW from a WTP in East Ireland	(Pender *et al*. 2015)
	Oestrone	0.256	Finished DW from a WTP in East Ireland	(Pender *et al*. 2015)
		33.2	Finished DW from a WTP in South-east of Ireland	(Pender *et al*. 2015)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
EDCs (synthetic and natural hormones) (cont.)	Progesterone	4.8	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
		0.93	DW from DWTPs in Abrera, Baix Llobregat, Spain	(Kuster *et al*. 2008)
	17 – α – Estradiol	6 – 38	Finished DW from a WTP in Windsor, Ontario, Canada	(Tabe *et al*. 2016)
	17 – α – Ethinyl oestradiol	5.73	Finished DW from a WTP in South-east of Ireland	(Pender *et al*. 2015)
		1.33	Finished DW from a WTP in East Ireland	(Pender *et al*. 2015)
	17 – α –oestradiol	4.18	Finished DW from a WTP in South-east of Ireland	(Pender *et al*. 2015)
	17 – α –oestradiol	1.87	Finished DW from a WTP in East Ireland	(Pender *et al*. 2015)
Food additive	Triethyl citrate	13	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)
Fragrance precursor	Acetophenone	580	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)
Heavy metals	Uranium	340	Finished DW from a WTP in East Ireland	(Pender *et al*. 2015)
		10470	Finished DW from a WTP in South-east of Ireland	(Pender *et al*. 2015)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Histamine H2-receptor antagonist	Ranitidine	5.6	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
Insect repellents	Bayrepel	10	DW from Galicia, Spain	(Rodil *et al*. 2012)
	N,N-Diethyl-m-toluamide	12	DW from Galicia, Spain	(Rodil *et al*. 2012)
Lipid regulators	Bezafibrate	0.31-0.85	Finished water from DWTPs in Beijing, China	(Cai *et al*. 2015)
	Clofibric acid	1.05	DW wells in Milan, Italy	(Riva *et al*. 2018)
		19	Tap water from Alzira, Burjassot, Picassent, Valencia and Aldaia, Spain	(Carmona *et al*. 2014)
		91.7	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)
	Gemfibrozil	2	Tap water from Alzira, Burjassot, Picassent, Valencia and Aldaia, Spain	(Carmona *et al*. 2014)
Musk fragrance	Cashmeran	9.9	Finished water from DWTP, Iowa, USA	(Wombacher and Hornbuckle 2009)
	Galaxolide	61	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
		61	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)
		2.2	Finished water from DWTP, Iowa, USA	(Wombacher and Hornbuckle 2009)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Musk fragrance (cont.)	Musk xylines	146	Finished DW from a WTP in East Ireland	(Pender *et al*. 2015)
		< 10	Finished DW from a WTP in South-east of Ireland	(Pender *et al*. 2015)
	Phantolide	0.06	Finished water from DWTP, Iowa, USA	(Wombacher and Hornbuckle 2009)
	Tonalide	0.51	Finished water from DWTP, Iowa, USA	(Wombacher and Hornbuckle 2009)
Nasal decongestant	Pseudoephedrine	3.75	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)
Neuro active drug	Carbamazepine	0.31 – 7.61	DW wells in Milan, Italy	(Riva *et al*. 2018)
		26.50 - 586	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
		26.5	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)
		3.34	Tap water from Northwest Portugal	(Barbosa *et al*. 2016)
		2.9 – 7.7	Treated DW from 5 DWTPs, Southern Ontario, Canada	(Metcalfe *et al*. 2014)
		6.0	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
		0.37 – 1.15	Finished water from DWTPs in Beijing, China	(Cai *et al*. 2015)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Nicotine and metabolites	Nicotine	28.3	DW wells in Milan, Italy	(Riva *et al*. 2018)
	Cotinine	0.5 – 1.9	DW wells in Milan, Italy	(Riva *et al*. 2018)
		15.8	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)
Non-steroidal anti-inflammatory drugs (NSAID)	Diclofenac	22	Effluent from DWTP in Manantiales e La Ayurá, Colombia	(Aristizabal-Ciro *et al*. 2017)
		18	Tap water from Alzira, Burjassot, Picassent, Valencia and Aldaia, Spain	(Carmona *et al*. 2014)
		114.3	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
		7.87	Tap water from Northwest Portugal	(Barbosa *et al*. 2016)
	Flufenamic acid	16	Tap water from Alzira, Burjassot, Picassent, Valencia and Aldaia, Spain	(Carmona *et al*. 2014)
	Ibuprofen	8 - 26	Effluent from DWTP in Manantiales e La Ayurá, Colombia	(Aristizabal-Ciro *et al*. 2017)
		17.17	Finished water from DWTPs in Beijing, China	(Cai *et al*. 2015)
		39	Tap water from Alzira, Burjassot, Picassent, Valencia and Aldaia, Spain	(Carmona *et al*. 2014)
		223.6	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Non-steroidal anti-inflammatory drugs (NSAID) (cont.)	Indomethacin	94	Finished DW from a WTP in Windsor, Ontario, Canada	(Tabe *et al*. 2016)
		4	Tap water from Alzira, Burjassot, Picassent, Valencia and Aldaia, Spain	(Carmona *et al*. 2014)
	Ketoprofen	22.5	Outlet of DWTP (Northern Italy)	(Mirasole *et al*. 2016)
		166.9	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
	Naproxen	11	Tap water from Alzira, Burjassot, Picassent, Valencia and Aldaia, Spain	(Carmona *et al*. 2014)
	Salicylic acid	38.7	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
Perfluorinated compounds (Fire retardants)	GenX	474	Finished DW from Cape Fear river (USA)	(Sun *et al*. 2016)
		11	Tap water, Netherlands	(Gebbink *et al*. 2017)
		257	Finished DW North Carolina	(Hopkins *et al*. 2018)
	PFBA	104	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
	PFBS	11.9	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Perfluorinated compounds (Fire retardants) (cont.)	PFCA long-chain	14	DW in Lambro basin, Italy (Industrialized area in North of Milan)	(Castiglioni *et al*. 2015)
	PFCA short-chain	44	DW in Lambro basin, Italy (Industrialized area in North of Milan)	(Castiglioni *et al*. 2015)
		18	DW in Lambro basin, Italy (Metropolitan area of Milan)	(Castiglioni *et al*. 2015)
	PFDA	24.70	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
	PFEA	200 - 400	Finished DW North Carolina	(Hopkins *et al*. 2018)
	PFHpA	177	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
	PFHxA	60.8	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
	PFHxS	38.4	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
	PFNA	38.60	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
	PFOA	8.56 – 11.48	Outlet of DWTP in Italy (Northwestern)	(Magi *et al*. 2018)
		0.03 – 1.59	DW wells in Milan, Italy	(Riva *et al*. 2018)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Perfluorinated compounds (Fire retardants) (cont.)	PFOA (cont.)	104	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
		4.2	Outlet of DWTP (Northern Italy)	(Mirasole *et al*. 2016)
		47	DW in Lambro basin, Italy (Industrialized area in North of Milan)	(Castiglioni *et al*. 2015)
		17	DW in Lambro basin, Italy (Metropolitan area of Milan)	(Castiglioni *et al*. 2015)
		< 25	Finished DW from a WTP in South-east of Ireland	(Pender *et al*. 2015)
		< 25	Finished DW from a WTP in East Ireland	(Pender *et al*. 2015)
	PFOS	0.08 – 1.29	DW wells in Milan, Italy	(Riva *et al*. 2018)
		3.99 – 9.39	Outlet of DWTP in Italy (Northwestern)	(Magi *et al*. 2018)
		36.90	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
		20.7	Outlet of DWTP (Northern Italy)	(Mirasole *et al*. 2016)
		< 25	Finished DW from a WTP in East Ireland	(Pender *et al*. 2015)
		< 25	Finished DW from a WTP in South-east of Ireland	(Pender *et al*. 2015)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Perfluorinated compounds (Fire retardants) (cont.)	PFPeA	514	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
	PFUnDA	1.85	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
	PFSA	32	DW in Lambro basin, Italy (Industrialized area in North of Milan)	(Castiglioni *et al*. 2015)
		29	DW in Lambro basin, Italy (Metropolitan area of Milan)	(Castiglioni *et al*. 2015)
	PFAS (diverse)	0.09 – 514	DWTP all over USA	(Boone *et al*. 2019)
		53 - 120	Public water systems in the USA	(Guelfo and Adamson 2018)
Pesticides	Atrazine	270	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
		1.14 – 2.24	Tap water from Northwest Portugal	(Barbosa *et al*. 2016)
	Carbofuran	23.8	DW from treatment plant in Rio Grande do Sul, Brazil	(Caldas *et al*. 2013)
	Chlorfenvinphos	2.46 – 6.50	Tap water from Northwest Portugal	(Barbosa *et al*. 2016)
	Clomazone	123.9	DW from treatment plant in Rio Grande do Sul, Brazil	(Caldas *et al*. 2013)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Pesticides (cont.)	Diuron	95.8	DW from a treatment plant in Rio Grande do Sul, Brazil	(Caldas *et al*. 2013)
	Epoxiconazole	83	DW from a treatment plant in Rio Grande do Sul, Brazil	(Caldas *et al*. 2013)
	Glyphosate	< 6	Finished DW from a WTP in South-east of Ireland	(Pender *et al*. 2015)
		< 6	Finished DW from a WTP in East Ireland	(Pender *et al*. 2015)
	Metaldehyde	11	Finished DW from a WTP in East Ireland	(Pender *et al*. 2015)
	Metolachlor	100	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
		100	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)
	Pichloram	< 20	Finished DW from a WTP in East Ireland	(Pender *et al*. 2015)
		50	Finished DW from a WTP in South-east of Ireland	(Pender *et al*. 2015)
	Simazine	1.45	Tap water from Northwest Portugal	(Barbosa *et al*. 2016)
	Tebuconazole	76.7	DW from a treatment plant in Rio Grande do Sul, Brazil	(Caldas *et al*. 2013)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Pesticides (cont.)	Bisphenol A	9.72 - 683	DW wells in Milan, Italy	(Riva *et al*. 2018)
		6	Tap water from Alzira, Burjassot, Picassent, Valencia and Aldaia, Spain	(Carmona *et al*. 2014)
	Tri-n-butyl phosphate	11 - 148	DW from Galicia, Spain	(Rodil *et al*. 2012)
Preservatives	Butylparaben	7	Effluent from DWTP in Manantiales e La Ayurá, Colombia	(Aristizabal-Ciro *et al*. 2017)
		28	Tap water from Alzira, Burjassot, Picassent, Valencia and Aldaia, Spain	(Carmona *et al*. 2014)
	Methylparaben	26 -80	Effluent from DWTP in Manantiales e La Ayurá, Colombia	(Aristizabal-Ciro *et al*. 2017)
		12	Tap water from Alzira, Burjassot, Picassent, Valencia and Aldaia, Spain	(Carmona *et al*. 2014)
	Methyl-p-hydroxybenzate	40	DW from Galicia, Spain	(Blanco *et al*. 2009)
	Propylparaben	135.5	DW from a treatment plant in Rio Grande do Sul, Brazil	(Caldas *et al*. 2013)
	Propylparaben (cont.)	9	Tap water from Alzira, Burjassot, Picassent, Valencia and Aldaia, Spain	(Carmona *et al*. 2014)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
Preservatives (cont.)	p- hidroxybenzoic acid	57	DW from Galicia, Spain	(Blanco *et al*. 2009)
Psychoactive stimulant	Caffeine	0.07 – 0.93	Outlet of DWTP in Italy (Northwestern)	(Magi *et al*. 2018)
		2.4 – 5.2	DW wells in Milan, Italy	(Riva *et al*. 2018)
		88	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
		5.4	Outlet of DWTP (Northern Italy)	(Mirasole *et al*. 2016)
		158.7	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)
Sedative	Diazepam	0.85	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)
Solvent	Isophorone	32	Effluent of 9 DWTP from 8 states across USA	(Glassmeyer *et al*. 2017)
		32	Treated water from 25 DWTP across USA	(Benson *et al*. 2017)
Surfactant	Nonyphenol	8 - 16	DW wells in Milan, Italy	(Riva *et al*. 2018)
UV filters	PBSA	4.0 – 50.0	DW wells in Milan, Italy	(Riva *et al*. 2018)
	Benzophenone	30 – 46	Effluent from DWTP in Manantiales e La Ayurá, Colombia	(Aristizabal-Ciro *et al*. 2017)

Contaminant class	Contaminant	Concentration detected (ng/L)	Location	Reference
UV filters (cont.)	Benzophenone - 3	295	Tap water from Barcelona, Spain	(Diaz-Cruz *et al*. 2012)
		7 – 29	Effluent from DWTP in Manantiales e La Ayurá, Colombia	(Aristizabal-Ciro *et al*. 2017)
	Benzophenone - 3	1.1 – 5.7	DW wells in Milan, Italy	(Riva *et al*. 2018)
	Benzophenone-4	62	DW from Galicia, Spain	(Rodil *et al*. 2012)
	Octocrylene	167	Tap water from Barcelona, Spain	(Diaz-Cruz *et al*. 2012)
	2- ethylhexyl 4-(dimethylamino) benzoate	115	Tap water from Barcelona, Spain	(Diaz-Cruz *et al*. 2012)
	2- ethylhexyl 4-methoxycinnamate	256	Tap water from Barcelona, Spain	(Diaz-Cruz *et al*. 2012)
	2-Phenylbenzimidazole-5-sulphonic acid	10	DW from Galicia, Spain	(Rodil *et al*. 2012)
	4-Methylbenzylidene camphor	10	DW from Galicia, Spain	(Rodil *et al*. 2012)
		35	Tap water from Barcelona, Spain	(Diaz-Cruz *et al*. 2012)
Vasodilator	Nafronyl	3.8	Treated water from DWTP in Gdańsk, Poland	(Kot-Wasik *et al*. 2016)

2.3 ECs consequences for the water microbiome

Conventionally, the screening of potential ecological risks of ECs is performed according to PBT (persistence, bioaccumulation and toxicity) criteria (Richmond *et al*. 2017). However, these criteria have demonstrated that some ECS, namely PPCPs only have toxic effects at high concentrations (higher than therapeutic doses). Therefore, these criteria may lead to the conclusion that many ECs are not dangerous for the environment based on the fact that the concentrations detected in water and soil are very low (ng/L or µg/L) (Richmond *et al*. 2017). However, it is of utmost importance to study the effects of trace levels of ECs on the biota. It is known that environmentally relevant concentrations may alter ecological interactions (Proia *et al*. 2011, Subirats *et al*. 2018, Thelusmond *et al*. 2018, Wang *et al*. 2017a). Several works described alterations in animal behaviour due to the exposure to some ECs (Barry 2014, Brodin *et al*. 2013). For example, tadpoles (*Bufo arabicus)* exposed to fluoxetine (at 3 µg/L) were more susceptible to predation from dragonfly larvae (*Anax imperator*) (Barry 2014). Brodin *et al*. (2013) also described changes in the behaviour of European perch after expsoure to oxazepan. Many other works have described the effects of ECs at environmentally relevant concentration in animal behaviour (Luis *et al*. 2016, Meador *et al*. 2018, Yeh *et al*. 2017). However, special attention should be taken on the microbiome, since it is known that ECs may alter microbial communities and function but also may be responsible for the spread of antibiotic resistance. Several works described the effects from the exposure to different ECs on the microbiome of the water environment (rivers, marine environment, wetlands, etc), soil and in engineered systems, like activated sludge from WWTPs (Aubenneau *et al*. 2010, Ma *et al*. 2016, Proia *et al*. 2013b, Wang *et al*. 2019a). However, the information about the effects on DW microbiome is still very limited (Wang *et al*. 2019a). The main

alterations caused by ECs in the microbiome may be divided in two main groups: consequences in the community diversity and function (ecological consequences), and in terms of antibiotic resistance.

2.3.1 Ecology

ECs have been detected in the most varied environment ecosystems. However, they are not included in routine monitoring programs and their ecotoxicological effects remains to be understood (Aristi *et al*. 2016). Natural microbiomes of river, marine environments, wetland and even soils have important role on the equilibrium of ecosystems. The exposure to ECs, even at trace levels, may alter the diversity of the community of microorganisms as well as their function in the ecosystem (Proia *et al*. 2011, Subirats *et al*. 2018, Thelusmond *et al*. 2018, Wang *et al*. 2017a). These alterations may have important impact on ecosystems function and biodiversity, since microbial community are responsible for rivers depuration and nutrients removal and for the nutrient cycle and plant interactions in soils (Besemer 2015, Harris 2009).

ECs are not found isolated, but complex mixtures of contaminants have been detected in the environment (del Carmen Salvatierra-Stamp *et al*. 2015, Peng *et al*. 2019, Snow *et al*. 2017). The mixture of ECs can have additive (*i.e.* response to multiple ECs is equal to the sum of their individual effects) or multiplicative effects (*i.e.* the response exceeds the sum of their individual effects) (Aristi *et al*. 2016). Multiplicative interactions can be synergistic (having a positive feedback) or antagonistic (having a negative feedback) (Brennan and Collins 2015). In this section the ecological effects of ECs on different microbiomes from water, soil and WWTPs is presented.

2.3.1.1 Water microbiomes

There are several works describing the effects of emerging contamination on the behaviour of natural microbiomes in different water sources, such as on rivers biofilms, in marine sediment biofilms and other aquatic ecosystems. The exposure to environment relevant concentrations of ECs caused significant alterations in the microbial community composition and function, such as alterations in respiration rate, extracellular polymeric substances (EPS) production and enzymatic activity (Proia *et al*. 2013a, Wang *et al*. 2019a).

Biofilms in aquatic environments are generally formed in sediments, not only in benthic substrata but also in floating macro and micro aggregates. These biofilms are composed by diverse microorganisms - bacteria, archaea, algae, fungi, protozoa and viruses (Besemer 2015). Aquatic biofilms play a crucial role on water purification, since they actively participate in organic matter and nutrient removal and have an important action in the biogeochemical cycle (Besemer 2015). Furthermore, aquatic biofilms can be used as indicators of the overall water quality (Balcázar *et al*. 2015). Therefore, several works aiming to understand the effects of ECs on aquatic biofilms have been published (Drury *et al*. 2013, Proia *et al*. 2011, Qiu *et al*. 2019, Subirats *et al*. 2018). Proia *et al*. (2011) evaluated the effects of two ECs, the herbicide diuron and the biocide triclosan on stream biofilms grown in the laboratory. Biofilms were exposed to short pulses of triclosan and diuron for 48 h and an immediate increase of the mortality of diatoms were observed after exposure to diuron. Furthermore, triclosan also increased the mortality of diatoms and bacteria. However, the effects on diatoms were only observed one week after exposure and bacterial mortality returned to the normal value one week after post-exposure. Also, algae were affected by triclosan and diuron, particularly the cell integrity of *Spirogyra* sp. was compromised in the presence of triclosan. Regarding biofilm function, authors

conclude that triclosan strongly inhibit phosphate uptake and this function was not recovered to normal until 2 weeks post-exposure (Proia *et al*. 2011). In a different study, Drury *et al*. (2013) found that triclosan influenced the diversity of benthic communities and was responsible for the increase of triclosan resistance. However, they did not found effects of triclosan on bacterial abundance and respiration rate. Latter, the effects of triclosan on the bacterial community of fish (fathead minnow) gut microbiome was assessed by Narrowe *et al*. (2015). Fathead minnows (*Pimephales promelas)* were exposed to two different concentration of triclosan for 7 days. The authors described that the exposure altered the gut microbiome community, however, these differences disappeared after two weeks of depuration. The exposure to triclosan shifted the gut microbial community and the main differences were observed in *Cetobacterium, Methylbacterium, Flavobacterium, Methylotenera, Hydrogenophaga* and CK-1C4-19. On its turn, Debenest *et al*. (2011) focused on the effects of tetrabromobisphenol A (TBBPA), a flame retardant, on freshwater microalgae. Single and multi-specie cultures of microalgae (*Pseudokirchneriella subcapitata* and *Nitzchia palea*) were exposed to TBBPA at 1.8, 4.8, 9.2, 12.9 and 16.5 µM for 72 h. These authors conclude that the three highest concentrations of TBBPA inhibited the growth of the two microalgae.

Pharmaceuticals also had significant impact in the dynamics of aquatic microbial communities (Aristi *et al*. 2016, Corcoll *et al*. 2014, 2015). The effects of antibiotics on the bacterial community of biofilms from a Mediterranean river were evaluated by Proia *et al*. (2013a). Biofilms were formed for 25 d and exposed to water contaminated with antibiotics (macrolides, quinolones, sulfonamides, tetracyclines and chemotherapeutic) for 9 days. These authors observed that the presence of antibiotics altered the bacterial community. For example, α-Proteobacteria were more abundant in biofilms exposed to more polluted water and Actinobacteria was highly associated with the exposure to the

16 different antibiotics detected in the more polluted water. In that work, the authors found that switching biofilms between environments with different pollution rate changed community composition, *i.e.* the communities become similar in composition to the autochthonous communities of the destination site (Proia *et al*. 2013a). The same work also reported that lower portion of live bacteria were detected in antibiotic contaminated water. The activity of extracellular enzymes is also affected by the exposure to antibiotics. Proia *et al*. (2013a) found a decrease in extracellular peptidase and phosphatase activity when biofilms were switched to more polluted water (higher concentrations of antibiotics). Stream biofilms were also studied by Rosi-Marshall *et al*. (2013) that focused their work on the effects of pharmaceuticals on algal growth, microbial respiration and bacterial community composition of aquatic biofilms. Pharmaceutical-diffusing substrates were used to assess the effects of six molecules (caffeine, cimetidine, ciprofloxacin, diphenhydramine, metformin, ranitidine and the mixture of each) on microbial community in streams in Indiana, Maryland and New York. Caffeine, ciprofloxacin, diphenhydramine and the mixture of pharmaceuticals caused a decrease on algal biomass. The biofilm respiration was also suppressed when biofilms were exposed to caffeine, cimetidine, ciprofloxacin, diphenhydramine and the mixture of all contaminants. Significant alterations in bacterial community were related to the presence of diphenhydramine, the abundance of *Pseudomonas* sp. in biofilms increased and the abundance of *Flavobacterium* sp. decreased for all the three streams studied. Corcoll *et al*. (2015) observed that stream biofilms were negatively affected by the exposure to a mixture of pharmaceuticals (ibuprofen, diclofenac, carbamazepine, sulfamethoxazole, erythromycin, metoprolol, atenolol, gemfibrozil and hydrochlorothiazide at a final concentration of 5000 ng/L), causing a decrease of algal biomass and of taxa richness. The same study also reported that exposure to the mixture of pharmaceuticals increased

the metabolic rates of biofilms, comparing with non-exposed biofilms. The effects of pharmaceuticals on biofilms may be influence by external factors, such as flow conditions/intermittency (Corcoll *et al*. 2015). Aristi *et al*. (2016) also studied the effects of a mixture of pharmaceuticals (ciprofloxacin, erythromycin, diclofenac, methylparaben and sulfamethoxazole) on stream biofilms but after 3 and 4 weeks of exposure only observed weak toxic effects.

Amphetamines have also been detected in water bodies. For instance, Lee *et al*. (2016) detected amphetamines in 6 streams in Baltimore (USA) at concentrations between 3 to 630 ng/L. They exposed stream communities to amphetamine at 1 µg/L and observed a decrease in biofilm chlorophyll a production, greater seston ash-free dry mass and lower seston community respiration (Lee *et al*. 2016). The bacterial and diatom community was also altered by the exposure to amphetamine. For example, the abundance of *Cloacibacterium*, *Cocconeis placentula* and *Reimeria uniseriata* increased and the abundance of *Luteimonas* decreased (Lee *et al*. 2016). Subirats *et al*. (2018) studied the effect of some pharmaceuticals (ciprofloxacin, erythromycin, sulfamethoxazole and diclofenac) and a preservative (methylparaben) on bacterial composition, abundance and antibiotic resistance profile of biofilms in artificial streams. However, significant alterations were not observed in terms of bacterial community composition and abundance. These authors also evaluated the effects of ECs exposure under high nutrient concentration and found synergy with increasing the abundance of *sul1* and *int1*. Qiu *et al*. (2019) evaluated 31 water and sediments samples from rivers in China and quantified the presence of 20 antibiotics in each sample. They evaluated the dissemination of ARGs in microbial communities and the profiling of microbial community. The presence of sulfamethoxazole was positively related with the abundance of *Fusobacteria* and *bla_d* gene.

Nanoparticles (NPs) may have significant impact in aquatic microbiomes. For example, recently, Zhai *et al*. (2018) evaluated the effects of silver nanoparticles on aquatic invertebrates and in the microbial community. For that, biofilms were formed in DECOTABs containing silver NPs in water. It was observed that silver nanoparticles at 0.8 µg/L inhibited and altered the metabolic activity of the organic matter-associated microorganisms and consequently affected the growth of invertebrates such as *Asellus aquaticus*. Silver NPs also caused structure alterations in marine biofilms and reduced its normal growth (Fabrega *et al*. 2011). Das *et al*. (2012) observed alteration of the bacterial community structure in natural waters (Ontario, Canada) after exposure to silver NPs. These authors found that bacterial community tolerated low levels of silver NPs, however, the bacterial community composition changed due to the exposure to silver NPs. In other work, Sendra *et al*. (2018) evaluated the effects of cerium oxide NPs (CeO_2NPs) and erythromycin on freshwater microalgae *Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum*. Erythromycin was responsible for the inhibition of microalgal growth and also reduced the quantum yield of photosystem II. However, the exposure to NPs and to erythromycin simultaneously demonstrated a protective effect of CeO_2NPs in both microalgae, preventing erythromycin toxic effects. Also, titanium oxide NPs caused alteration in freshwater microbial community, reducing the abundance of *Bacillus subtilis* and *Aeromonas hydrophila* and stimulating the growth of *Arthrobacter* sp. and *Klebsiella* sp. (Binh *et al*. 2014). However, there is a lack of information regarding the effects of ECs in DW natural microbiomes. Apparently, only one recent work studied this topic, evaluating the response of DW biofilms to sulfadiazine and ciprofloxacin (Wang *et al*. 2019a). These two antibiotics were responsible for the increase of *Hyphomicrobium* abundance and were related to the promotion of ARGs. The production of EPS was also favored by the exposure to sulfadiazine and ciprofloxacin. Both antibiotics combined had

higher impact in biofilms than when applied alone, namely an increase in the number of bacteria in biofilms, promotion of ARGs, activation of proteases and dehydrogenases and higher content of proteins in biofilms' EPS.

The effect of non-antibiotic ECs and the mixtures of ECs, at environmental levels, have not yet been explored in the DW microbiome. This is an important aspect that is lacking and could give useful information about DW safety and potential risks for consumers.

2.3.1.2 Microbiomes from wastewater treatment plants

Conventional activated sludge process (ASP) consists in a flocculated microbial community responsible for the treatment of wastewater (WW) under an aeration process. Nevertheless, the use of conventional clarifiers to separate biomass from treated water may be a limitation of ASP in cases of settleability problems due to the formation of undesired biomass (Mutamim *et al*. 2013). Therefore, a membrane can be used to separate biomass from treated water, this technology is designated as membrane bioreactors (MBR). MBR is a WW treatment technology that combines activated sludge treatment with microfiltration (MF) or ultrafiltration (UF) membrane for solid liquid separation (Besha *et al*. 2017). These microbial communities are the primary receptacles of contaminants in WW and are exposed to higher concentrations of ECs than other communities from different ecosystems (Goyal *et al*. 2010). Therefore, several studies reported ECs effects in WWTP microbiome in terms of the EPS production, bacterial community and (bio)fouling behaviour (Aubenneau *et al*. 2010, Delgado *et al*. 2010a, Kraigher *et al*. 2008).

Several authors evaluated the effects from the presence of pharmaceuticals in activated sludge community and function (Aubenneau *et al*. 2010, Avella *et al*. 2010a, Delgado *et al*. 2010a). For example, Kraigher *et al*. (2008) studied the effects of ibuprofen, naproxen,

ketoprofen, diclofenac and clofibric acid in bacterial community structure of activated sludge. In general, activated sludge containing 50 µg/L of each selected pharmaceuticals had lower community diversity than those observed in non-exposed activated sludge, the abundance of *Nitrospira* sp. was also reduced in the presence of the selected pharmaceuticals. Aubenneau *et al*. (2010) found that carbamazepine at 1 µg/L caused an immediate increase on the endogenous respiration of activated sludge in a pilot scale MBR. Nevertheless, carbamazepine was also related to the decrease of exogenous oxygen demand and the reduction of the mean size of sludge particles. Nevertheless, carbamazepine did not cause significant changes in chemical oxygen demand (COD) removal and in the sludge production (Aubenneau *et al*. 2010). Several works assessed the effects of cytostatic drugs, especially the cyclophosphamide, on microbial community in MBR (Avella *et al*. 2010a, Delgado *et al*. 2010a, Delgado *et al*. 2010b). Avella *et al*. (2010a) found that cyclophosphamide induced EPS production in biological sludge. The endogenous and exogenous respiration of activated sludge was also influenced by the presence of cyclophosphamide that stimulated endogenous respiration in activated sludge but inhibited the exogenous respiration (Delgado *et al*. 2010a, Delgado *et al*. 2010b). These authors also found that biofouling in membrane of MBR occurred more quickly in the presence of the referred pharmaceutical (Delgado *et al*. 2010a). However, a decrease in sludge production was associated to the presence of the cytostatic drug (Delgado *et al*. 2010b). Wu *et al*. (2017) also evaluated the consequences from the exposure to 8 cytostatic drugs (azathioprine, cyclophosphamide, doxorubicin, epirubicin, flutamide, methotrexate, mitotane and tamoxifen) on microbial community of an osmosis anaerobic MBR. The presence of these drugs was associated with an inhibition on microbial metabolism and an increase on EPS concentration (Wu *et al*. 2017). The exposure to cytostatic drugs was also associated with a higher dominance of biofilm-forming and

siderophore producing bacteria in the microbial community (Wu *et al*. 2017). The effects of antibiotics (erythromycin, roxithromycin, amoxicillin, tetracycline and sulfamethoxazole) on municipal sludge was also reported by Avella *et al*. (2010b). The main interference in the activated sludge function and structure was caused by erythromycin and roxithromycin - both induced a significant increase of EPS in activated sludge flocs. Nevertheless, the exposure to erythromycin inhibited the ability of sludge to remove COD and nitrogen. Recently, Dong *et al*. (2019) found that the presence of the plasticizer bisphenol A and the antiseptic pentachlorophenol altered the activity of activated sludge enzymes. Bisphenol A increased the activity of urease and invertase, however, inhibited dehydrogenase activity. The effects of pentachlorophenol on enzymatic activity was dependent on its concentration and exposure time. The exposure to pentachlorophenol at 100 mg/L inhibited the urease activity, however, the exposure to the same contaminant at 40 mg/L also inhibited dehydrogenase. Oppositely, the invertase activity was improved after exposure to pentachlorophenol for 0.5 h, but the exposure for 2.5 h had an opposite effect (Dong *et al*. 2019). Triclocarban, an antimicrobial used in personal care products, increased the microbial diversity in activated sludge (Wang *et al*. 2017b). The presence of this contaminant in WWTP promoted acidogenesis, acetogenesis and homoacetogenesis processes; nonetheless, the methanogenic ability of activated sludge was inhibited (Wang *et al*. 2017b). Boni *et al*. (2018) reported that the presence of methamphetamine was not related with the inhibition of activated sludge activity.

WWTPs receive water contaminated with highly complex mixtures of ECs. Harb *et al*. (2016) examined the effects of a cocktail of ECs from different classes (pharmaceuticals, neuroactive stimulants, plasticizers, preservatives, insect repellent, personal care products, artificial sweeteners and flame retardants). The presence of these mixture of ECs were responsible for the alteration of microbial community, changing keystone

bacterial populations and also significantly impacted biodegradation-associated gene expression levels. Nevertheless, the overall reactors performance was not affected by the presence of the ECs.

Nanomaterials also reach WWTPs with consequences for activated sludge community composition and performance. Goyal *et al*. (2010) reported that single walled carbon nanotubes (SWCNTs) impacted activated sludge community. SWCNTs were responsible for community shift, affecting differentially microbial species. The effects of the presence of silver NPs in MBRs were studied, especially the effects on activated sludge microbial community and in their performance (Tan *et al*. 2015, Zhang *et al*. 2014). Zhang *et al*. (2014) did not report significant alterations in activated sludge performance and on the membrane fouling in the presence of silver nanoparticles. Nevertheless, Tan *et al*. (2015) reported a decrease of activated sludge community accompanied with a decrease on the nitrifying efficiency of activated sludge from 98% to 15 % when silver NPs were available. Despite these controversial results, both works described that the EPS production was promoted by the exposure to silver NPs (Tan *et al*. 2015, Zhang *et al*. 2014).

The literature consistently reports consequences from the presence of ECs in microbial communities of WWTPs and it is known that these contaminants accumulate in the sludge during the treatment process (Rajasulochana and Preethy 2016). So, the treatment of sludge with accumulated ECs is very important to avoid the continuous accumulation of contaminants. Anaerobic digestion of sludge is one of the treatments that can be applied to stabilize sludge, allowing the simultaneous production of methane. Some works examined the consequences from the presence of NPs and antibiotics for anaerobic digestion of sludge efficiency and for sludge community diversity. Recently, Huang *et al*. (2019) evaluated the impact of copper and zinc oxides NPs on sludge anaerobic digestion.

The presence of these NPs was associated to a shift on bacterial community and to the enrichment of *Acidovorax*, *Burkholderia*, *Pseudomonas* and *Rhodobacter*. The presence of CuO and ZnO NPs also stimulated two-component regulatory system that is responsible for quorum-sensing, pili synthesis and metal tolerance (Huang *et al*. 2019). The presence of zinc oxide NPS and antibiotics simultaneously has negative impact on methanogenesis during sludge digestion and are also responsible for alteration in bacterial and archaeal communities (Zhao *et al*. 2019, Zhao *et al*. 2018).

2.3.1.3 Soil microbiomes

Although one of the main focus of this work is on the consequences for selected DW microorganisms from exposure to ECS, it is also important to understand the effects of these contaminants in the soil microbiota. Soil contamination and the effects on soil microbiome may be reflected in water ecosystems. ECs may reach soil through different ways: applying manure or sewage sludge as fertilizer, using treated WW for irrigation, due to the ECs leaching from landfills or due to the direct application of pesticides. Information about the effects of ECs on soil microbiome is still limited. Nevertheless, some works studied the presence of ECs in soils and some attention was given to the effects on soil microbiomes (Christou *et al*. 2017, Thelusmond *et al*. 2018, Tong *et al*. 2007). Ma *et al*. (2016) studied the effects of the exposure to oxytetracycline in soil microbial community for 120 days. A stimulation of the microbial carbon mass and the nitrification process was observed in association with the presence of the antibiotic. Enzymes activity was also evaluated and an increase in the activity of dehydrogenase was reported after 14 d of exposure to oxytetracycline (Ma *et al*. 2016). McNamara and Krzmarzick (2013) found that triclosan impacts anaerobic microbial community in soils, namely causing an increase in the abundance of *Dehalococcoides*-like *Chloroflexi.*

Thelusmond *et al*. (2018) found that the presence of diclofenac in soils increased the abundance of *Proteobacteria*, *Gemmatimonadales* and *Actinobacteria* and benefited propanoate, lysine, fatty acid and benzoate metabolism. Furthermore, the presence of carbamazepine increased the abundance of *Bacteroidetes, Actinobacteria, Proteobacteria* and *Verrucomicrobia*, while triclocarban was responsible for an increase on the abundance of *Proteobacteria* and *Actinobacteria* in soils.

NPs may also be found in soil and have been recognized to affect microbial community depending on the soil characteristics, such as soil type, organic matter and salt contents (Mohanty *et al*. 2014). Different nanomaterials (carbon-based NPs, iron NPs, cobalt NPs, titanium oxide NPs and others) have been detected in soils and had some impact on soil microbial community and function (Chung *et al*. 2011, Jin *et al*. 2014, Jin *et al*. 2013, Khodakovskaya *et al*. 2013, Pawlett *et al*. 2013, Shah *et al*. 2014). For example, the effects of carbon nanotubes on soil microbiome were described (Chung *et al*. 2011, Khodakovskaya *et al*. 2013). Jin *et al*. (2014) concluded that the presence of SWCNTs altered the composition of microbial community in contaminated soils. SWCNTs caused a decrease of bacterial and fungal biomass, however, the relative abundance of bacteria increased in the presence of the SWCNTs. Jin *et al*. (2013) found that exposure of soil urban microorganisms to SWCNTs for 3 weeks altered their enzymatic activity. For example, activities of β-1,4-glucosidase, cellobiohydrolase, β-1,4-xylosidase, acid phosphatase, and β-1,4-N-acetylglucosaminidase significantly decreased under 1000 μg SWCNT g^{-1} soil. Chung *et al*. (2011) found that multi-walled carbon nanotubes (MWCNT), commonly used in industrial products and easily reaching soils, had significant impact on soil microbial activity since most enzymes were repressed in the presence of MWCNT and the N and C in the microbial biomass also decreased. Khodakovskaya *et al*. (2013) also described the effects of carbon nanotubes in soil

microbial community. They found that the relative abundance of *Bacteroidetes* and *Firmicutes* increased while the abundance of *Proteobacteria* and *Verrucomicrobia* decreased in the presence of increasing concentrations of carbon nanotubes (Khodakovskaya *et al*. 2013). Pawlett *et al*. (2013) found that zero-valent iron NPs affected microbial communities depending on the soil mineral type and the abundance of organic matter. Iron NPs negatively affected the microbial biomass in soils amended with straw. The microbial community also seemed to be more susceptible to iron NPs in sandy soil than in clay soils. Pawlett *et al*. (2013) also described bactericidal effects of iron NPs in Gram-negative bacteria as well as potential reduction of arbuscular mycorrhizal fungi, which may have negative implications for land remediation. On the other hand, Shah *et al*. (2014) did not observe significant alterations of microbial community richness in soils contaminated with metal (cobalt, nickel, iron and silver) NPs. However, individual analysis by genera demonstrated a reduction of *Sphingomonas* and *Lysobacter* as well as an increase of *Flavobacterium* and *Niastella.*

2.3.2 Tolerance to antimicrobials

ARB and ARG occur in nature and are already considered contaminants of emerging concern. The mechanisms responsible for bacterial resistance to antimicrobials are inherent in the quickly alteration of bacterial genome. However, bacterial genome alterations are not only consequence of mutations or genome rearrangements during bacterial life cycle, but it may also be related to the acquisition of exogenous genes through the exchange between microorganisms or by the gene capture in integrons by horizontal gene transfer (HGT) (Baharoglu *et al*. 2013, Ye *et al*. 2017). Integrons are natural gene expression systems that can act as reservoir of silent genes mobilizable when

necessary. These mobilization by site-specific recombination transform silent genes in functional genes (Baharoglu *et al*. 2013).

The presence of ARG and ARBs in different ecosystems worldwide is of particular concern (Ma *et al*. 2019). DW is recognized as a reservoir of ARB and ARG and it is known that the abundance of ARG and ARB is higher in tap water than in finished water (Farkas *et al*. 2013, Xi *et al*. 2009). Therefore, the study of the effects of ECs on antibiotic resistance in DW microbiome is of utmost importance. Antibiotic resistance in DW may threat human health by three different ways: (1) human may be contaminated with an antibiotic resistant pathogen through direct ingestion of water; however, no human to human transmission occurs; (2) human to human transmission occurs after a direct infection after consumption of contaminated DW; (3) horizontal ARG transfer to human pathogens (Sanganyado and Gwenzi 2019). There are few works reporting the occurrence of disease outbreaks attributed to the presence of ARB in DW (Ma *et al*. 2017, Qamar *et al*. 2018, Sanganyado and Gwenzi 2019).

Usually the spread of antibiotic resistance is believed to be the consequence of the use of antibiotics, however, the importance of other non-antibiotic chemicals as responsible for cross-resistance has been overlooked (Ye *et al*. 2017). Qiu *et al*. (2019) analyzed 31 water and sediment samples from rivers in China and observed positive correlations among *bla*D gene, *Fusobacteria* and sulfamethoxazole. Therefore, the authors suggested that antibiotic exposure may be positively linked to the expression of ARG in certain bacteria (Qiu *et al*. 2019). Wang *et al*. (2019a) reported significant effects from the antibiotic exposure on the spread of ARG in DW biofilms. The exposure to sulfadiazine and ciprofloxacin enhanced the abundance of resistance genes for those antibiotcs. However, the exposure to ciprofloxacin also induced *mex* A, a gene that confers bacterial resistance to antibiotics by pumping them out. The exposure to ciprofloxacin and sulfadiazine

simultaneously had higher impact on the abundance of *mex* A and *int* 1 (class I integrase gene) (Wang *et al*. 2019a). Nevertheless, in the environment bacteria are exposed to a miscellaneous of contaminants and have to develop mechanisms to tolerate them (Ye *et al*. 2017). There are several works relating the exposure to different non-antibiotic contaminants with the development of antibiotic resistance in environmental microbiomes. Wang *et al*. (2017a) found that the exposure of coastal water microbiome to polycyclic aromatic hydrocarbons accelerated the propagation of ARGs. These authors reported that naphthalene at 100 mg/L and phenanthrene at 10 mg/L enhanced the abundance of *intl* 1, *sul* 1 (sulfanilamide resistance gene) and *aad* A2 (aminoglycosides resistance genes) in coastal microbiome. *Intl* 1 and *sul* 1 were also induced in stream biofilms after the exposure to the mixture of different antibiotic and non-antibiotic ECs (ciprofloxacin, erythromycin, sulfamethoxazole, diclofenac and methylparaben) (Subirats *et al*. 2018). However, the availability of high concentrations of nutrients favored the spread of the referred ARGs when the microbial community was also exposed to the cocktail of ECs (Subirats *et al*. 2018). Lv *et al*. (2015) evaluated the effects of DBPs on the development of antibiotic resistance in *Pseudomonas aeruginosa* PAO1. The exposure of *P. aeruginosa* to bromoacetamide, trichloroacetonitrile or to tribromonitromethane increased the resistance to ciprofloxacin, gentamycin, polymyxin B, rifampin, tetracycline, ciprofloxacin + gentamicin and ciprofloxacin + tetracycline. The authors demonstrated that these alterations in antibiotic resistance profile was caused by mutagenesis, associated with an overexpression of efflux pumps (Lv *et al*. 2015). On the other hand, several other works also reported that some non-antibiotic ECs promote the horizontal gene transfer intra and across bacterial genera. Some examples are triclosan (Lu *et al*. 2018), carbamazepine (Wang *et al*. 2019b), disinfectants such as free chlorine,

chloramine and hydrogen peroxide (Zhang *et al*. 2017b) and heavy metals (copper, silver, chromium and zinc) (Zhang *et al*. 2018).

2.4 Strategies to reduce DW microbiome exposure to ECs

The information reported before proves the complexity of the presence of ECs in the environment: the alteration of natural microbiomes and their function in soil and aquatic environments as well as the worrying impact in antibiotic resistance spread. Therefore, it is of utmost importance to reduce the exposure of natural microbiomes to ECs. Special attention should be given to DW microbiome, since the consequences in terms of antibiotic resistance spread may constitute a direct threat to consumers. Therefore, these questions also reinforce the importance of improved strategies to better control biofilm formation in DWDS and plumbing systems.

Two strategies can be followed in order to reduce the exposure of microbial communities to ECs: (1) reduce the entrance of ECs in DWDS; (2) reduce the development of biofilms in DWDS. However, it is important to taken into account that there are no strategies able to eradicate biofilms in DWDS and the complete removal of ECs from DW is also not feasible.

2.4.1 Removal of emerging contaminants from drinking water

There are several treatments that are applied and that can remove partially or completely some ECs. Nevertheless, the presence of complex mixtures of ECs with different physical and chemical properties in the environment highlights the complete removal of ECs a harder challenge. Conventional treatments applied in WWTPs have also been studied in ECs removal, however, their efficiency is limited. There are several works reporting the

use of conventional and/or advanced processes to remove ECs from WW, however, the information about the removal of ECs in DWTPs is still limited.

DWTPs are a multi-barrier process composed by different strategies to produce safe DW. Among this strategies, the most practiced are oxidation processes (chlorination, ozonation and other advanced oxidation processes), filtration (including biofiltration), coagulation, flocculation, settling and also secondary disinfection (NRC 1987, Prest *et al*. 2016). However conventional DWTPs are not effective in the removal of ECs and the concentration of these contaminants remains relatively unchanged in finished DW (Tabe *et al*. 2016).

2.4.1.1 Coagulation, flocculation and settling

Coagulation and flocculation are not very effective in the removal of ECs. These processes are commonly designed to remove suspended solids. Therefore, ECs can only be removed if they are able to adsorb on the flocs formed (Gomes *et al*. 2017). Boiteux *et al*. (2017) and Sun *et al*. (2016) evaluated conventional DW treatments on the removal of PFAS (per- and polyfluoroalkyl substances) and observed that coagulation followed by sedimentation did not cause PFAS removal. Flocculation using iron (II) chloride was unable to eliminate bezafibrate, clofibric acid, carbamazepine and diclofenac (Ternes *et al*. 2002). Petrovic *et al*. (2003) also evaluated the efficiency of different processes on the removal of estrogenic short-chain ethoxy nonylphenolic compounds and found that settling, flocculation and sand filtration process only removed 7% of the ECs presented in water. On its turn, Westerhoff *et al*. (2005) evaluated the removal of 62 different contaminants (endrocine disruptors compounds (EDCs) and PPCPs). The use of aluminum sulfate and ferric chloride coagulants removed some polyaromatic hydrocarbons, however, the removal of most of the ECs was lower than 25% (Westerhoff

et al. 2005). More recently, Su *et al*. (2018) investigated the occurrence and diversity of ARGs in DW treatment processes and observed that sedimentation is an important process able to effectively reduce the abundance of ARGs.

2.4.1.2 Filtration processes

Filtration is an important process in DWTP and can be divided in two main applications: rapid filtration processes – aiming to separate solid particles in water and that usually represent the last clarification steps following processes like coagulation, flocculation or sedimentation processes; slow filtration process - formed by filters with smaller grains and consequently lower pore size, improve general water quality even in terms of microbial content (Brandt *et al*. 2017). There are several filter media commonly used such as sand, activated carbon and anthracite, and the removal of contaminants will be driven by the pore size and the adsorption to the selected media. There are several works evaluating the ability of conventional filtration (sand, activated carbon and anthracite) on the removal of different emerging contaminants (Chen *et al*. 2007, Flores *et al*. 2013, Gomes *et al*. 2017, Westerhoff *et al*. 2005). Sand filtration is not effective in the removal of ECs (Boiteux *et al*. 2017, Chen *et al*. 2007, Flores *et al*. 2013, Ternes *et al*. 2002). However, it may have an important effect on the reduction of ARGs abundance in DW (Su *et al*. 2018). Oppositely, activated carbon have a more variable performance, since it only removes dissolved ECs able to adsorb on its surface. Activated carbon efficiency on ECs removal is dependent of several factors: water matrix, type of carbon used; activated carbon usage rate and loading (Flores *et al*. 2013). ECs octanol-water partition coefficient may drive their adsorption to activated carbon (Gomes *et al*. 2017, Westerhoff *et al*. 2005). Boiteux *et al*. (2017) evaluated the removal of PFAS in three DWTPs, and observed that activated carbon filtration was inefficient in PFAS removal after sand filtration, however, the use of activated carbon filtration applied after ozonation decreased

the concentration of a PFAS. Flores *et al*. (2013) reported a removal of 64 and 45% of PFOS and PFOA through GAC, respectively. Kleywegt *et al*. (2011) collected water in DWTPs and analyzed for the presence of 48 ECs. Different removal efficiencies for different contaminants were reported when water passed through GAC filters: 71 to 93% for carbamazepine, 44 to 55% for gemfibrozil and 80% of bisphenol A. On its turn, Westerhoff *et al*. (2005) described that the use of powder activated carbon removed 50-98% of the volatile contaminants and 10 -95% of polar contaminants. Petrovic *et al*. (2003) also obtained a significant removal (73%) of the ethoxy nonylphenolic compounds using GAC filtration in a DWTP. Ternes *et al*. (2002) also demonstrated that GAC was very effective in the removal of different pharmaceuticals (benzafibrate, carbamazepine and diclofenac), however, clofibric acid was recalcitrant and remained unchanged after this process. Moreover, the use of GAC in DWTP seems to have a negative impact in the removal of ARGs. Su *et al*. (2018) observed an increase in the abundance of some ARGs after GAC filtration. Anthracite is also a common filter matrix applied in DWTP. Chen *et al*. (2007) obtained significant elimination of estrogens (84 – 99%) due to the adsorption of these contaminants on anthracite.

Biofiltration consists in the development of biofilms in media filters (sand, activated carbon, etc) surface. This process has also been studied in terms of ability to remove ECs in DWTPs (Borges *et al*. 2016, Sun *et al*. 2016, Zearley and Summers 2012, Zhang *et al*. 2017a). Borges *et al*. (2016) evaluated the removal of diclofenac, ibuprofen, naproxen and amoxicillin using activated carbon containing biofilms and was able to remove more than 80% of the selected pharmaceuticals. Zearley and Summers (2012) used sand substrate to form biologically activate filters and obtained variable removals for different contaminants. Gemfibrozil and trimethoprim were contaminants easily removed by those biofilters (> 85% of removal). Zhang *et al*. (2017a) evaluated biologically active filters

formed on GAC and obtained significant ECs removal (> 80 %). Oppositely, Sun *et al*. (2016) did not obtain significant removal of PFAS using biologically active carbon filters.

Membrane filtration processes are advanced treatments that have significant impact in DWTPs, including in the improvement of ECs removal. These methods employ a semi-permeable membrane to separate materials according to their physical and chemical properties, through pressure differential or electrical potential difference (Brandt *et al*. 2017, Zhang *et al*. 2017a). Nanofiltration (NF), UF and reverse osmosis (RO) are the most commonly applied in DWTPs and their performance has been important in the removal of different ECs (Boiteux *et al*. 2017, Flores *et al*. 2013, Yoon *et al*. 2006). NF is an important strategy to remove PFAS from water (Boiteux *et al*. 2017) as well as RO (> 99% removal). UF demonstrated to be an irrelevant process to remove PFAS (Flores *et al*. 2013). Flores *et al*. (2013) also demonstrated that RO was more efficient on the removal of some PFAS than reverse electrodialysis. Yoon *et al*. (2006) studied the efficiency of NF and UF membranes on the removal of 52 ECs with different physico-chemical properties (size, hydrophobicity and polarity). NF was able to retain many ECS due to hydrophobic adsorption and size exclusion. On the other hand, UF retained only hydrophobic ECs due to hydrophobic adsorption to membrane (Yoon *et al*. 2006).

The available literature has been demonstrating the inefficiency of settling processes on the removal of ECs. However, such process can be detrimental in the filtration efficiency. Yang *et al*. (2014) evaluated the removal of eight phthalate esters (PAEs) and thirteen pharmaceuticals from DW using a complex system of simultaneous electrocoagulation and electrofiltration (EC/EF). Therefore, authors used a tubular carbon nanofiber/carbon/alumina composite membrane (TCCACM) for DW filtration. The conventional crossflow filtration test without application of an external electric field removed 20-45% of the selected contaminants. The application of an electric field

improved the removal of PAEs up to 78% and the pharmaceuticals up to 77%. In the conventional systems the removal was caused by steric exclusion based on the pore size/network microstructure and due to the adsorption to carbon nanotubes. However, the application of an electric field reduces the formation of filter cake on the membrane surface, improving the permeate flux, therefore, the removal efficiencies. The overall disadvantage of these physical methods is the expensive treatment of the contaminated adsorbent material and of the waste generated (Pereira *et al*. 2011).

2.4.1.3 Conventional and advanced oxidation processes

Oxidation process are of utmost importance in DW treatment for the control of microbial load. Conventional oxidation processes use strong oxidant reagents to inactivate microorganisms, such as free chlorine, chlorine dioxide, chloramines, hydrogen peroxide, ozone. Nevertheless, the reaction of these oxidant reagents with organic matter can form carcinogenic halogenated DBPs. Therefore, advanced oxidation processes (AOPs) emerged as an important technology for DW treatment without the formation of DBPs (Rodriguez-Chueca *et al*. 2015).

The oxidation power of conventional and advanced processes is very important in the degradation of ECs in DWTPs (Acero *et al*. 2013, Boiteux *et al*. 2017, Flores *et al*. 2013, Gomes *et al*. 2017, Petrovic *et al*. 2003, Sichel *et al*. 2011). Chlorine was able to degrade some ECs such as methyl indole and in lower extent chlorophene and nortriptyline (Acero *et al*. 2013). However, benzatriazole and N,N-diethyl-m-toluamide were recalcitrant and were not altered by chlorine (Acero *et al*. 2013). Oxidation processes such as chlorination, chlorine dioxide treatment, peroxidation and ozonation were not able to remove PFAS in DWTPs (Boiteux *et al*. 2017, Flores *et al*. 2013, Sun *et al*. 2016) probably due to the strength of the C-F bond. Su *et al*. (2018) also reported inefficiency of ozonation in

DWTPs since the abundance of some ARGs increased after water treatment with ozone. On the other hand, Westerhoff *et al*. (2005) reported that chlorine and ozone were able to transform part of the ECs present in water and observed that ECs that are easily oxidized by chlorine are always oxidized at least as efficiently by ozone. Between the 62 contaminants analyzed only six were poorly oxidized by chlorine and ozone. However, the results demonstrated, in general, that the use of ozone may improve ECs removal (Westerhoff *et al*. 2005).

Sichel *et al*. (2011) presented an important work comparing conventional and advanced oxidation processes applied in DW treatment. Sulfamethoxazole was easily degraded by free chlorine and chlorine dioxide both at 6 mg/L for 15 min of contact. However, benzatriazole, tolyltriazole, carbamazepine and iopamidole were not degraded by these conventional processes. H_2O_2/UV (5 mg/L), HOCl/UV (1 mg/L) and ClO_2/UV (0.4 mg/L) were the AOPs evaluated by Sichel *et al*. (2011). Chlorine AOPs completely removed 17α-ethinylestradiol (EE2), H_2O_2/UV also had important action on its removal. Sulfamethoxazole and diclofenac were readily degraded by all the evaluated AOPs. The higher removal efficiency (40-60%) for desethylatrazine and carbamazepine were obtained through H_2O_2/UV. Benzatriazole and iopamidole were highly removed by HOCl/UV, with yields of 60-80% and 80-100%, respectively. Benotti *et al*. (2009) demonstrated that a membrane pilot system employing TiO_2/UV was able to reduce the concentration of 32 ECs including pharmaceuticals and EDCs.

2.4.2 Controlling microbial growth in drinking water

The DW microbial community is mainly presented as biofilms, particularly 95% of microbial communities adhered on the surfaces of DWDS (Flemming *et al*. 2002). Biofilms confer several advantages for microbial growth under conditions of low nutrient

content and of environmental stress, as found in DWDS. The main advantages are related to the protection conferred by the EPS matrix that retains and stores nutrients and also binds to and inactivates disinfectants. The EPS matrix also confers limitations to mass transfer avoiding and/or retarding the diffusion of disinfectants and other stressors through the matrix. Cells in biofilms have low metabolizing rate and may acquire vegetative states which can be an important strategy for bacterial survival under stress conditions. Biofilms also confers the possibility of metabolic interaction between the bacteria with different physiological requirements (Berry *et al*. 2006, Liu *et al*. 2016, Simões and Simões 2013). The development of biofilms in DWDS may be responsible for alterations in the organoleptic characteristics of the delivered water (Zhou *et al*. 2017). It is also known that biofilms have impact on metal corrosion, accelerating the degradation of pipes and reducing their lifetime. Furthermore, biofilms are a reservoir of microorganisms and may be also a source of pathogens. Portions of biofilms are often released into the bulk water and potentially reach the consumers tap. More recently, DW biofilms have also been described as reservoirs of ARGs (Liu *et al*. 2016).

There are several strategies in DWTP and along the DWDS and plumbing systems (Figure 2-2) to avoid microbial proliferation.

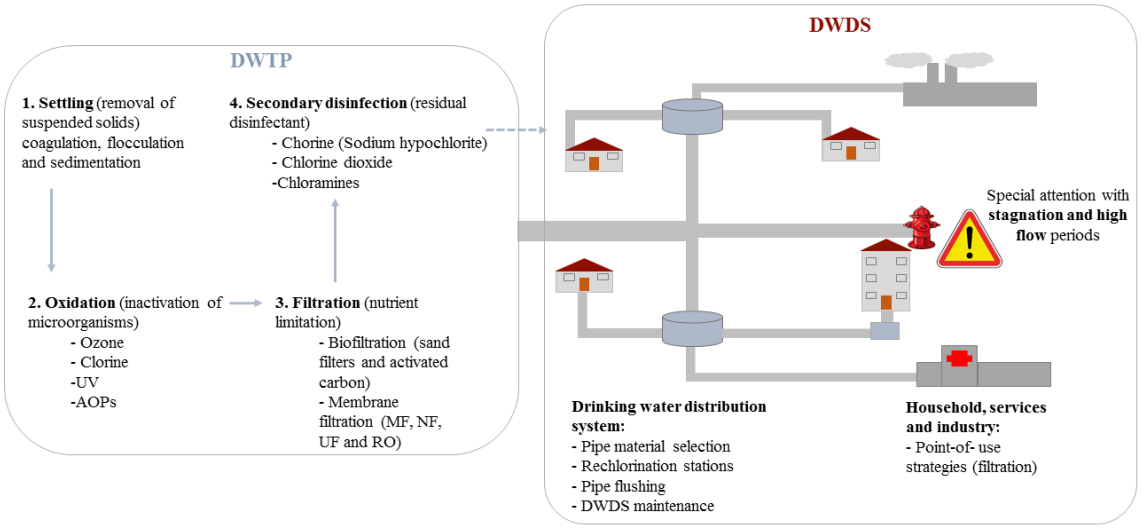

Figure 2-2. Strategies applied in DWTP or DWDS to control microbial growth along the system (based on Simões and Simões 2013)

2.4.2.1 Microbial growth control in DWTP

A DWTP comprises several processes aiming to guarantee the delivery of chemically and biologically safe water. All the processes referred previously (settling, oxidation, filtration and secondary disinfection) have an important action on the control of microbial and nutrient load of the finished water. Oxidation using ozone, chlorine or AOPs is an important step for initial microbial inactivation. However, this process can alter water composition, increasing the availability of nutrients. Therefore, water generated after the oxidation process is very unstable which imposes the need of following steps able to reduce nutrient load (Prest *et al*. 2016). The use of sand or activated carbon filters is fundamental on the removal of nutrients. Microbial communities may adhere on the filters, forming a biofilter that will have important action on nutrient consumption. To improve the nutrient removal, successive biofiltration processes may be applied. For example, humic substances of low molecular weight are removed during rapid sand filtration while polysaccharides may be degraded during slow sand filtration

(Lautenschlager *et al*. 2014). The control of the availability of nutrients in distributed water is of utmost importance to better control microbial growth along DWDS and consequently avoid biofilm development (Prest *et al*. 2016). The use of membrane filtration, such as microfiltration (MF), NF, UF or RO is very common in DWTPs to remove bacteria and suspended particles without the formation of by-products potentially harmful for the human health. Membrane filtration processes are important to avoid particles in DWDS and consequent deposition. Nevertheless, any of these membrane systems are able to efficiently remove organic matter from water. Therefore, the use of ion exchange in DWTP is very important to remove low molecular weight organic matter, reducing nutrient availability along the DWDS. The final step before the entrance of the finished water in DWDS is the secondary disinfection. This process aims to inactivate the remaining microorganism and avoid bacterial growth in DWDS by keeping a residual concentration of disinfectant. The most commonly used disinfectants are free chlorine (mainly in the form of hypochlorite), chloramines and chlorine dioxide (Prest *et al*. 2016).Ozone, hydrogen peroxide and UV are also applied for secondary disinfection, nevertheless, these methods are not suitable to keep residual disinfectants along DWDS. Table 2-2 presents the main advantages and disadvantages of the main disinfectants applied in DW that allows the use of residual disinfectant along DWDS.

Table 2-2. Main advantages and drawbacks of disinfectants applied in DWTPs that allow residual concentrations along DWDS.

Disinfectant	Advantages	Drawbacks	References
Free chlorine	- High effectiveness - High solubility - Easy to use - Low cost - Relatively stable	- Organoleptic problems (high dosages) - Production of carcinogenic DBPs -Selection of resistant microorganisms - Decay (high temperatures, easily reacts with pipes, organic matter, bacteria, particles and deposits)	(Liu *et al*. 2016, Prest *et al*. 2016)
Chloramines	- Relatively stable - Maintain residual level for longer periods	- Generates DBPs (in lower extent than chlorine) - Associated with the growth of nitrifying bacteria	(Liu *et al*. 2016, Prest *et al*. 2016)
Chlorine dioxide	- Low levels of DBPs are formed	- More expensive - Relatively unstable - Hazards of chlorine and chlorine dioxide gas release - Incompatibility with several pipe materials (decreasing material life time)	(Schwartz *et al*. 2003a, Simões and Simões 2013, van der Stok *et al*. 2018)

2.4.2.2 Microbial growth control along DWDS

The complexity of DWDS impairs the control of biofilm development, since it can be affected by several factors: temperature, nutrient availability, pH, materials used, hydrodynamic conditions, disinfectant type and concentration. Therefore, there are

several measures that may play an important role in biofilm development and may be crucial in the prevention of the spread of waterborne diseases or pathogens. A synthesis of the strategies commonly applied in DWDS design, along DWDS and at the point-of-entrance or at the point-of-use is presented below.

2.4.2.2.1 Pipe material

DWDS and plumbing systems may be composed by a wide variety materials: plastics (polyvinyl chloride – PVC, polyethylene – PE, high density PE – HDPE, etc), metals such as stainless steel, iron, galvanized iron, copper and cement pipes. The selection of materials used along the DWDS and plumbing systems have a significant impact in biofilm development. Biofilm development is favored on iron and cement pipes compared to plastic materials (Niquette *et al*. 2000). Iron pipes are also more susceptible to corrosion which will facilitate biofilm development (Fish *et al*. 2016, Liu *et al*. 2016, Morton *et al*. 2005). On the other hand, plastic pipes are not affected by corrosion, but leaching typically occurs (Bucheli-Witschel *et al*. 2012, Mercea *et al*. 2019, Skjevrak *et al*. 2003). Copper has well known antimicrobial properties and already demonstrated potential to reduce or retard biofilm formation (Gulati and Ghosh 2017, Lehtola *et al*. 2004, Rhoads *et al*. 2017). For example, Lehtola *et al*. (2004) found that biofilm development on PE was higher than the growth observed on copper pipes. For longer periods (more than 200 days) the biofilms formed on copper pipes was not significantly different from those formed on PE (Lehtola *et al*. 2004). More recently, Rhoads *et al*. (2017) observed an important role of copper on *L. pneumophila* biofilm control in a stagnant DWDS. On the other hand, a previous work of Gião *et al*. (2015) demonstrated that the abundance of *L. pneumophila* was higher in DW biofilms grown on copper materials than on PVC. Gulati and Ghosh (2017) reported that *Sphingomonas paucimobilis* biofilms were not developed on copper after 90 h of incubation. The

development of 90 d-old DW biofilms was lower on copper materials than on other metals or plastics and *E. coli* growth in DW biofilms was also diminished on copper surfaces (Yu *et al*. 2010). Although being an interesting material, due to the resistance to corrosion and its attractive antimicrobial properties, copper is a high cost material and for this reason has been replaced by plastic pipes in plumbing systems (Cooper and Hanlon 2010).

2.4.2.2.2 Hydrodynamic conditions and pipe flushing

The water flow inside pipes has huge impact in biofilm growth and behaviour. Stagnation zones, such as storage tanks or pipe sections that commonly are not used (firefights pipeline, new buildings or buildings closed for significant periods), stimulate microbial growth and biofilm development due to the accumulation of deposits, the increase of residence time and consequent disinfectant decay (Zlatanović *et al*. 2017). Nevertheless, the sudden use of high water flow will motivate biofilm detachment and consequently increase the abundance of microorganisms in the delivered water. Therefore, these both conditions should be carefully evaluated in order to avoid DW deterioration and potential risks from consumption of contaminated water.

Pipe flushing is a strategy commonly used in DWDS for pipe cleaning, acting by removing sediments, contaminants and biofilms. Flushing consists in running water at high flow through pipes to replace water in DWDS or plumbing systems (Ragain *et al*. 2019). This strategy can be used in plumbing systems in cases of water contamination, in order to remove contaminated water and restore safe DW in buildings, preventing the exposure to chemical and/or biological contamination (Casteloes *et al*. 2015, Katner *et al*. 2018, Ragain *et al*. 2019). Pipe flushing can also be an important strategy to restore the levels of residual disinfectant along the DWDS (Cohn *et al*. 2015). However, the protocols used are controversial, and several adjustments in flushing time have been

studied in order to avoid an increase of exposure to contaminations, if short time flushing is applied (Casteloes *et al*. 2015, Katner *et al*. 2018, Ragain *et al*. 2019).

2.4.2.2.3 Booster disinfectant stations

Chlorine is the most used disinfectant in DW treatment and is kept at a residual concentration along the DWDS in order to avoid microbial regrowth. Nevertheless, chlorine decay along the pipeline is a real problem that may impair the quality of the transported water. The concentration of free chlorine in finished water leaving DWTP should be between 0.5 and 1.0 mg/L in order to keep residual concentration available along the distribution system (WHO 2011a). Chlorine easily reacts with organic matter, corrosion by-products, pipes materials and is also susceptible to increased temperatures. It is challenging to ensure that enough free chlorine is available in the DWDS to reach the tap at a minimum concentration of 0.2 mg/L (WHO 2011a). The implementation of booster chlorination stations along the DWDS allows to reapply chlorine within the network at strategic locations (Goyal and Patel 2018).

The presence of rechlorination stations at the point- of- entry (POE) of critical buildings (hospitals or healthcare units) can be an important strategy to avoid waterborne outbreaks, such as of *Legionella pneumophila* (Cohn *et al*. 2015). The use of located equipment for copper-silver ionization at the POE of private networks is also an important strategy for water decontamination, avoiding biofilm formation and the proliferation of pathogens. Copper-silver ionization systems have emerged as a long-term disinfection method for *Legionella* spp. in hospital water systems. It consists in an electrolytic generation of copper and silver ions that are dispersed into the water and have antimicrobial activity, reducing the risks of *L. pneumophila* exposure and outbreaks (WHO 2018).

2.4.2.2.4 Point-of-use strategies

There are several technologies for application in the water point-of-use (POU) aiming to avoid the intake of chemically or microbiologically contaminated water. These strategies can have a significant impact on public health if applied in areas with low water quality control. The application of this strategies in some high risk healthcare units can have significant impact avoiding the possible exposure of immune-compromised people to some pathogens present in the plumbing system (Totaro *et al*. 2017). Between these POU strategies, filtration is of the main applied. Filters can be made of different materials: ceramic, sand, activated carbon, textile, membrane and hollow fiber filters and polyurethane foams (Hunter 2009, Jain and Pradeep 2005, Liu *et al*. 2014, Totaro *et al*. 2017, WHO 2018). Silver ions and nanoparticles have important antimicrobial properties, therefore, they have been incorporated in the referred filters in order to avoid biofouling and filter clogging, improving the water quality and the filter lifetime (Jain and Pradeep 2005, Lalley *et al*. 2014, Liu *et al*. 2014, WHO 2018).

Chapter 3

Materials and methods

This chapter describes the materials and methods used to perform the work presented in Chapters 4 to 8.

3.1 Bacteria and culture conditions

Acinetobacter calcoaceticus and *Stenotrophomonas maltophilia* were isolated from a DWDS in a previous work (Simões *et al*. 2007a) and were chosen as microbial models for DW biofilm formation. These two bacteria were selected for this work taking into account their frequent presence in DW and their ability to infect immunocompromised consumers. *Acinetobacter* spp. and *S. maltophilia* were already described by the WHO (Glasmacher *et al*. 2003) as causative agents of infections in immunocompromised patients through tap water. The bacteria were grown overnight at 25 ºC and under agitation (120 rpm) in Reasoner's 2A (R2A) broth medium (see section 3.2.1).

3.2 Media Composition

3.2.1 R2A

R2A broth medium for bacterial growth is composed by 0.5 g/L peptone (Oxoid, Hampshire, England), 0.5 g/L glucose (CHEM-LAB, Zedelgem, Belgium), 0.1 g/L magnesium sulfate heptahydrate (Merck, Darmstadt, Germany), 0.3 g/L sodium pyruvate (Fluka, Steinheim, Germany), 0.5 g/L yeast extract (Merck, Darmstadt, Germany), 0.5 g/L casein hydrolysate (Oxoid, Hampshire, England), 0.5 g/L starch (Sigma) and 0.4 g/L di-potassium phosphate trihydrate (Aplichem Panreac, Darmstadt, Germany).

3.2.2 Synthetic tap water

Synthetic tap water (STW) was used in order to mimic the tap water and is composed by 100 mg/L $NaHCO_3$ (Fisher Scientific, Leicestershire, UK), 13 mg/L $MgSO_4.7H_2O$ (Merck, Darmstadt, Germany), 0.7 mg/L K_2HPO_4 (Aplichem Panreac, Darmstadt,

Germany), 0.3 mg/L KH_2PO_4 (CHEM-LAB, Zedelgem, Belgium), 0.01 mg/L $(NH_4)_2SO_4$ (Labkem, Barcelona, Spain), 0.01 mg/L NaCl (Merck, Darmstadt, Germany), 0.001 mg/L $FeSO_4.7H_2O$ (VWR PROLABO, Leuven, Belgium), 1 mg/L $NaNO_3$ (Labkem, Barcelona, Spain), 27 mg/L $CaSO_4$ (Labkem, Barcelona, Spain), 1 mg/L humic acids (Sigma-Aldrich, Steinheim, Germany) (EPA 2011).

3.3 Substrates for biofilm formation

3.3.1 Polyvinyl chloride

Polyvinyl chloride (PVC) coupons (Neves & Neves, Portugal) with 1 cm × 1 cm were used as substratum for biofilm formation. PVC was selected as a representative pipe material from DW networks (SDWC *et al*. 1982). In order to clean and sterilize PVC for biofilm formation, coupons were immersed in a solution of a commercial detergent (Sonasol Pril, Henkel Ibérica S.A.) in distilled water for 30 min. Afterwards, the coupons were rinsed in distilled water and subsequently immersed in ethanol at 70% for 30 min. After that, coupons were rinsed three times with distilled sterile water and dried overnight at 60 ºC (Simões *et al*. 2007a). Then PVC coupons were placed in 48 well microtiter plates and exposed to ultra-violet (UV) light for 30 min before being used for biofilm formation.

3.3.2 Copper

Four copper alloys were tested (Table 3-1). Elemental copper (100% Cu) was used as positive control and stainless steel AISI 316 (SS) was used as negative control (0% Cu). Coupons (1 × 1 cm) or cylinders (sampling area of 39.27 cm^2; d = 2.5 cm; l = 5 cm) of the referred surface materials were used as substratum for biofilm formation. In order to

clean and sterilize copper materials, coupons and cylinders were degreased in absolute ethanol (Panreac Applichem, Darmstadt, Germany) and cleaned for 5 min in an ultrasound bath (70 W, 35 kHz, Ultrasonic Bath T420, Elma, Singen, Germany). To remove surface oxides, each coupon was exposed to HCl (39% sp gr 1.19 from Fisher, Leicestershire, UK) diluted two times in water, with 2 min exposure. To conclude oxides removal, coupons were washed in distilled water, dried with paper towel and abraded with abrasive paper P1000 until achieving a homogeneous appearance (ASTM 1999). Afterwards, the coupons were rinsed thoroughly with distillated water and dried with paper. After this process, coupons were washed for 20 min with ethanol at 70% (v/v) and further sterilized by UV for 45 min on each coupon face

Table 3-1. Composition of materials used as substrate for biofilm formation.

	Surface material composition (%)								
Material (US Standard)	Cu	Fe	Ni	Mn	Sn	Al	Zn	Cr	Other
AISI 316	-	62	10-14	2	-	-	-	16-18	C, Si, P, S, Cr Mo, N
C11000	100	-	-	-					-
C18000	96	-	4	-					-
C90800	83	-	-	-	17				-
C95500	79	-	5	1		10			-
C38500	57	5	-	-			39		Pb (3 %)

3.4 Emerging contaminants on *Stenotrophomonas maltophilia* behaviour

3.4.1 Selected emerging contaminants

Three nonsteroidal anti-inflammatory drugs (NSAIDs) (antipyrine - ANTP - from Alfa Aesar (Karlsruhe, Germany); diclofenac sodium salt - DCF - from Fluka (Steinheim, Germany) and ibuprofen – IBP - from Alfa Aesar (Karlsruhe, Germany)), two musk fragrances (galaxolide – GAL - and tonalide - TON - both from Sigma-Aldrich (Steinheim, Germany)), one neuro-active drug (carbamazepine - CBZ - from Acros Organics, New Jersey, USA), one lipid regulator (clofibric acid - CA - from Acros Organics, New Jersey, USA) and one veterinary antibiotic (tylosin - TY - from Sigma-Aldrich, Steinheim, Germany) were selected as ECs for the experiments. Stock solutions (from 800 to 1000 mg/L) were prepared in dimethyl sulfoxide (DMSO) (Fisher Scientific, Leicestershire, UK) and stored at -20 ºC until use (See Annex B for additional information about solvent selection). The concentrations tested were prepared from the stock solutions using STW (EPA 2011) (prepared as described in 3.2.2) DMSO final concentration in each solution was 1% (v/v). Table 3-2 shows information on the ECs tested and the respective concentrations found in DWDS and used in the present study. Two concentrations were tested, an environmental concentration similar to those found in DW ([DW] - Table 3-2) and a concentration 100 times higher than those detected in DW (100 × [DW]). When ECs were used in combination, the concentration of each ECs was the same used to test the compound alone ([DW] and 100 × [DW])

Table 3-2. Selected ECs and respective concentration detected in DWDS. The concentration detected in DWDS and a concentration 100 × higher were tested in the present work.

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

3.4.2 Experimental design: from biofilm colonization to exposure to ECs and bacteria characterization

The experimental set-up is constituted of four main steps (Figure 3-1). Firstly, *S. maltophilia* biofilms were developed on PVC coupons for 24 h using 48 wells microtiter plates (Section 3.4.3 – Figure 3-1.A). Afterwards, coupons with biofilms were exposed to ECs for 26 d in a new 48 wells microtiter plate (Section 3.4.4 – Figure 3-1.B). Biofilms exposed to ECs were characterized in terms of numbers of colony forming units (CFU) (Section 3.4.4). Moreover, these coupons were used to inoculate fresh R2A broth (Figure 3-1.C) to characterize the bacteria exposed to ECs (Section 3.4.5 – Figure 3-1.D) in terms of planktonic behaviour: determination of the minimum bactericidal

concentration of NaOCl (Section 3.4.5.1); and susceptibility to antibiotics (Section 3.5.4.2), and biofilm productivity (Section 3.4.5.3) and susceptibility to NaOCl (Section 3.4.5.4).

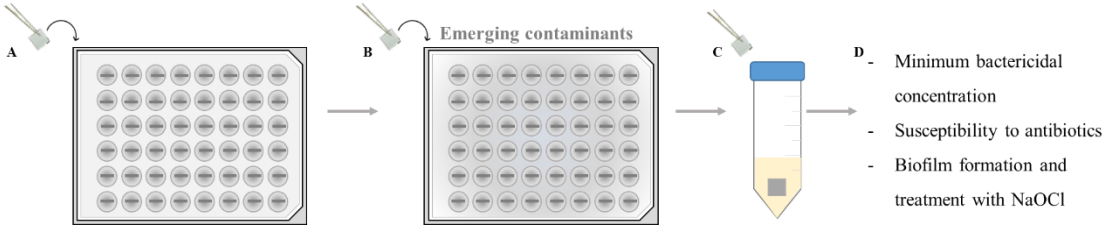

Figure 3-1. Experimental set-up. A - Biofilm formation for 24 h in PVC coupons (25 ºC, 120 rpm). B - Biofilm exposure to ECs for 26 d in STW (25 º C, 120 rpm). C - Inoculation of R2A using a colonized coupon exposed to ECs (25 ºC, 120 rpm, overnight). D - Characterization of bacteria after biofilm exposure to ECs.

3.4.3 Biofilm pre-establishment on PVC coupons

S. maltophilia biofilms were formed on PVC coupons sterilized as described in Section 3.3.1.For that, 1 mL of *S. maltophilia* suspension in R2A broth (2 × 10^8 CFU/mL) was added to each well. Microtiter plates were incubated for 24 h at 25 ºC and under agitation (120 rpm) – Figure 3-1.A.

3.4.4 *S. maltophilia* biofilms exposure to ECs

After 24 h, colonized PVC coupons (prepared as described in Section 3.4.3), were carefully removed from the wells and placed in new microtiter plates with STW in order to remove the weakly and non-adherent bacteria. Then, *S. maltophilia* biofilms were exposed to ECs for 26 d (Figure 3-1.B). For that, colonized coupons were inserted in other microtiter plates with the ECs solutions (prepared as described previously) using STW at environmental concentrations found in DWDS ([DW]) and at 100 times the [DW] (100 × [DW]). ECs solutions were renewed every two days in order to ensure the continuous exposure to a constant amount of ECs for 26 d. Before being placed in new ECs solutions,

colonized coupons were carefully washed in STW to remove non-adherent bacteria and the ECs solution. Two controls were performed: biofilm in contact with STW and biofilm in contact with 1% DMSO (v/v) in STW. After 26 d of continuous exposure to ECs, the numbers of CFU of *S. maltophilia* biofilms were determined. The planktonic behaviour of bacteria grown from biofilms exposed to ECs was also evaluated. For that, colonized coupons were used to inoculate R2A broth (Figure 3-1.C) in order to characterize bacterial susceptibility to sodium hypochlorite (NaOCl) and antibiotics (levofloxacin and trimethoprim-sulfamethoxazole) and its ability to form biofilms.

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

3.4.5 Characterization of bacteria exposed to ECs

Coupons with biofilms exposed to ECs were inserted in 50 mL centrifuge tube containing 10 mL of R2A broth (Figure 3-1.C). Bacteria were naturally released from coupons to the bulk medium (a more favourable environment with high availability of nutrients) and were grown overnight at 25 ºC and 120 rpm for further evaluation of the effects of ECs on *S. maltophilia* behaviour. These bacteria were subsequently characterized in terms of susceptibility to NaOCl (3.4.5.1) and to antibiotics (3.4.5.2), as well as biofilm formation ability (3.4.5.3) and biofilm susceptibility to NaOCl (3.4.5.4) – Figure 3-1.D.

3.4.5.1 Minimum bactericidal concentrations

A pre-culture grown as described previously (Section 3.1.1) was centrifuged (12 min, 3777 g) and ressuspended in STW to achieve a concentration of 3×10^5 CFU/mL in order to mimic the number of cells present in DWDS (Prest *et al*. 2016). NaOCl (Acros Organics, New Jersey, USA) was prepared at different concentrations (10, 20, 30, 40, 50,

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

3.4.5.2 Susceptibility to antibiotics

S. maltophilia grown as described in Section 3.1 were characterized on its susceptibility to antibiotics by the disk diffusion susceptibility methods according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2007). Levofloxacin (LEV) at 5 µg and trimethoprim-sulfametoxazole (TMP-SMX) at 1.25/23.75 µg were the selected antibiotics, according the CLSI guidelines for *S maltophilia* (CLSI 2007). A bacterial suspension (6×10^8 CFU/mL) (from an inoculum prepared as described in Section 3.4.5) was spread on Mueller-Hinton agar (MHA) plates containing antibiotic disks. MHA plates were incubated at 37 ºC for 24 h before measuring the diameter of growth inhibition. Each condition was tested in duplicate with three independent experiments.

3.4.5.3 S. maltophilia biofilm formation after exposure to ECs

Bacteria obtained from biofilms exposed to ECs were characterized in terms of biofilm production. Biofilms were developed according to the modified microtiter plate test proposed by Stepanović *et al*. (2000). Briefly, sterile 96 wells microtiter plates were filled with 200 µL of bacterial suspension (2×10^8 CFU/ml in R2A broth). Negative control

wells were filled with sterile R2A. The plates were incubated for 24 h at 25 ºC and agitated at 120 rpm. Biofilm production was assessed in terms of CFU and by crystal violet (Merck, Darmstadt, Germany) staining.

3.4.5.3.1 CFU enumeration

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

3.4.5.3.2 Crystal violet staining

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

3.4.5.4 NaOCl effects on biofilms formed by bacteria exposed to ECs

Biofilms developed as described previously (Section 3.4.5.3) were treated with NaOCl to evaluate biofilm susceptibility to this disinfectant. After 24 h of biofilm formation, the bulk suspension was discarded and each well was washed with NaCl solution at 8.5 g/L. Then, 180 µL of STW and 20 µL of NaOCl solution at 10 × MIC (minimum inhibitory

concentration) were added to each well for 30 min at 25 ºC and 120 rpm. NaOCl was used at a final concentration of 130 mg/L as this value corresponds to the MIC for *S. maltophilia* in R2A broth (data not shown). Negative controls corresponded to the use of 200 µL of STW. After 30 min of exposure, NaOCl was removed from each well and the remaining biocide was neutralized with sodium thiosulfate at 0.50% (w/v) for 10 min (Gomes *et al*. 2016). NaOCl effects on biofilms were assessed by CFU enumeration and crystal violet (Merck, Portugal) staining according to 3.4.5.3.1 and 3.4.5.3.2, respectively.

3.5 Clofibric acid effects on *Stenotrophomonas maltophilia* virulence and tolerance to antimicrobials

3.5.1 Clofibric acid

Stock solutions of clofibric acid (CA; Acros Organics, New Jersey, USA) were prepared in dimethyl sulfoxide (DMSO) (Fisher Scientific, Leicestershire, UK) and stored at -20 ºC until use. Stock solutions were prepared at 0.017 mg/L and 1.7 mg/L in order to use low DMSO volume in biofilms. Stock solutions were diluted 100 times in synthetic tap water (STW) in order to obtain the final concentrations to be tested: 170 and 17000 ng/L. The concentration of DMSO in the final solution was 1%.

3.5.2 Experimental set-up and biofilm formation

S. maltophilia was grown as described in Section 3.1. PVC coupons (Neves & Neves, Portugal) with 1 cm × 1 cm were used as substratum for biofilm formation. Before use, the coupons were cleaned and sterilized as described in Section 3.3.1.

The experimental set-up was similar to the described in Section 3.4.2 and in Figure 3-1. *S. maltophilia* biofilms were formed as detailed in Section 3.4.3.

3.5.3 *S. maltophilia* biofilms exposure to CA

Colonized PVC coupons were exposed to CA for 12 weeks in 48 wells microtiter plates. Biofilms were formed for 12 weeks. This incubation period was based on the fact that DW biofilms are developed under oligotrophic conditions, taking several weeks/months to reach a mature state (Abberton *et al*. 2016, Abe *et al*. 2012). The biofilms were formed under continuous exposure to DW-containing trace levels of ECs. Two controls were performed: (1) biofilms were grown only in the presence of STW for 12 weeks; (2) biofilms were exposed to 1% DMSO (solvent control) for 12 weeks. The exposure to CA was performed at two concentrations - an environmental concentration similar to those found in DW distribution systems (DWDS) ([DW] - 170 ng/L) (Jones *et al*. 2005) and a concentration one hundred times higher (100×[DW] - 17000 ng/L). The solutions were replaced every week, in order to guarantee a constant exposure to trace CA concentrations.

3.5.4 *S. maltophilia* characterization after prolonged exposure to CA

After biofilm exposure to CA for 12 weeks, *S. maltophlia* cells from biofilms were recovered in R2A agar (Oxoid, Hampshire, England) plates before further tests to characterize susceptibility to chlorine, antibiotic tolerance, biofilm formation and susceptibility to chlorine, bacterial motility, siderophores production and the adhesion and invasion of HT29 cells.

3.5.4.1 Minimum bactericidal concentrations

The bacterial susceptibility to free chlorine was assessed as described in Section 3.4.5.1.

3.5.4.2 Susceptibility to antibiotics

Antibiotics from six different classes were prepared using the solvents listed in Table 3-3. Amoxicillin – AMO (Sigma- Aldrich, Steinheim, Germany); ciprofloxacin – CIP (Fluka, Steinheim, Germany); erythromicin – ERY (AppliChem, Darmstadt, Germany); kanamicin – KAN (Eurobio, Courtaboeuf, France); levofloxacin – LEV (Alfa Aesar, Karlsruhe, Germany); oxacillin – OXA (Sigma- Aldrich, Steinheim, Germany); spectinomycin – SPE (Sigma-Aldrich, Steinheim, Germany); tetracycline – TET (Sigma-Aldrich, Steinheim, Germany); trimethoprim - sulfamethoxazole - TMP-SMX (Alfa Aesar, Karlsruhe, Germany). Bacterial suspensions were adjusted to 6×10^8 cells/mL and the disk diffusion test was applied according the CLSI guidelines (CLSI 2007) to understand the effects of CA exposure on *S. maltophilia* susceptibility to antibiotics. Negative controls were performed with the solvents used (DMSO, NaOH and HCl). Three independent experiments with duplicates were performed for each condition tested.

Table 3-3. Antibiotic class, content per disk and solvent used.

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

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

S. maltophilia from 12 weeks biofilms was grown in R2A broth overnight at 25 ºC and 120 rpm. Then biofilms were formed as described in Section 3.4.5.3. Biofilm production was assessed in terms of culturability (enumeration of CFU) and by crystal violet (Merck, Darmstadt, Germany) staining as described in Sections 3.4.5.3.1 and 3.4.5.3.2, respectively.

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

The 24 h-old biofilms (Section 3.5.4.3) were treated with 130 mg/L NaOCl (the minimum inhibitory concentration for *S. maltophilia* in R2A broth) for 30 min, as described in Section 3.4.5.4. Negative controls were obtained from exposure to 200 µL of STW for 30 min. After exposure, chlorine was removed from each well and the remaining biocide was neutralized with sodium thiosulfate at 0.50% (wt/v) for 10 min (Gomes *et al*. 2018b). Chlorine effects on biofilms were assessed by CFU enumeration and crystal violet staining as previously described in Sections 3.4.5.3.1 and 3.4.5.3.2, respectively.

3.5.4.5 Motility

S. maltophilia motility was assessed after CA exposure for 12 weeks. Swimming, swarming and twitching were the three types of motility evaluated as described by Gomes *et al*. (2016). Briefly, bacteria isolated from CA-exposed biofilms were grown overnight in LBB (Luria-Bertani broth) and the cell density was adjusted to 10^6 CFU/mL in fresh LBB. A volume of 15 µL of bacterial suspension was then dropped in the centre of agar plates. These agar plates were prepared with tryptone at 10 g/L (Fisher Bioreagents, New Jersey, USA), NaCl at 2.5 g/L and agar (VWR Chemicals, Leuven, Belgium) at 3 g/L (for swimming motility), 7 g/L (for swarming motility) or 15 g/L (for twitching motility).

Plates were then incubated at 25ºC for 72 h. After the incubation period, the colony growth was measured. Three independent assays were performed with three replicates.

3.5.4.6 Siderophores production

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

3.5.4.7 Adhesion and internalization of S. maltophilia exposed to CA to HT29 cells line

Human colorectal adenocarcinoma cell line HT29 (ATCC® HTB-38™) (kindly provided by the Institute for Research and Innovation in Health Sciences - I3S, Porto) was selected as model to assess the potential effects from the ingestion of DW contaminated with *S. maltophilia* in the interaction with mammalian intestinal cells. These cells were cultured in RPMI 1640 medium (Biowester, Nuaillé - France) containing 10% (v/v) fetal bovine serum (FBS) (Biowester, Nuaillé - France) and 1% (v/v) antibiotics solution (Biowester, Nuaillé - France). Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere. HT29 cells were seeded in 24 well plates at concentration of 4.0×10^4 cells per well obtaining a semiconfluent monolayers in 48 h. Cell cultures were replenished with RPMI

1640 without antibiotics 24 h before the assay was performed. All cell cultures were incubated at 37°C in a 5% CO_2 atmosphere.

The protocol of bacterial adhesion and internalization was performed according to Gagnon *et al*. (2013). *S. maltophilia* grown as described in Section 2.1 were centrifuged at 3777 g for 15 minutes, washed twice in saline solution (0.85% NaCl). A final concentration of bacteria of 10^6 per mL was prepared in RPMI 1640 medium. A volume of 500 µL of this suspension was then added to each well of the 24 well plate containing a monolayer of HT29. The procedure was validated with *Salmonella* sp. (isolated from DW), a bacterium with known ability to internalize human colon cells (Gagnon *et al*. 2013). Two independent assays were performed with three replicates.

3.5.4.7.1 *S. maltophilia* adhesion on HT29 cells

To determine bacterial adhesion on HT29, plates were incubated for 30 minutes at 37 ºC in a 2.5 L AnaeroJar™ with microaerophilic conditions assured by Oxoid™ CampyGen™ sachet. Afterwards, bacterial suspension was discharged from each well and HT29 cells were washed twice with phosphate buffered saline (PBS), in order to remove non-adhered cells. A volume of 250 µL of trypsin-EDTA (ethylenediamine tetraacetic acid) (Sigma-Aldrich, Steinheim, Germany) was added to each well to detach HT29 cells and adhered bacteria. After incubation for 10 min at 37 ºC, trypsin was inactivated by adding 250 µL of RPMI 1640 with FBS. The bottom of each well was scrapped with the pipette tip to guarantee the maximum removal of HT29 monolayer. The suspension was then plated in tryptic soy agar (TSA) (Merck, Darmstadt, Germany) plates and incubated for 24 h at 37 ºC in order to assess the number of bacteria adhered on HT29 cells.

3.5.4.7.2 *S. maltophilia* internalization in HT29 cells

Bacteria was incubated with HT29 cells for 90 min at 37 ºC in an 2.5 L AnaeroJar™ with microaerophilic conditions assured by Oxoid™ CampyGen™ sachet, in order to assess the ability of *S. maltophilia* to internalize these cells. After incubation, the supernatant was discharged and cells were washed twice with 500 µL PBS. Afterwards, 1 mg/L of levofloxacin (prepared in RPMI 1640 medium) was added to each well in order to kill adhered bacteria and incubated for 1 h. Cells were washed twice with PBS after treatment with the antibiotic. A volume of 250 µL of trypsin was added to each well for 10 min, in order to detach cells from the plate (3.5.4.7.1), followed by 250 µL of ice cold Triton X - 100 (Sigma-Aldrich, Steinheim, Germany) at 2% (v/v) in order to lise the HT29 cells and release the internalized bacteria. The bottom of each well was scrapped with a pipette to guarantee maximum cell removal. Suspensions were collected to 1.5 mL microtubes, vortexed and incubated for 10 min in order to improve homogenization and achieve complete cell lyses. The number of internalized bacteria was then assessed by plating the suspension in TSA followed by incubation for 24 h at 37 ºC.

3.6 Understanding the role of copper materials on biofilm control in plumbing systems

3.6.1 Biofilm formation

A. calcoaceticus and *S. maltophilia* were used for biofilm formation and were grown as described in Section 3.1. Afterwards, bacterial cells were harvested by centrifugation (Eppendorf centrifuge 5810R) at 3777 *g*, 15 min, and resuspended in STW. The cell density was adjusted to 10^6 CFU/ml for further experiments. Biofilms were formed in six

different materials with different content of copper (Table 3-1). Coupons were cleaned and sterilized as described in Section 3.3.2.

Single and dual species biofilms were formed on coupons of each material inserted in 24 wells microtiter plate. Six coupons of each material were inserted in 24 wells microtiter plates and bacterial suspension prepared as described in previously was added to each well. Two coupons of each material was inserted in 24 wells microtiter plates and fresh R2A broth was added to assess the efficiency of sterilization. For single species biofilms, 1 ml of bacterial suspension was added to each well. To prepare dual species biofilms, 500 µL of each bacterial suspension was added to each well in order to keep the same final bacterial concentration. Microtiter plates were then incubated at 25 ºC and 120 rpm for 24 h. After this period of incubation, two coupons of each material were removed to assess biofilm culturability. The bulk phase with bacteria in STW from the remaining coupons was replaced by new sterile STW, and incubated for additional 24 h at 25 ºC and under agitation (120 rpm). After the 48 h of biofilm formation, two additional coupons of each material were removed to assess biofilm culturability and viability. The STW from the bulk was removed and R2A was added in order to evaluate the ability of bacteria to regrowth from a sudden increase in nutrients availability. For this, coupons in R2A broth were incubated for additional 24 h at 25 ºC and 120 rpm. Three independent experiments with duplicates were performed for each condition tested.

3.6.1.1 Biofilm culturability

Culturability were assessed for 24 and 48 h biofilms. Colonized coupons were washed in 1 ml of STW in new 24 wells microtiter plates in order to remove weakly or non-adhered bacteria. Each coupon was inserted in a 50 ml centrifuge tube containing 3.5 ml of saline water (8.5 g/l of NaCl) after being scrapped with a micropipette tip while 1 ml of saline

water was dispensed on the coupon to help washing the scrapped cells (Gomes *et al*. 2018b). Then, 0.5 ml of thiosulphate (0.5% w/v) was added to the tube containing the scrapped suspension in order to neutralize the release of copper ions (Lea 2016). Tubes containing coupons were then vortexed for 2 min to complete the removal of adhered bacteria and to dissociate possible bacterial aggregates (Gomes *et al*. 2018b). Serial dilutions from the obtained suspension were then performed and plated in R2A agar (Oxoid, Hampshire, UK) plates. CFU were enumerated after incubation for 48 h at 25 ºC. For dual species biofilms, the number of CFU per cm^2 was also assessed for each bacterium. This was possible because the selected bacteria had clearly distinct colony morphologies when grown in R2A agar.

3.6.1.2 Biofilm viability

The viability of 48 h-old biofilms was assessed through the Live/Dead Bac light bacterial viability kit (Invitrogen Life Technologies, Alfagene). This kit is composed of two nucleic acid-binding stains: SYTO 9™ that penetrates all bacterial membranes and stains cells green and propidium iodide (PI) that only penetrates bacteria with damaged membranes and stains bacteria red. 500 µL of biofilm samples, ressuspended as described in 3.6.1.1, were filtered through a Nucleopore (Whatman, Middlesex) black polycarbonate membrane with 0.22 µm pore size. Filtered samples were stained with 250 µl of SYTO 9 ™ and 50 µl of PI and left in the dark for 10 min. Samples were analyzed using a LEICA DM LB2 epifluorescence microscope connected to a Leica DFC300 FX camera (Leica Microsystems Ltd, Heerbrugg). The optical filter combination for optimal viewing of the stained preparations consisted of a 515-560-nm excitation filter combined with a dichromatic mirror at 580 nm and a suppression filter at 590 nm. Viable (green stained) and non-viable (red stained) cells were assessed from counts of a minimum of

20 fields of view. The total number of cells corresponds to the sum of viable (green stained) and non – viable (red stained) cells.

Viable but non-culturable bacteria (VBNC) were determined by the difference between the viable bacteria (green stained) and the numbers of CFU grown on R2A plates for the same sample.

3.6.1.3 Biofilm regrowth

The regrowth of 48 h biofilm in terms of CFU numbers was evaluated after the replacement of STW by a medium with higher content of nutrients: R2A broth. The biofilm in R2A were incubated for additional 24 h at 25 º C under agitation (120 rpm) and regrowth was assessed in terms of culturability in R2A agar plates, sampled as described in section 3.6.1.1.

3.6.2 Reactive oxygen species

The formation of reactive oxygen species (ROS) was evaluated when *A. calcoaceticus* and *S. maltophilia* were in contact with the different surface materials for 24 h. Dichloro-dihydro-fluorescein diacetate, H_2DCFDA, (from Sigma – Aldrich, Steinheim, Germany) was used to determine ROS formation. This molecule is oxidized by ROS inside cell membrane resulting in a fluorescent molecule - 2'7'-dichlorofluorescein (Jambunathan 2010). Bacteria were grown overnight at 25 ºC and 120 rpm. Bacterial suspensions were centrifuged (3777 g for 15 min), resuspended in PBS and the bacterial concentration adjusted to 2×10^8 CFU/ml. H_2DCFDA prepared in absolute ethanol, was added to the suspensions to a final concentration of 10 µM, and incubated in the dark for 20 min. Afterwards, bacteria were washed by centrifugation (3777 g for 15 min) to remove the excess of H_2DCFDA and resuspended in STW (Dwivedi *et al*. 2014). A volume of 1 mL

of bacterial suspension was added to each well of a 24 wells microtiter plate, containing coupons of the different materials. Two controls were performed: only with STW (a negative control without bacterial growth) and with bacteria not marked with H_2DCFDA (to evaluate the possible autofluorescence of bacteria unrelated to the formation of ROS). Plates were incubated for 24 h at 25 ºC and 120 rpm. Then coupons were vortexed in 5 ml of PBS for 2 minutes and the bacteria were sampled and analyzed in a 96 well microtiter plate using a FluoStar Omega (BMG Labtech, Madrid, Spain) microtiter plate reader under fluorescence (excitation wavelength: 485 nm and emission: 530 nm).

3.6.3 Copper materials corrosion and leaching

Corrosion of copper and copper alloys was evaluated according to the standard practice for preparing, cleaning and evaluating corrosion test specimens (ASTM 1999). Coupons were cleaned as described in 3.3.2 and dried at 60 º C for 30 min. Corrosion was evaluated for 6 months in STW without chlorine and in STW with 1 mg/L of free chlorine (residual concentration commonly present in DW (WHO 2003a)). Several time points were analyzed in this period: 14 d, 21 d, 1 month, 2 months, 3 months, 4 months, 5 months and 6 months. Coupons were inserted in 12 wells microtiter plates and incubated at 25 ºC under static conditions. Two coupons were removed in each time point, sonicated for 10 min in order to remove corrosion products. Then, the remaining corrosion product were removed with HCl (39% VWR chemicals, Fontenay-sous-Bois) diluted twice in water for 2 min at 25 ºC ((ASTM 1999). The coupons were weighed after being washed with distillate water and dried. This procedure was repeated three times until complete removal of corrosion products. The corrosion rate is given by equation 3-1.

Corrosion rate $(\mathbf{CR}) = \frac{\mathbf{3.45 \times 10^6 \times W}}{\mathbf{A \times T \times D}}$ Equation 3-1

where CR is defined in mils/year, W is the mass loss in g, A is the coupon area in cm^2, T is the exposure time in hours and D is the density of the material in g/cm^3.

To assess copper leaching, bulk water was collected for each time point and copper concentration was determined by flame emission and atomic absorption spectroscopy with hollow-cathode lamp using a GBC AAS 932 plus device with GBC Avante 1.33 software.

3.7 Influence of surface copper content on biofilm control using chlorine and mechanical stress

3.7.1 Bacterium and culture conditions

S. maltophilia was selected for this study since it was more tolerant to copper than *A. calcoaceticus*. *S. maltophilia* was grown in two 1 L Erlenmeyer containing 250 mL of R2A broth and was incubated overnight at 25 º C and 120 rpm.

3.7.2 Substrate for biofilm formation

Four different substrate with different content in copper were tested: stainless steel (SS) with no copper in its composition was used as negative control, elemental copper (100% of Cu) as positive control and two alloys with 96 and 57 % of copper. Material composition is presented in Table 3-1. Three cylinders from each material with a sampling area of 39.27 cm^2 were used for biofilm formation. Cylinders were cleaned and sterilized as described in Section 3.3.2.

3.7.3 Biofilm formation

A rotating cylinder reactor (RCR) was used for biofilm formation under conditions mimicking DWDS. The RCR (Figure 3-2) was composed by a 5 L vessel where three cylinders were immersed in a bacterial suspension. The cylinders were connected with a synchronizing belt, guaranteeing that all the cylinders were continuously rotating at the same speed and direction through an overhead stirrer. The 5 L vessel was inoculated with 500 mL of bacterial suspension prepared as referred before and diluted to a final volume of 5 L in synthetic tap water (STW, prepared as described by Gomes *et al*. (2018b), to a final concentration of 2×10^7 cells/mL. The RCR operated under batch conditions for 2 h in order to promote initial bacterial adhesion. Afterwards, the RCR was continuously fed with 10 times diluted R2A broth at 0.5 L/h, ensuring a constant dilution rate of 0.1 h^{-1}. Biofilms were formed for 7 days in order to obtain mature biofilms (Simões *et al*. 2005), under constant liquid flow of 0.1 m/s on the cylinder surface (equivalent to 0.1 Pa of shear stress) - corresponding to typical water velocity in plumbing systems (Husband and Boxall 2011, Neilands *et al*. 2012, Ragain *et al*. 2019).

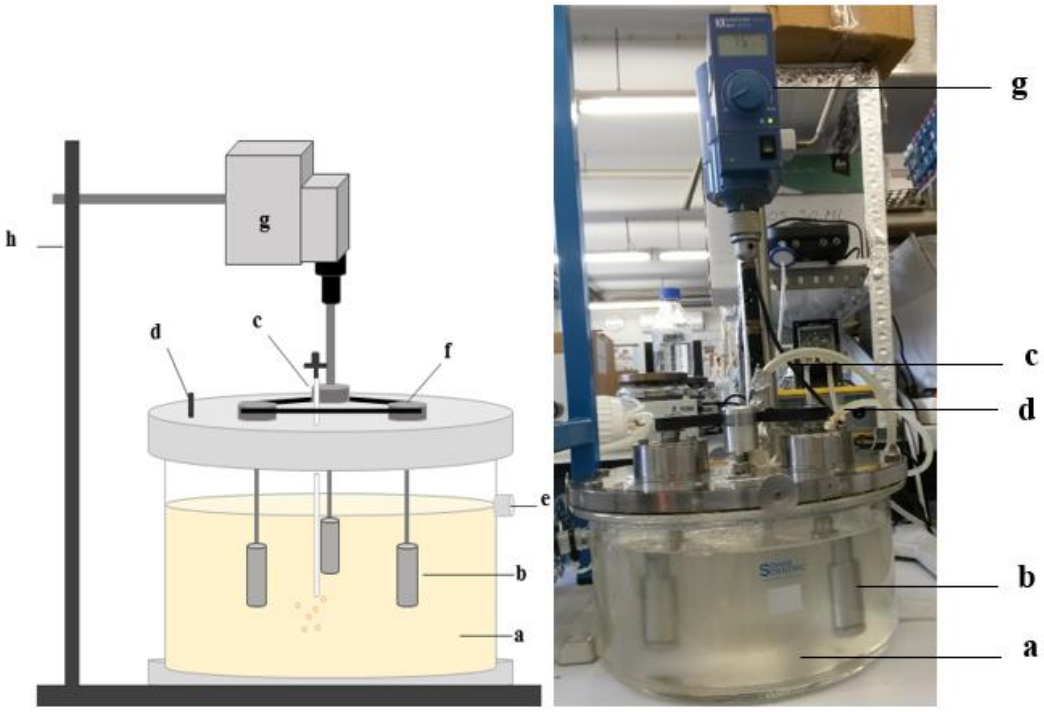

Figure 3-2. Rotating cylinder reactor representation at left and a picture of the RCR during an experiment with SS cylinders at right. a - 5 L vessel with bacteria suspension; b- rotating cylinders for biofilm formation; c – inlet of sterilized air; d – inlet of diluted R2A broth; e- outlet of residues (bacterial suspension and medium), f – Reactor lid with synchronizing belt; g – stirring overhead; h – support for stirring system.

The shear stress (τ_W) on the cylinder surface was assessed according to equation 3-2 (Altman *et al*. 2009):

$$\mathbf{f = \frac{2\tau_W}{\rho V^2}} \qquad \text{Equation 3-2}$$

f is the Fanning factor, ρ is the fluid density (kg/m^3) and *V* is the fluid velocity (m/s).

The linear velocity of fluid (m/s) on the cylinder surface is given by equation 3-3, where *N* is the rotational speed of cylinder (rpm) and *D* is the cylinder diameter (m):

$$\mathbf{V = N\pi D} \qquad \text{Equation 3-3}$$

The Fanning factor is assessed according to equation 3-4 (Gabe and Walsh 1983):

$$\mathbf{f = 0.158 Re_a^2} \qquad \text{Equation 3-4}$$

Where the Reynolds number of agitation (Re_a) is obtained from equation 3-5:

$$\mathbf{Re_a} = \frac{\mathbf{D^2 N \rho}}{\boldsymbol{\mu}}$$ Equation 3-5

μ is the fluid dynamic viscosity (kg/m.s).

After 7 d of biofilm formation each cylinder was removed from the RCR and carefully washed in saline water (0.85% of NaCl) in order to remove non-adhered bacteria. Then, one cylinder was used for biofilm characterization and the other two cylinders were exposed to one of the treatments tested: sodium hypochlorite (NaOCl) at 10 mg/L of free chlorine for 10 min; high shear stress exposure (10 Pa for 30 s – equivalent to a flushing situation), or the combination of both chemical and physical treatments. The assay was performed at least three times for each material and for each treatment, with duplicates.

3.7.4 Biofilm characterization

Biofilms formed in the RCR were characterized in terms of wet and dry mass, cell density, culturability and content of extracellular proteins and polysaccharides. The biofilms were removed from the cylinders surface using a stainless steel scraper and resuspended in 20 mL of extraction buffer (0.76 g/L $Na_3PO_4 \cdot H_2O$, 0.36 g/L $Na_2HPO_4 \cdot H_2O$, 0.53 g/L NaCl, 0.08 g/L KCl) (Frølund *et al*. 1996). The suspension was homogenized for two minutes by vortexing (VV3 model,VWR).

3.7.4.1 Biomass quantification

The wet mass was obtained by the difference between the mass of the cylinder with biofilm (before scrapping) and the mass of the cylinder without biofilm (after scrapping). The dry biofilm mass was assessed by the total volatile solids (TVS). A volume of 10 mL of the homogenized biofilm suspension was placed in crucibles and dried for 24 h at 105 ºC. After that, crucibles were weighted and placed in a furnace at 550 ºC for 2 h and

weighted again in order to assess the TVS (WEF 1989). The water content was estimated as the difference between the wet mass and the dry mass.

3.7.4.2 Cellular density

The biofilm cellular density was evaluated in terms of culturable, viable and total bacteria. To assess culturable bacteria, serial dilutions of biofilm suspension were prepared and plated in R2A agar plates. The plates were incubated at 25 ºC for 48 h before CFU enumeration. For enumeration of viable and total cells, 500 µL of biofilm suspension was filtered through a 0.22 µm black polycarbonate membrane Nucleopore® (Whatman, Middlsex, UK). The membrane was stained with Live/Dead BacLight bacterial viability kit (Invitrogen Life Technologies, Alfagene) and the procedure was performed as described in Section 3.6.1.2.

3.7.4.3 EPS quantification – proteins and polysaccharides

A biofilm suspension was used for extraction of extracellular polymeric substances (EPS) using a Dowex® Marathon © resin (Na^+ form, strongly acidic, 20–50 mesh, Sigma-Aldrich, Steinheim, Germany) at 4º C for 4 h and under constant agitation (400 rpm) (Frølund *et al*. 1996). After the extraction process, the suspension was centrifuged at 3700 g for 5 minutes in order to harvest the EPS and the supernatant was used for quantification of extracellular proteins and polysaccharides. Extracellular polysaccharides were quantified according the phenol-sulfuric method (Dubois *et al*. 1951) using glucose as standard. The quantification of extracellular proteins was performed by the Bradford microassay (Sedmak and Grossberg 1977), using bovine serum albumin as standard.

3.7.5 Biofilm treatment with free chlorine

Sodium hypochlorite from Sigma-Aldrich (Acros Organics, New Jersey, USA) was used to prepare a solution with 10 mg/L of free chlorine in STW. Free chlorine concentrations were adjusted using a photometer from Hanna Instruments using the N,N-diethyl-p-phenylenediamine (DPD) method (test kit from Hanna Instruments, Woonsocket, USA). Cylinders containing biofilms were washed in STW to remove weakly adhered cells. Then, biofilms were exposed to 200 mL of chlorine solution at 10 mg/L, for 10 minutes, in a 250 mL glass beaker, under constant rotation speed - corresponding to a linear velocity of 0.1 m/s. After exposure, the cylinders were immersed in a solution of sodium thiosuphate at 0.50% (wt/v) for 10 minutes, in order to neutralize the residual chlorine and remove the non-adhered cells. Afterwards, the biofilm was scrapped and resuspended in extraction buffer as described previously. The sample was analyzed in terms of culturability, viability and total cell number as described before. The chlorine solution used for biofilm treatment was also analyzed in term of cells viability in order to characterize the bacterial status in the bulk phase. Therefore, it was possible to evaluate the viability of biofilm released bacteria. This information is of utmost importance as these bacteria could reach the consumers tap.

3.7.6 Biofilm treatment by hydrodynamic stress

Cylinders containing biofilms were immersed in a beaker containing 200 mL of STW to remove weakly adhered cells. Then, the biofilms were transferred to 200 mL of sterile STW in a 250 mL glass beaker for mechanical treatment. For that, the rotation speed under which the biofilm was exposed was increased in order to guarantee a linear velocity on the cylinder surface of 1.5 m/s (10 Pa) for 30 s, mimicking flushing situations in

DWDS (Ellison 2003). After exposure, the cylinders were immersed in STW in order to remove non-adhered cells. The biofilm was scrapped and resuspended in extraction buffer as described previously and further analyzed in terms of culturability, viability and total cell numbers. The STW solution used for biofilm treatment was also analyzed in term of cell viability.

3.7.7 Biofilm combined treatment

Cylinders containing biofilms were also treated with the combination of both chemical and mechanical treatments. The chemical and mechanical treatments were applied sequentially, following the conditions described before. The STW used for biofilm treatment was also analyzed in term of cell viability in order to evaluated biofilm removal and cell numbers and viability in the bulk phase.

3.8 Impact of copper leachate on virulence, cytotoxicity and genotoxicity

3.8.1 Bacteria and culture conditions

S. maltophilia was selected for this study since it was more tolerant to copper than *Acinetobacter calcoaceticus. S. maltophilia* was grown overnight as described in Section 3.1. and afterwards, harvested by centrifugation (Eppendorf centrifuge 5810R) at 3777 *g*, 15 min, and resuspended in STW. The cell density was adjusted to 1×10^6 CFU/ml for further experiments. *S. maltophilia* was exposed to copper materials (Section 3.3.2) and to copper solutions with different concentrations for 24 h in 24 wells microtiter plate. Copper ($CuCl_2$) solutions were prepared in STW, 10 times concentrated. The

concentrations tested correspond to the minimum and the maximum detected in copper leachates (0.8 and 2.8 mg/L) and to the maximum value recommended for DW by EPA and WHO (1.3 and 2.0 mg/L, respectively).

3.8.2 Copper effect on virulence factors production

The impact of the exposure to copper materials and solutions on *S. maltophilia* was evaluated in terms of production of virulence factors, namely siderophores, proteases, and gelatinases, and by verifying the interference of these exposures in the motility of *S. maltophilia.*

3.8.2.1 Siderophores

The ability of *S. maltophilia* to produce siderophores was evaluated in exposed and non-exposed bacteria. Ten µL of microbial suspension from each well were plated in CAS plates prepared in R2A (in triplicates) as described in Section 3.5.4.6.

3.8.2.2 Proteases

Ten µL of the microbial suspension from each well were plated in PCA (Oxoid, Hampshire, England) with 10 g/L of skim milk powder (in triplicates). Plates were incubated for 72 h at 25 ºC. Protease producers formed a clearance zone in agar medium.

3.8.2.3 Gelatinase

Ten µL of the microbial suspension from each well were plated in gelatin agar plate composed by 5 g/L of peptone, 3 g/L of yeast extract, 30 g/L of gelatin, 15 g/L of agar and the pH 7. Plates were incubated at 25 ºC for 48 h. After this period, plates were flooded with a saturated solution of ammonium sulphate (2.84 M). In the presence of

gelatinase production, gelatin in solid medium will precipitate, therefore a transparent halo could be measured (Lopes M *et al*. 2006).

3.8.2.4 Motility

S. maltophilia motility after exposure to copper materials and solutions was evaluated as described in Section 3.5.4.5.

3.8.3 Cell culture

Human colorectal adenocarcinoma cell line HT29 (ATCC® HTB-38™) (kindly provided by the Institute for Research and Innovation in Health Sciences - I3S, Porto) were cultured in RPMI 1640 medium (Biowester, Nuaillé - France) containing 10% (v/v) fetal bovine serum (FBS) (Biowester, Nuaillé - France) and 1% (v/v) antibiotics solution (Biowester, Nuaillé - France). Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere. HT29 cells were seeded in 96 well plates at a concentration of 1.0×10^4 cells per well (for cytotoxicity assays) and in 24 well plates at concentration of 5.0×10^4 cells per well (for genotoxicity assays) obtaining confluent monolayers in 72 h.

3.8.3.1 Exposure to copper materials leachate

Leachates were obtained after the exposure of different copper materials (Section 3.3.2) to STW and to STW with chlorine (1 mg/L) for 180 days as described in 3.6.3. Solutions were autoclaved previous to be used, guaranteeing the degradation of the possible residual chlorine present in leachates.

HT29 cells were exposed to leachates from copper materials and, concurrently, to copper solutions at different concentrations (0.8, 1.3, 2.0 and 2.8 mg/L of $CuCl_2$). HT29 cell were

seeded and the exposure started when complete confluence was achieved (after 3 days of incubation). Cells were exposed to copper and to leachates for 30 minutes for 3 consecutive days. Leachates were diluted in RPMI 1640 (1:1) and copper solutions were prepared in RPMI 1640 to avoid cell death due to nutrient deprivation. Negative and positive controls were performed, RPMI 1640 with 50% of STW (v/v) was used as negative control and ethanol at 70% was used as positive control.

3.8.4 Cytotoxicity assay

After the exposure, leachates or copper solutions were discharged and 100 µL of MTT (0.5 mg/mL in RPMI 1640) were added to each well and incubated for 4 h at 37 ºC in dark conditions. After the incubation period, MTT was removed and the produced formazan was solubilized in 200 µL of DMSO. Then the absorbance was measured in a microplate reader (Cambrex ELx808, Biotek KC4) at 570 nm with a reference wavelength of 630 nm (Bessa *et al*. 2017). Three independent assays were performed with triplicates.

3.8.5 Genotoxicity assay

Trypsin-EDTA (250 µL) was added to each well of the 24 wells plate and incubated for 5 minutes at 37ºC. Then, 500 µL of PBS was added to stop trypsin activity. Cells were scrapped with the pipette tip for further cryopreservation until being used for genotoxicity assay.

Alkaline comet assay was performed to assess leachates and copper solutions genotoxicity. Cells were embedded in 100 µL of 0.6% of low-melting point (LMP) agarose. 5 µL drops were placed on microscope slides precoated with 1% of normal melting point (NMP) agarose, using a medium throughput system 12 gel comet assay

unit, Severn Biotech Ltd®. Slides with solidified agarose were immersed in a copplin jar containing lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-base, 10 M NaOH, Triton-X 100 at 1% and pH 10) for 1 h. Then, slides were immersed in electrophoresis solution (1 mM Na_2EDTA and 0.3 M NaOH at pH 13) for 40 min in order to unwind DNA. Afterwards, electrophoresis run for 20 min (1.15 V/cm). After electrophoresis, slides were immersed in cold PBS (pH 7.2) for 10 minutes and then in cold deionized water for additional 10 minutes. Then, slides were fixed in ethanol at 70 and 96% for 15 minutes each, at room temperature. Mini-gels were dried overnight for further staining with SyberGold® in TE buffer (10 mM Tris-HCl and 1 mM EDTA). Genotoxicity was evaluated through visualization in a Nikon Eclipse E400 microscope with an epifluorescence attachment and 250× magnification. Comet Assay IV Software (Perceptive Instruments) was used for the analysis and results were presented as the percentage of DNA in comet tail (% tDNA). Gels were prepared in duplicate and at least 50 cells were scored per gel.

3.9 Statistical analysis

IBM® SPSS® Statistics (Statistical Package for the Social Sciences) version 25.0 was used for the statistical analysis of data applying the one-way analysis of variance (ANOVA). The comparisons between and within experimental groups were carried out using Tukey test, which is applied for the simultaneous comparison of independent groups with homogeneity of variance and normally distributed. Statistical calculations were based on confidence level $\geq$ 95% ($P < 0.05$) which was considered statistically significant.

Chapter 4

Emerging contaminants on *Stenotrophomonas maltophilia* behaviour

The present chapter comprises the evaluation of the effects of prolonged exposure of planktonic and sessile *S. maltophilia* to trace concentrations of selected ECs on its tolerance to sodium hypochlorite and resistance to antibiotics.

4.1 Results

4.1.1 Effect of ECs on pre-established *S. maltophilia* biofilms

Figure 4-1 presents the log CFU/cm^2 of *S. maltophilia* biofilms grown on PVC coupons in the presence of ECs (alone and combined) for 26 d. The exposure to GAL slightly increased the log CFU/cm^2, even if no significant differences were observed from the other situations ($P > 0.05$). In general, no statistical significant differences were observed on *S. maltophilia* log CFU/cm^2 from the exposure to ECs at [DW] and at 100 × [DW] ($P > 0.05$).

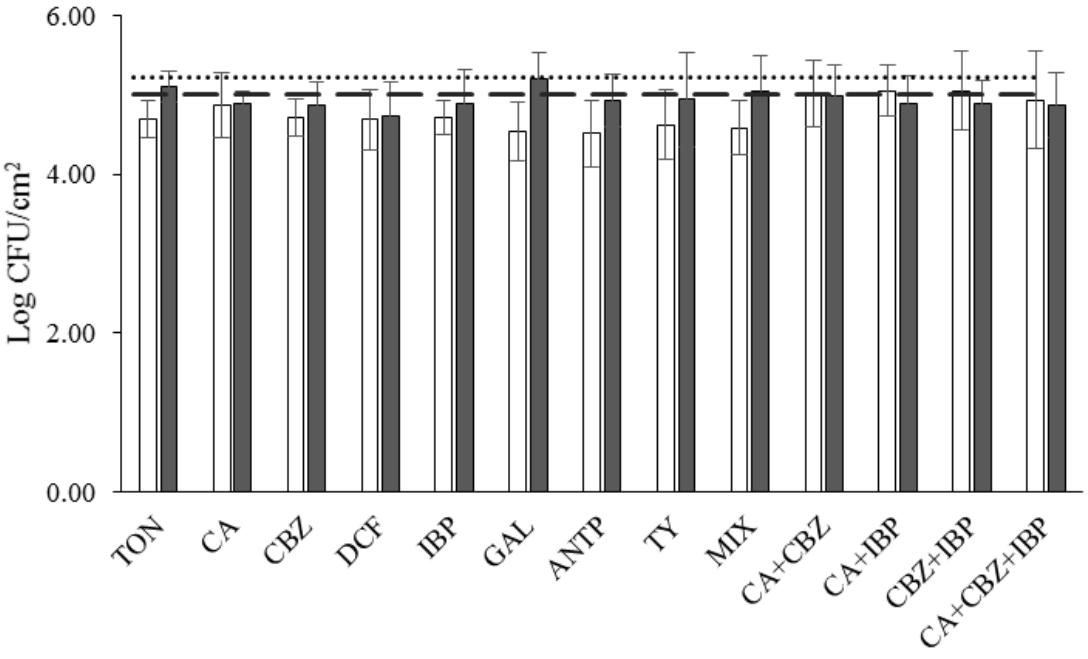

Figure 4-1. Log CFU/cm^2 of *S. maltophilia* biofilms after growing for 26 d in the presence of the selected ECs at [DW] (□) and 100 × [DW] (■). … - biofilm not exposed to ECs (only in STW), ----- Solvent control (biofilms only exposed to DMSO at 1%(v/v)). *

* Ton – tonalide, CA – clofibric acid, CBZ – carbamazepine, DCF – diclofenac, IBP – ibuprofen, GAL – galaxolide, ANTP – Antypiryne, TY – tylosin, MIX – Mixture of all compounds.

4.1.2 Effects of ECs on NaOCl action against planktonic *S. maltophilia*

Bacteria from the 26 d ECs exposed biofilms were evaluated for their susceptibility to NaOCl (Table 4-1). The MBC of NaOCl varied from 2.0 to 5.0 mg/L. The presence of 1% (v/v) of DMSO did not cause significant differences in the MBC (3.5 – 5.0 mg/L), showing that the solvent at the concentration used had no influence on *S. maltophilia* tolerance to NaOCl ($P > 0.05$). CBZ at [DW] and the combination of CBZ and IBP at [DW] caused a slight increase of MBC, however, not statistically significant ($P > 0.05$). The increase of ECs concentration to 100 × [DW] caused no significant changes in *S. maltophilia* susceptibility to NaOCl ($P > 0.05$).

4.1.3 Effects of ECs on *S. maltophilia* susceptibility to antibiotics

S. maltophilia was susceptible to the selected antibiotics (inhibition halo ≥ 1.7 cm for LEV and ≥ 1.6 cm for TMP-SMX) (Table 4-2). No significant differences in antibiotic susceptibility were observed when using *S. maltophilia* grown in the absence and presence of ECs at [DW] or 100 × [DW] ($P > 0.05$).

Table 4-1. Minimum bactericidal concentration of NaOCl (mg/L) after *S. maltophilia* exposure to ECs for 26 d.

Exposure conditions*	MBC (mg/L)	
	[DW]	100 × [DW]
STW	2.0-5.0	
DMSO	3.5-5.0	
ECs		
TON	3.0 - 6.0	3.0 - 4.5
CA	3.0 – 5.0	3.0 – 5.0
CBZ	4.5 – 6.0	2.0 - 6.0
DCF	3.0 - 5.0	3.0 – 5.0
IBP	3.0 – 5.0	3.0 – 5.0
GAL	3.0 – 5.0	3.0 – 5.0
ANTP	2.5 – 6.0	3.0 – 6.0
TY	2.0 – 5.0	3.5 – 4.0
MIX	4.0 – 5.0	3.5 – 5.0
CA+CBZ	4.0 - 5.5	4.5 – 5.0
CA+IBP	4.0 - 5.5	2.5 - 5.5
CBZ+IBP	4.0 - 6.5	2.5 - 5.5
CA+CBZ+IBP	4.0 – 5.0	3.5- 4.0*

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

Table 4-2. Inhibition halo diameter (cm) for LEV and TMP-SMX, after *S .maltophilia* exposure to ECs for 26 d.

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

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

4.1.4 Effect of ECs on *S. maltophilia* biofilm production

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

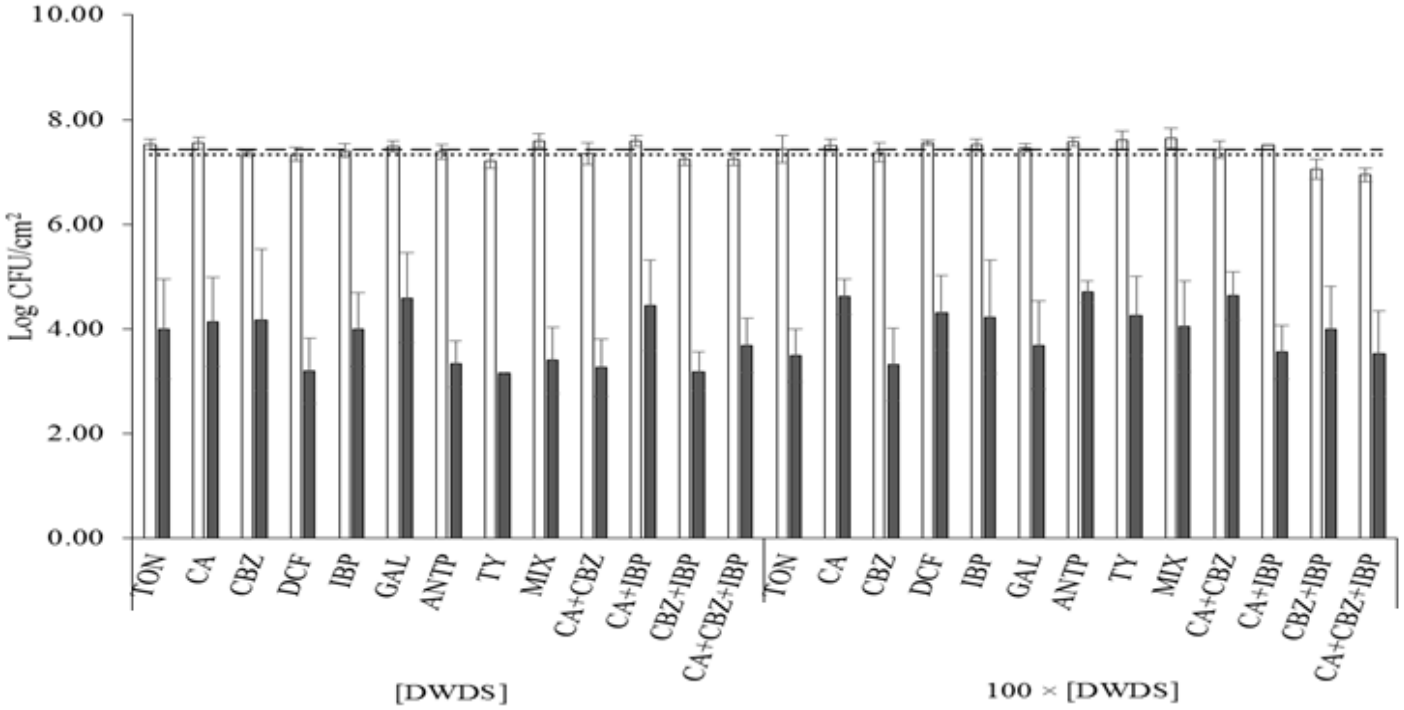

Figure 4-2. Log CFU/cm^2 of 24 h *S. maltophilia* biofilms formed after previous exposure to ECs at [DW] and 100 × [DW] for 26 d to form biofilms and treated with NaOCl at 130 mg/L for 30 min. □ – 0 mg/L of NaOCl (biofilm formation); ■ – 130 mg/L of NaOCl (biofilm inactivation); … - biofilm not exposed to ECs (only in STW), ----- Solvent control, biofilms only exposed to DMSO. *

* Ton – tonalide, CA – clofibric acid, CBZ – carbamazepine, DCF – diclofenac, IBP – ibuprofen, GAL – galaxolide, ANTP – Antypiryne, TY – tylosin, MIX – Mixture of all compounds

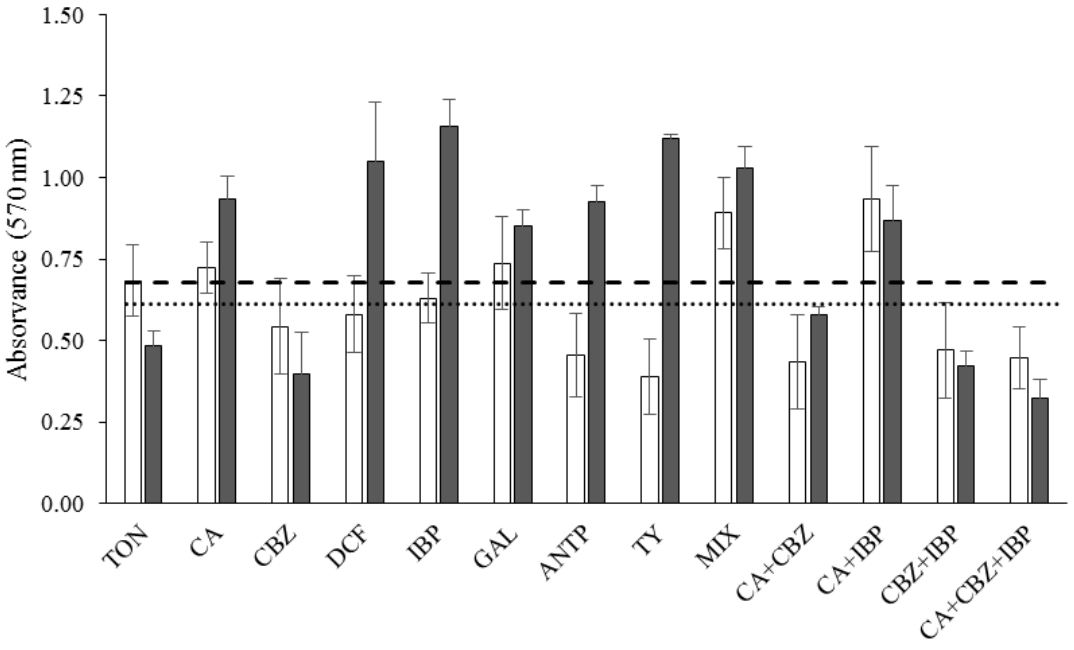

Figure 4-3. Biofilm formation for 24 h after *S. maltophilia* exposure to ECs for 26 d in terms of total biomass assessed by crystal violet staining (absorvance at 570 nm). □ – [DW]; ■ - 100 × [DW]; … - biofilm not exposed to ECs (only in STW), ----- Solvent control, biofilms only exposed to DMSO.*

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

The susceptibility of *S. maltophilia* biofilms to NaOCl was evaluated in terms of CFU (Figure 4-2) and biomass removal (Figure 4-4). The treatment with NaOCl at 130 mg/L for 30 min was not enough to completely inactivate or remove biofilms. NaOCl had no effects on biofilm CFU reduction, regardless the ECs and the concentration under which the biofilms were grown (Figure 4-2). In terms of removal, the biofilms formed by bacteria grown in the presence of CA+CBZ at [DW] and to CA+CBZ+IBP at 100 × [DW] were more resistant to removal by NaOCl ($P < 0.05$). The exposure to TY and MIX at

* Ton – tonalide, CA – clofibric acid, CBZ – carbamazepine, DCF – diclofenac, IBP – ibuprofen, GAL – galaxolide, ANTP – Antypiryne, TY – tylosin, MIX – Mixture of all compounds.

[DW] and at 100 × [DW] slightly decreased biofilm removal, although without statistical significance ($P > 0.05$).

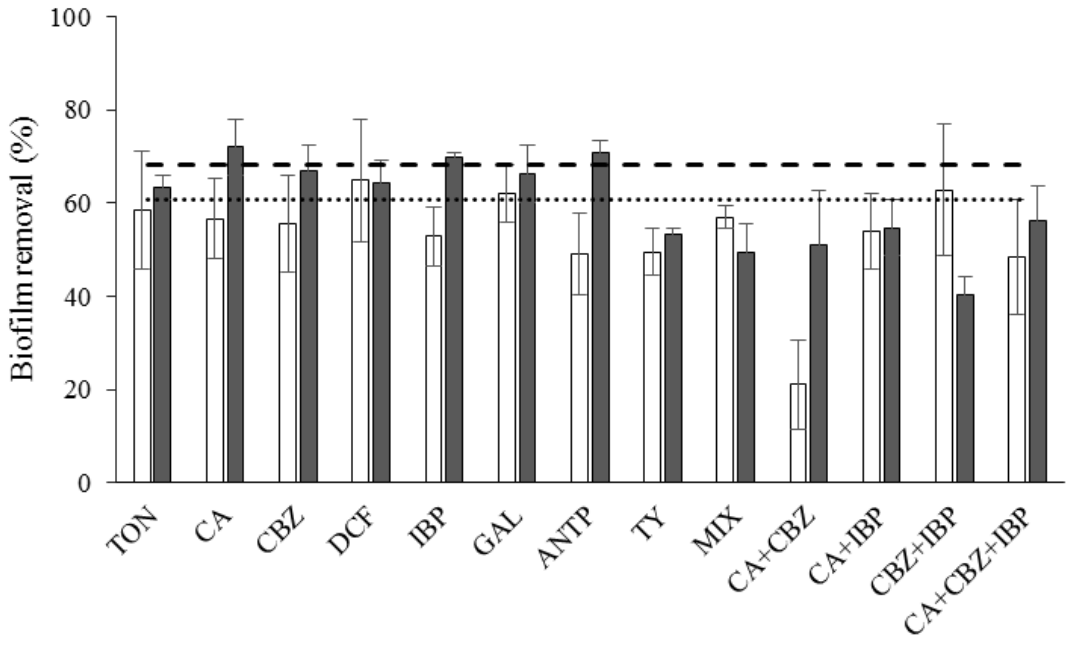

Figure 4-4. Biofilm removal after treatment with NaOCl at 130 mg/L for 30 min. □ - [DW]; ■ - 100 × [DW]; … - biofilm not exposed to ECs (only in STW), ----- Solvent control, biofilms only exposed to DMSO. *

4.2 Discussion

Recent studies have demonstrated the presence of ECs in DWDS highlighting their potential public health risks. In fact, there is the potential toxicological risk from the intake of chemically contaminated water (Schriks *et al*. 2010). However, DWDS are highly colonized by microorganisms. This is particularly relevant when looking to the surface materials of DWDS where the microorganisms can account for 95% of the cells

* Ton – tonalide, CA – clofibric acid, CBZ – carbamazepine, DCF – diclofenac, IBP – ibuprofen, GAL – galaxolide, ANTP – Antypiryne, TY – tylosin, MIX – Mixture of all compounds.

present in the system (Flemming *et al*. 2002). These cells are inevitably exposed to trace concentrations of ECs. Nevertheless, to our knowledge no studies are available on the effects of ECs on the behaviour of DW bacteria and on their susceptibility to disinfection. The study of ECs mainly focus human health and aquatic life, including the study of fluvial biofilms (Brodin *et al*. 2014, Lei *et al*. 2015, Luis *et al*. 2016). In this study, a *S. maltophilia* strain isolated from a DWDS (Simões *et al*. 2007a) was selected to assess the role of ECs on biofilm formation and antimicrobial susceptibility. *S. maltophilia* is considered an emerging pathogen and strains of this species are encountered in DW (Brooke 2012, Guyot *et al*. 2013, Vincenti *et al*. 2014).

This study demonstrates that ECs (alone and combined) at the concentrations used had no antimicrobial effects on *S. maltophilia* biofilms. Also, they had no noticeable action in stimulating biofilm development when *S. maltophilia* was inoculated with ECs (biofilm CFU counts were not affected after 26 d exposure to ECs). Nevertheless, the presence of ECs at higher concentrations in rivers was found to change the composition of biofilm communities, depending on the ECs present in the environment (Bonnineau *et al*. 2010, Corcoll *et al*. 2015, Proia *et al*. 2011, Proia *et al*. 2013b, Shaw *et al*. 2015). For example, Bonnineau *et al*. (2010)) exposed fluvial biofilms to β-blockers at different concentrations (0.9 µg/L to 9000 µg/L for propranolol and metoprolol, 0.9 µg/L to 900 000 µg/L for atenolol) and described that higher concentrations of metoprolol were toxic for bacteria and propranolol was responsible for the inhibition of algal photosynthesis. On the other hand, Proia *et al*. (2013b)) studied the effect of polluted and highly polluted water on fluvial biofilms and concluded that the presence of high concentrations of ibuprofen and paracetamol in river waters may be responsible for a decrease in algal photosynthetic capacity. Other studies reported bacterial death on fluvial biofilms caused mainly by the presence of antimicrobial contaminants, particularly antibiotics (erythromycin,

trimethoprim and clindamycin) (Waiser *et al*. 2016) and triclosan (Proia *et al*. 2011, Ricart *et al*. 2010, Shaw *et al*. 2015, Waiser *et al*. 2016). Also, Osorio *et al*. (2014) observed a decrease of bacterial viability when biofilms were translocated from a less to a more polluted site of the river. Therefore, biofilms can be an important way to evaluate the impact of ECs on water systems (Aubertheau *et al*. 2017). Nevertheless, it is important to mention that fluvial biofilms are significantly different from DWDS biofilms, as they are composed by algae, bacteria, protozoa, cyanobacteria and fungi and are not formed in the presence of chronic residual concentrations of disinfectant (Corcoll *et al*. 2015). These differences limit any accurate comparison between the results obtained in the present study and the available literature.

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

DWDS are a recognized pool of antibiotic resistant bacteria and their genes (Bergeron *et al*. 2015, Schwartz *et al*. 2003b, Xi *et al*. 2009, Xu *et al*. 2016). In this study, LEV and TMP-SMX were tested on their antimicrobial action against cells of *S. maltophilia* obtained from biofilms grown in the presence for ECs for 26 d. The development of antibiotic resistance by bacteria found in aquatic environments exposed to trace levels of antibiotics has been already reported (Baquero *et al*. 2008, Hong *et al*. 2013, Martinez 2009). More recently,Subirats *et al*. (2017) and Subirats *et al*. (2018) demonstrated that water sources polluted with ECs are responsible for bacterial antibiotic resistance in stream biofilms. However, reduced information is available about the possible effects of non-antibiotic compounds on bacterial tolerance to antibiotics. In the present study, trace levels of TY (a veterinary antibiotic) did not cause significant changes on *S. maltophilia* tolerance to LEV and to TMP-SMX. It is important to highlight that studies correlating the exposure to antibiotics in the environment with the spread of resistance used antibiotics at least at 1 µg/L (Bengtsson-Palme *et al*. 2016, Henderson-Begg *et al*. 2006, Jutkina *et al*. 2016, López and Blázquez 2009, Lundstrom *et al*. 2016), which is a concentration significantly higher than those tested in the present work (0.17 to 10.7 µg/L for TY - the only antimicrobial EC tested). Also, the short-term exposure of *S. maltophilia* to non-antibiotic compounds did not alter significantly bacterial tolerance to LEV and TMP-SMX.

The exposure to ECs at [DW] did not alter the ability of *S. maltophilia* to form biofilms in a significant manner. Nevertheless, the exposure to TY, MIX and CA+CBZ at [DW] led to the formation of biofilms more tolerant to NaOCl. The exposure to higher concentrations of ECs (DCF, TY and CA+CBZ+IBP at 100 × [DW]) altered biofilm formation. After being exposed to CA+CBZ+IBP at 100 × [DW] *S. maltophilia* formed lower amounts of biofilm. Moreover, this biofilm was more tolerant to NaOCl. On the

other hand, the exposure to DCF and TY at 100 × [DW] increased *S. maltophilia* biofilm production. The biofilms formed after exposure to TY and CA+CBZ and MIX were also more tolerant to NaOCl. It is known that some compounds, even when not specific to target bacteria may change bacterial behaviour, as happened in the current study. In fact, previous works reported that NSAIDs such as DCF and IBP are responsible for changes in the gut microbiome (Guslandi 2012, Rogers and Aronoff 2016). Cycoń *et al*. (2016) also found that NSAIDs are responsible for biochemical and microbiological changes in soil.

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

4.3 Conclusions

The presence of ECs in DWDS can constitute a cause of concern for consumers and DW companies regarding their effects on the behaviour of the DW-colonizing microbiota. In this study, it was found no clear evidence of the exposure to ECs and changes in planktonic *S. maltophilia* susceptibility to NaOCl and antibiotics. Nevertheless, some ECs (DCF, IBP, TY and CA+CBZ+IBP at 100 × [DW]) were responsible for changes in

S. maltophilia ability to form biofilms and on their tolerance to NaOCl (CA+CBZ at [DW] and CA+CBZ+IBP at 100 × [DW]). Therefore, the simultaneous presence of different compounds, even if at trace concentrations, altered *S. maltophilia* biofilm behaviour and can potentially hinder the disinfection of biofilms in DWDS.

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Chapter 5

Clofibric acid effects on *Stenotrophomonas maltophilia* virulence and tolerance to antimicrobials

The motivation for this chapter is based on the principles of the "One Health" initiative and encloses the effects of clofibric acid (CA), a lipid regulator, on the behaviour of a selected bacterium isolated from DW.

5.1 Results

5.1.1 Characterization of *S. maltophilia* from biofilms exposed to CA

S. maltophilia cells were isolated from biofilms exposed to CA during 12 weeks. These cells were characterized in terms of susceptibility to chlorine (Section 5.1.2), susceptibility to nine antibiotics (AMO, CIP, ERY, KAN, LEV, OXA, SPE, TET, TMP-SMX - Section 5.1.3) using planktonic cells. Biofilm formation ability and susceptibility to chlorine (Section 5.1.4) were further assessed. The effects of CA were also evaluated on bacterial motility (Section 5.1.5), siderophores production (Section 5.1.6) and on the ability of *S. maltophilia* to adhere and internalize the human colorectal adenocarcinoma cell line HT29 (Section 5.1.7).

5.1.2 Effects of CA exposure on *S. maltophilia* susceptibility to chlorine

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

5.1.3 Effects of CA exposure on *S. maltophilia* susceptibility to antibiotics

S. maltophilia isolated from 12 weeks biofilms was resistant to AMO, OXA and SPE, regardless the conditions under which biofilms were exposed (Table 5-1). The exposure to CA did not alter bacterial susceptibility to these antibiotics ($P > 0.05$). *S. maltophilia* was susceptible to CIP, KAN, LEV, TET and TMP-SMX, when bacteria obtained from biofilms exposed to STW, DMSO and CA at [DW] and at 100 × [DW] were used. *S. maltophilia* had intermediate susceptibility to ERY. Bacteria obtained from biofilms exposed to CA at 100 × [DW] had higher tolerance to this antibiotic ($P < 0.05$). On the other hand, exposure to CA at 100 × [DW] increased susceptibility to CIP ($P < 0.05$).

Table 5-1. Zone of inhibition (mm) for the selected antibiotics. Mean values and the corresponding standard deviation are presented.

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

[†] AMO- amoxicillin, CIP- ciprofloxacin, ERY – erythromycin, KAN- kanamycin, LEV – levofloxacin, OXA – oxacylin, SPE- Spectinomycin, TET – tetracycline, TMP – SMX– trimethoprim-sulfamethoxazole.

[**] Zone of inhibition statistically different from those obtained after exposure to the solvent (DMSO) – $P < 0.05$.

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

S. maltophilia from CA-exposed biofilms were used to assess their ability to form biofilms and the susceptibility of these biofilms to chlorine was assessed in terms of total biomass (crystal violet method –Figure 5-1) and culturability (log CFU/cm^2 – Figure 5-2). It was found that exposure to 1% DMSO (solvent control) for 12 weeks increased *S. maltophilia* ability to form biofilms on PS compared to *S. maltophilia* exposed to STW for 12 weeks ($P < 0.05$). However, exposure to CA at [DW] and 100 × [DW] did not cause significant changes in biofilm formation when compared to the solvent control ($P > 0.05$) (Figure 5-1.A).

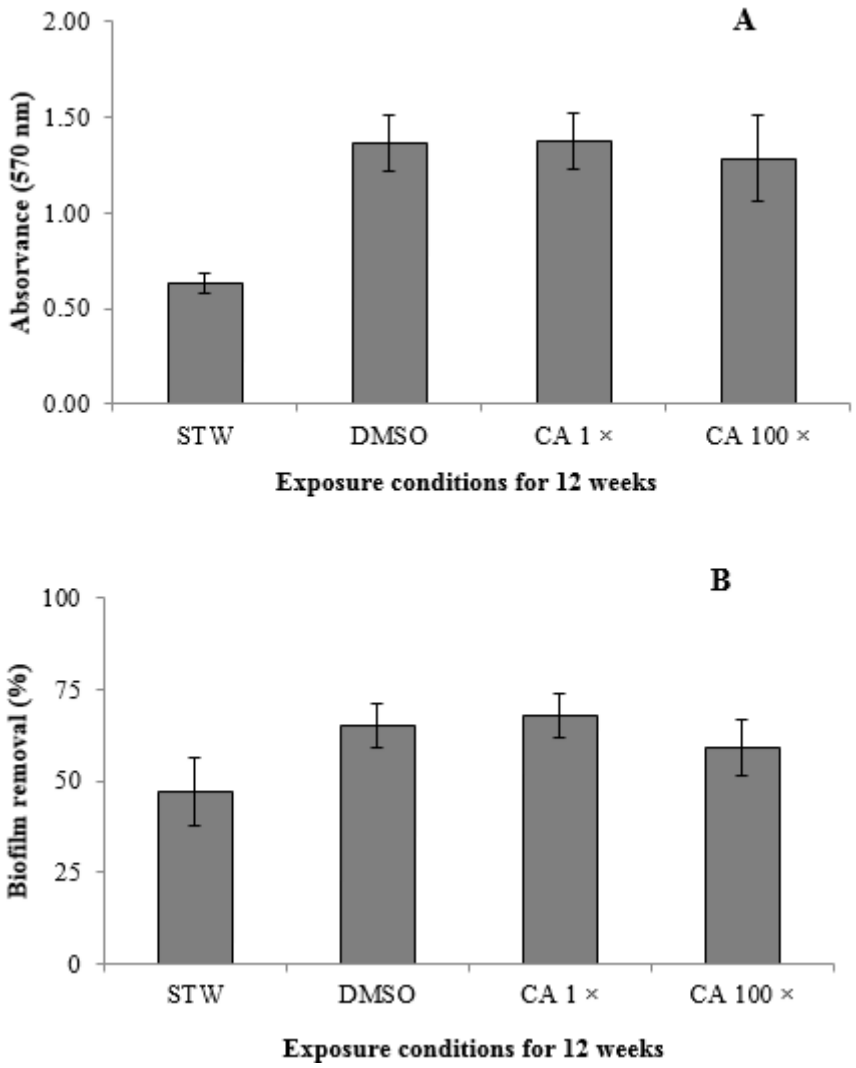

Figure 5-1. . Biofilm formation for 24 h after *S. maltophilia* exposure to STW, DMSO and CA at [DW] and 100 × [DW] for 12 weeks in terms of total biomass as assessed by crystal violet staining (absorvance at 570 nm) – A. Biofilm removal after treatment with free chlorine (130 mg/L) for 30 min in terms of total biomass – B.

Complete biofilm removal was not obtained after treatment with 130 mg/L of free chlorine for 30 min (Figure 5-1.B). Biofilms formed after exposure to DMSO were removed in a higher extent (65% biofilm removal) than biofilms formed after exposure to STW (47% biofilm removal). *S. maltophilia* exposed to CA at [DW] formed biofilms with similar susceptibility to these obtained from *S. maltophilia* exposed to DMSO ($P > 0.05$). Although, statistically significant differences were not observed, the biofilms

formed after *S. maltophilia* exposure to CA at 100 × [DW] showed a decrease in removal from chlorine exposure.

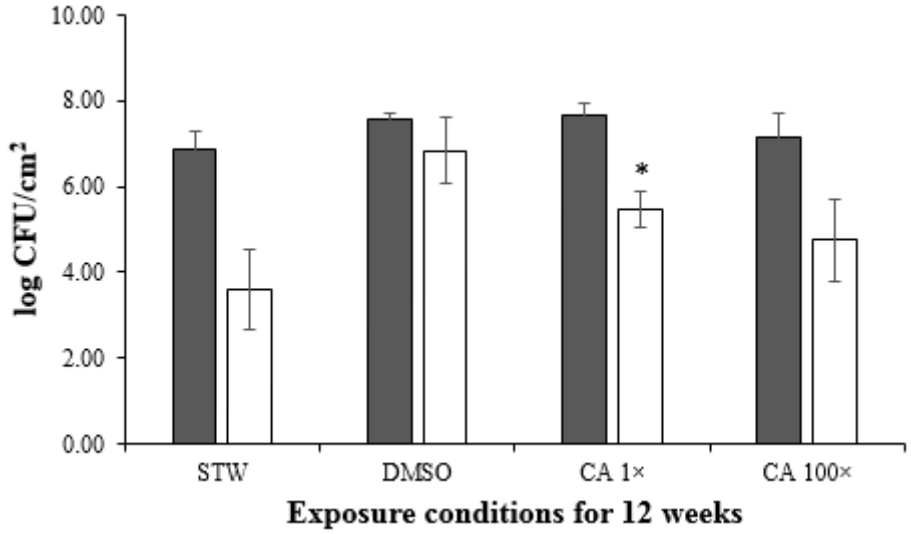

Figure 5-2. The effects of the exposure to STW, DMSO at 1%, CA at [DW] and 100 × [DW] for 12 weeks on the ability of *S. maltophilia* to form biofilms, with data presented as log CFU/cm^2 (■). Biofilm susceptibility to free chlorine at 130 mg/l for 30 min, with data presented as log CFU/cm^2 reduction (□).* - mean values statistically different from those obtained after exposure to the solvent (DMSO) – $P < 0.05$.

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

5.1.5 Effects of CA exposure on *S. maltophilia* motility

The exposure to CA at both concentrations tested did not cause significant changes (Table 5-2) in *S. maltophilia* swimming and twitching motilities ($P > 0.05$). The previous exposure to CA at [DW] slightly increased *S. maltophilia* swarming motility, even if not statistically significant ($P > 0.05$).

Table 5-2. Motility of *S. maltophilia* from biofilms grown in the presence of CA at [DW], 100×[DW], DMSO and STW. Motility halos were measured 24 h after incubation. Diameter of the initial drop was 7.2 ± 0.4 mm.

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

5.1.6 Effects of CA exposure on *S. maltophilia* siderophores production

S. maltophilia was found to be able to produce siderophores for iron acquision when this metal is limiting in the environment (Table 5-3). The results further demonstrate that the diameter of the orange halo formed around the growing colony was very similar for all the CA-exposed cells and the control ($P > 0.05$).

Table 5-3. Siderophores production after exposure to CA. Diameter of the orange halo around the grown colony (mm).

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

5.1.7 Effects of CA exposure on *S. maltophilia* ability to adhere and internalize HT29 cells

S. maltophilia demonstrated to be able to adhere and internalize HT29 cells (Figure 5-3). The exposure to CA did not affect the ability of *S. maltophilia* to adhere on HT29 cells. For both CA concentrations 5.19 – 5.29 log CFU of *S. maltophilia* was able to adhere ($P > 0.05$). Following adhesion, *S. maltophilia* was able to invade HT29 cells. However, internalization was significantly lower for *S. maltophilia* obtained from CA-exposed biofilms ($P < 0.05$) (Figure 5-3).

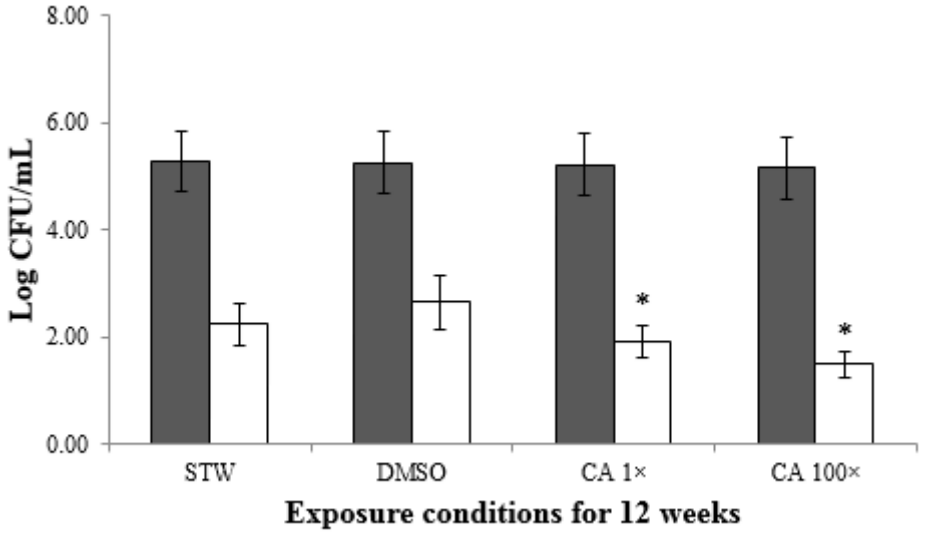

Figure 5-3. Effects of CA exposure on *S. maltophilia* interaction with HT - 29 cells. ■ - *S. maltophilia* adhesion on HT29 cells; □ - *S. maltophilia* internalization in HT29 cells. * - mean values statistically different from those obtained after the exposure to the solvent (DMSO) – $P < 0.05$.

5.2 Discussion

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

CA is a metabolite of clofibrate, etofibrate, and etofyllinclofibrate, pharmaceuticals commonly used as lipid regulators (Stumpf *et al*. 1999). CA is also an isomer of the herbicide mecoprop (Buser *et al*. 1998) and was the first pharmaceutical detected in the environment as contaminant (Evangelista *et al*. 2010). Although it has been discontinued as pharmaceutical and new drugs have been used to replace it, this molecule is still a

concern, due to its widespread occurrence and its environmental persistence (Emblidge and DeLorenzo 2006). CA was selected for this study based on previous results (Gomes *et al*. 2018a) on ECs effect on bacteria, where CA was found to be present in all the combinations of ECs causing changes in bacterial behaviour. For instance, *S. maltophilia* exposure to CA at 1700 ng/L or to the combination of CA (170 ng/L) and ibuprofen - IBP (3 ng/L) increased biofilm formation ability. On the other hand the exposure to the combination of CA (1700 ng/L) with carbamazepine (CBZ at 2580 ng/L) and IBP (30 ng/L) reduced biofilm productivity (Gomes *et al*. 2018b). The biofilms formed after exposure to the combination of CA (170 ng/L) and CBZ (258 ng/L) or CA (1700 ng/L), CBZ (2580 ng/L) and IBP (30 ng/L) were also more tolerant to disinfection with chlorine (Gomes *et al*. 2018b). Based on these results (Gomes *et al*. (2018b) and on CA environmental persistence (Schmidt *et al*. 2017), this EC was selected for the present study.

The tolerance of planktonic *S. maltophilia* to free chlorine was not affected from CA exposure. Concentrations of free chlorine, 2 – 4 mg/L, slightly higher than those recommended for chlorinated DWDS (0.5 – 1 mg/L) (WHO 2011a) were needed to cause bactericidal effects. Therefore, *S. maltophilia* may be able to survive in chlorinated systems, and other works already described the presence of *S. maltophilia* in chlorinated DWDS (Amoli *et al*. 2017a, Silbaq 2009). The use of chlorine is a widespread strategy used to avoid bacterial growth along DWDS. However, it is not completely efficient and the presence of ARBs has been consistently reported (Li and Gu 2018, Xi *et al*. 2009). The use of sub-inhibitory concentrations of disinfectants is known to be responsible for the increase of incidence of ARBs and ARGs in DWDS (Li and Gu 2018). Previous studies propose that the presence of antibiotic residues in the environment are responsible for the selection and spread of ARBs and ARGs (Baquero *et al*. 2008, Martinez 2009, Xie

et al. 2019). However, information on the effects of non-antibiotic contaminants on bacterial resistance to antimicrobials is scarce. Subirats *et al*. (2017) studied the effects of contaminated wastewater on the resistome of biofilms. Using a variety of contaminants they found that biofilms exposed to wastewater had higher levels of ARBs and ARGs than biofilms exposed to treated wastewater. Wang *et al*. (2019a) also verified that exposure to ciprofloxacin and sulfadiazine promoted ARGs spread in drinking water biofilms. The present results demonstrated that *S. maltophilia* was resistant to AMO, OXA and SPE, even without being exposed to CA. *S. maltophilia* is commonly described as an opportunistic pathogen with intrinsic multidrug-resistance (Adegoke *et al*. 2017, Rizek *et al*. 2018, Sánchez 2015, Zhang *et al*. 2000). However, it is also known that *S. maltophilia* can acquire resistance (Furlan *et al*. 2018, Rizek *et al*. 2018, Song *et al*. 2010). This study demonstrates that exposure to CA at 100 × [DW] increased *S. maltophilia* tolerance to ERY. Some works described that *S. maltophilia* strains may use efflux pump systems to pump out ERY such as the SmDEF, SmeOP, EmrCABsm and MexCD-OprJ, and in some cases the influx of this antibiotic through the bacterial membrane is not favored due to the reduced permeability of the outer membrane (Adegoke *et al*. 2017, Alonso *et al*. 2000, Chang *et al*. 2015, Safdar and Rolston 2007). It is possible CA exposure may increase the activity of some *S. maltophilia* efflux systems or alter bacteria membrane permeability, therefore increase tolerance to ERY. Balagué and Véscovi (2001) demonstrated that exposure to CA, ethacrynic acid (EA) and 2,4-dichlorophenoxyacetic acid (2,4-D) altered the outer membrane permeability of *Escherichia coli*. *E. coli* exposure to these aryloxoalcanoic agents - CA, EA and 2,4-D – caused the induction of the MarAB regulatory system (Balagué and Véscovi 2001). Balague *et al*. (2006) further studied the effects from exposure of *E. coli* to CA, EA and 2,4-D (three structurally analogue compounds) on ERY susceptibility. No significant

effects from CA exposure were observed on the antibiotic action of ERY. On the other hand, *E. coli* was more tolerant to ERY when exposed to the other two CA analogs. It is important to emphasize that the concentrations used in that study are the therapeutic doses (CA – 28.6 mg/kg/d, EA – 2.9 mg/kg/d and 2,4-D – 70 mg/kg/d) which are significantly higher than the trace concentrations detected in environment (170 - 17000 ng/L). TMP-SMX is the most common therapeutic option used to treat *S. maltophilia* infections (Emblidge and DeLorenzo 2006, Sánchez and Martínez 2015) and tolerance to this combination of antibiotics was not observed despite the exposure of *S. maltophilia* to CA. CA had no remarkable effects on *S. maltophilia* ability to form biofilms. Significant changes in biofilm formation ability were observed after exposure to DMSO, particularly the increase in biofilm formation. Lim *et al*. (2012) also observed an increase in *E. coli* biofilm formation after exposure to 4% DMSO. This emphasizes the importance of adequate control testing for a reliable analysis of results. Biofilms formed by *S. maltophilia* exposed to CA were more tolerant to chlorine action. The changes in biofilm tolerance to chlorine were mostly in bacterial inactivation, and not in biofilm removal. This altered tolerance can result from changes on the bacterial membrane structure or associated proteins caused by the prolonged exposure to CA, as proposed by Balague *et al*. (2006). Therefore, it is important to take into account that prolonged exposure of DW bacteria to CA may hinder DW disinfection with chlorine. For a shorter exposure to CA (26 d), Gomes *et al*. (2018a) did not observe changes in biofilm susceptibility to chlorine. Siderophores are molecules produced by microorganisms whose main function is to chelate the ferric iron (Fe^{3+}) from different environments making it available for the producer microorganism. Siderophores production is considered a virulence factor as during the infectious process microbes secrete siderophores to acquire and solubilize ferric iron from the host (Wilson *et al*. 2016). Iron is essential to host cells, being regulated

by iron transport systems. Nevertheless, the affinity of bacterial siderophores to iron are generally much higher than those of host proteins/molecules, allowing pathogens to outcompete the host in iron acquisition (Wilson *et al*. 2016). This study shows that *S. maltophilia* produces siderophores regardless the conditions tested. Nas and Cianciotto (2017) also described the production of siderophores by *S. maltophilia*, suggesting a unique siderophore structure for iron acquisition for this species. However, no changes in siderophores production were observed from CA exposure.

S. maltophilia motility was also studied in the present work as it is an important factor affecting bacterial ability to form biofilms as well as the interaction with host cells (Trifonova and Strateva 2018). In pathogens, motility and virulence are often linked by a complex regulatory system (Josenhans and Suerbaum 2002). It is known that the ability of *S. maltophilia* to adhere on host tissues and infect host cells is related to motility (Adegoke *et al*. 2017, Trifonova and Strateva 2018). Swimming motility is usually related to the ability of bacteria to move in liquid medium due to the presence of functional flagella (Ha *et al*. 2014). Swarming is related to the flagellum-mediated movement of bacteria, responsible for biofilm spread over a surface (Vanderleyden *et al*. 2004). In this study, the modest increase in swarming motility caused by CA did not cause changes in biofilm formation. Twitching motility is mediated by type IV pilli, a polar fimbriae responsible for bacterial adhesion on eukaryotic cells and abiotic surfaces, also allowing its movement over surfaces (Deziel *et al*. 2001). The presence of fimbriae is a virulence factor described for *S. maltophilia* - essential for colonization of the host and crucial in the infection process (Trifonova and Strateva 2018). The results demonstrated no significant changes in bacterial twitching motility after CA exposure. Therefore, it is possible to predict that *S. maltophilia* adhesion on intestinal cells may not be influenced by CA exposure. In fact, the results corroborate this prediction and no significant effect

was observed on the ability of CA exposed *S. maltophilia* to adhere on HT29 cells. CA exposure slightly decreased *S. maltophilia* ability to internalize HT29 cells. This can be attributed to changes in *S. maltophilia* invasion mechanisms caused by CA. Two mechanisms of bacterial invasion of hosts cells are recognized: the zipper mechanism of cell invasion (outer membrane proteins of bacteria will interact with the host membrane and force the cytoskeletal rearrangement for invasion); the trigger mechanism (is a consequence of the production of effectors that will rearrange the host cytoskeletal structure enabling invasion) (Ribet and Cossart 2015). Balague *et al*. (2004) observed that CA was responsible for a decrease in the expression of membrane proteins and fimbriae in *E. coli*, with significant impact on bacterial ability to invade host cells.

5.3 Conclusions

The presence of ECs in DW is a problem of potential environmental and public health concern. Their impacts on the DW microbiome and as drivers affecting resistance evolution/selection is far from being understood. This is particularly critical for non-antibiotic drugs. The present work showed that the presence of trace concentrations of CA did not affect planktonic *S. maltophilia* tolerance to chlorine. Nevertheless, the presence of CA in DW was responsible for the formation of biofilms with higher tolerance to chlorine disinfection. *S. maltophilia* ability to produce virulence factors and the ability to form biofilms was also not altered from CA exposure. Of additional concern was the changes observed in *S. maltophilia* susceptibility to antibiotics. After exposure to CA at 17000 ng/L *S. maltophilia* was more tolerant to ERY. Moreover, changes were observed on the ability of *S. maltophilia* to internalize HT29 cells. In general, the results propose that CA influenced *S. maltophilia* behaviour. It is important to highlight that CA exposure was carried out for 12 weeks - real environmental conditions where accumulation and

exposure periods higher than 12 weeks can occur will certainly increase the impact of CA on *S. maltophilia* planktonic and biofilm behaviour.

Chapter 6

Understanding the role of copper materials on biofilm control in plumbing systems

In this chapter the ability of selected copper alloys in biofilm control is investigated, without disregarding the mode of antimicrobial action (oxidative stress and membrane damage), leaching and corrosion phenomena.

6.1 Results

6.1.1 Copper effect on single and dual species biofilms

6.1.1.1 Culturable bacteria

Biofilm formation was found in all the surface materials tested. However, copper and copper alloys decreased significantly the culturability of single and dual species biofilms ($P < 0.05$). *S. maltophilia* in single species biofilms (Figure 6-1B) were more tolerant to copper contact than *A. calcoaceticus* (Figure 6-1A). In fact, no CFU of *A. calcoaceticus* single species biofilms were found on copper materials after 24 and 48 h incubation (Figure 6-1A). On the other hand, significant reductions (> 3.5 log CFU/cm^2) were observed in *S. maltophilia* culturability for 24 h-old biofilms formed on copper alloys ($P < 0.05$). Nevertheless, after 48 h it was observed increased *S. maltophilia* biofilm control from the use of the copper materials. Alloys with 57 and 79% copper completely reduced the culturability of 48 h-old *S. maltophilia* single species biofilms. All the other copper materials had similar ability to reduce culturability of 48 h-old *S. maltophilia* single species biofilms (> 3.9 log CFU/cm^2) ($P > 0.05$).

Regarding dual species biofilms (Figure 6-1C-D), all copper surfaces were very effective in reducing 24 and 48 h-old *A. calcoaceticus* culturability (> 5.6 log CFU/cm^2). For *S. maltophia,* all reductions obtained were > 5 log CFU/cm^2. The exception was the alloy with 79% copper that reduced *S. maltophilia* culturability from 24 h-old dual species biofilms in 3.93 log CFU/cm^2. Also, in dual species biofilms it was possible to observe an overtime increase of copper efficiency. Higher reductions of *S. maltophilia* culturability were observed after contact with copper alloys for 48 h.

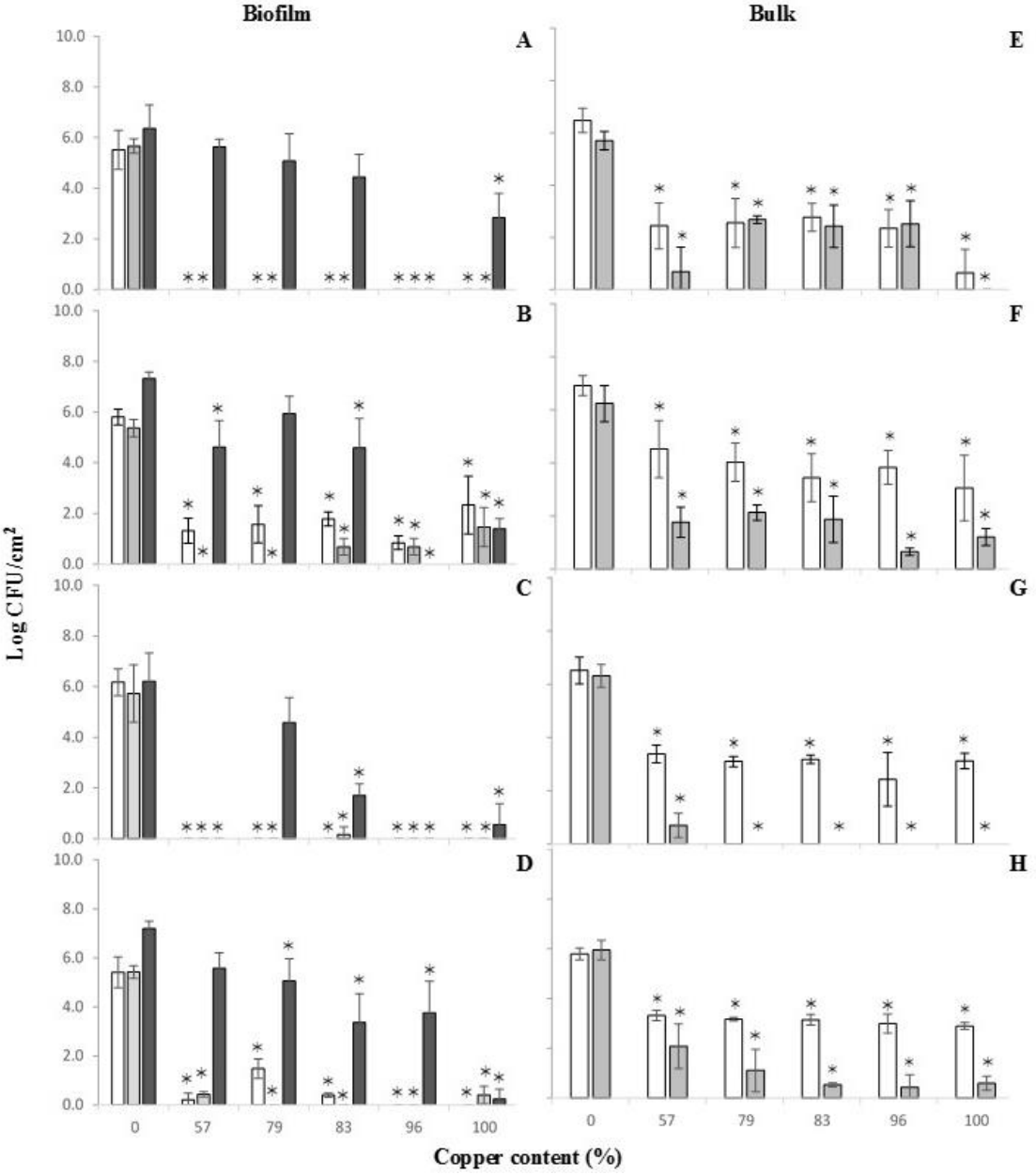

Figure 6-1. Log CFU/cm^2 of biofilms formed on surfaces with different copper content (0, 57, 79, 83, 96 and 100 % Cu) and the corresponding bulk phase culturability. □ – 24 h biofilms formed using STW, ■ – 48 h biofilm formed using STW, ■ – biofilm regrowth in the presence of high nutrient levels (R2A broth). Left figures (A, B, C and D) correspond to biofilm culturability. Right figures (E, F, G and H) correspond to bulk phase culturability. * - Differences statistically significant ($P < 0.05$) in comparison to SS (0% of copper). *A. calcoaceticus* in single species biofilms (A), *S. maltophilia* in single species biofilms (B), *A. calcoaceticus* in dual species biofilms (C), *S. maltophilia* in dual species biofilms (D), *A. calcoaceticus* present in bulk phase from single species biofilms (E), *S. maltophilia* present in bulk phase from single species biofilms (F), *A. calcoaceticus* in bulk phase from dual species biofilms (G) and *S. maltophilia* in bulk phase from dual species biofilms (H).

6.1.1.2 Viable and total bacteria

The viability of 48 h-old biofilms grown on the copper alloys was further characterized, in addition to the total number of adhered cells. The total number of adhered *A. calcoaceticus* in single species biofilms slightly decreased on copper materials ($P < 0.05$) (Figure 6-2A). Viable *A. calcoaceticus* was observed in all the biofilms formed. However, all the copper materials significantly reduced the number of viable *A. calcoaceticus* compared to SS ($P < 0.05$) (Figure 6-2A). The lowest reduction (2.08 log cells/cm^2) was obtained with the 57% copper alloy ($P < 0.05$). On the other hand, the highest reduction of *A. calcoaceticus* viability (4.68 log cells/cm^2) was obtained using the 79% copper alloy.

The total number of *S. maltophilia* in 48 h-old single species biofilms was not affected by the surface material used (Figure 6-2B) ($P > 0.05$). Nevertheless, reductions of viable cells were observed for all the copper materials, even if at a lower extent than the observed for *A. calcoaceticus* ($P < 0.05$). The higher reductions were obtained with 57% copper alloy (2.29 log cells/cm^2) and elemental copper (100% Cu) (2.08 log cells/cm^2) ($P < 0.05$).

A slight decrease in the number of total adhered bacteria caused by copper materials was observed for *A. calcoaceticus* in 48 h-old dual species biofilms (Figure 6-2C) ($P < 0.05$). The exception was the 57% copper alloy ($P > 0.05$). High viability reductions of *A. calcoaceticus* in dual species biofilms (> 5.2 log cells/cm^2) were obtained for all copper materials tested.

The total number of *S. maltophilia* in dual species biofilms was slightly reduced when the copper surface materials were used (Figure 6-2D) ($P < 0.05$). *S. maltophilia* in dual species biofilms was also more susceptible than in single species biofilms ($P < 0.05$). The higher reduction in viable *S. maltophilia* was obtained using 96% copper alloy (5.31 log

cells/cm^2) (P < 0.05), followed by alloys with 83, 79, 100 and 57% copper, with reductions of 4.01, 3.94, 3.82 and 3.16 log cells/cm^2, respectively.

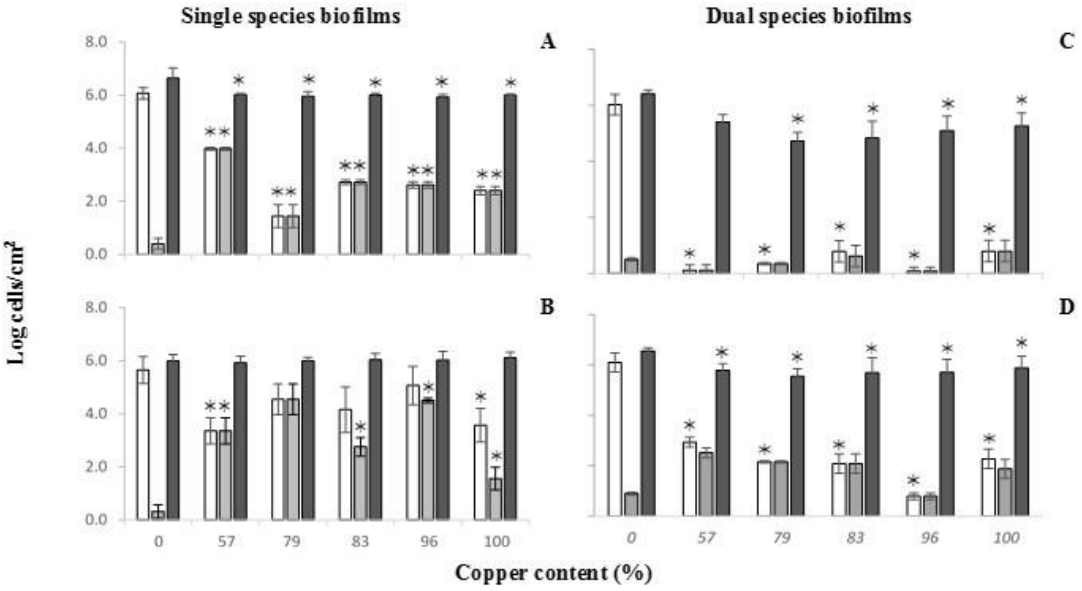

Figure 6-2. Viability (log cells/cm^2) of 48 h biofilms formed on surface materials with different copper content (0, 57, 79, 83, 96 and 100 % Cu). □ – viable cells (VC), ■ – viable but non-culturable (VBNC) bacteria, ■ –Total cells (TC). Figures A and C correspond to *A. calcoaceticus* data. Figures B and D correspond to *S. maltophilia* data. * - Differences statistically significant (P < 0.05) in comparison with SS (0% copper).

6.1.1.3 Viable but-non culturable bacteria

The presence of VBNC bacteria was determined from the difference between viable cells and CFU. In general, copper surface materials increased the numbers of VBNC of *A. calcoaceticus* in single species biofilms compared to SS. The higher amount of VBNC was found when using 57% copper alloy (P < 0.05). It was observed that, regardless the copper alloy, the number of VBNC follows the same tendency found for viable cells. Also, in *S. maltophilia* biofilms it was observed an increase in VBNC counts from biofilm growth on the copper materials in comparison to SS. The higher numbers of VBNC (4.55

and 4.50 log cells/cm^2) were found on 79 and 96% copper alloys, respectively. Also, in dual species biofilms the numbers of *A. calcocaeticus* in VBNC state was similar to the number of viable cells ($P > 0.05$). However, in this particular case, the numbers of *A. calcoaceticus* in VBNC state was similar on SS and on the copper materials. It was also observed that VBNC *A. calcoaceticus* in dual species biofilms was much lower than the detected in single species biofilms. On the other hand, dual species biofilms formed on copper materials were composed of higher amounts of VBNC *S. maltophilia* than these biofilms formed on SS ($P < 0.05$), with the exception of 96% copper alloy where the numbers of VBNC *S. maltophilia* was similar to these observed on SS ($P > 0.05$). Also, in this situation the VBNC follows the same tendency observed for viable cell counts ($P > 0.05$). In general, the number of VBNC bacteria was lower in dual species biofilms formed on copper alloys than in single species biofilms.

6.1.1.4 Bacterial regrowth on copper materials

It was observed that *A. calcoaceticus* single species biofilms (Figure 6-1A) were unable to recover its culturability when biofilms formed on 96% copper alloy were exposed to a sudden increase of nutrients level (R2A broth). On the other hand, the regrowth of *A. calcoaceticus* culturability was similar on SS (0% Cu) and on 57 and 79% copper alloys ($P > 0.05$). The elemental copper surface (100% Cu) was able to reduce the *A. calcoaceticus* regrowth in 3.52 log CFU/cm^2 compared to SS.

Regarding *S. maltophilia* single species biofilms, it was possible to observe that only 96% copper alloy was able to completely inhibit regrowth (Figure 6-1B). Elemental copper had also an important role on the impairment of *S. maltophilia* regrowth with a difference of 5.93 log CFU/cm^2 compared to SS ($P < 0.05$). Alloys with 57 and 83% of copper

caused impairment in *S. maltophilia* biofilm regrowth of 2.71 and 2.74 log CFU/cm^2, respectively ($P > 0.05$).

In dual species biofilms, regrowth of *A. calcoaceticus* only was found on materials with 79, 83 and 100% copper (Figure 6-1C). These alloys reduced *A. calcoaceticus* regrowth in 1.64, 4.51 and 5.66 log CFU/cm^2 compared to SS 316, respectively. No regrowth was found on alloys with 57 and 96% copper. *S. maltophilia* regrowth in dual species biofilms occurred in all the copper materials tested (Figure 6-1.D). The lowest *S. maltophilia* regorwth was observed for elemental copper (100% Cu) with a reduction of 6.96 log CFU/cm^2 compared to SS ($P < 0.05$). Reductions of 1.61, 2.14, 3.83, and 3.43 log CFU/cm^2 on the ability of *S. maltophilia* to recover were observed when using 57, 79, 83 and 96% copper alloys, respectively ($P > 0.05$).

6.1.2 Copper effect in bulk bacteria

The culturability of bacteria present in the bulk phase was also analyzed at 24 and 48 h. Bacteria detected in the bulk phase after 24 h of incubation in the presence of copper materials, correspond essentially to culturable non-adhered bacteria. On the other hand, bacteria detected in the bulk phase at 48 h correspond to culturable biofilm-released bacteria. All the copper materials reduced the numbers of *A. calcoaceticus* CFU detected in the bulk phase (Figure 6-1E) ($P < 0.05$). Regarding the effects on released bacteria for 48 h, no *A. calcoaceticus* CFU in the bulk phase were observed when elemental copper (100 % Cu) was used. The 57% copper alloy caused 5.02 log CFU/cm^2 reduction ($P < 0.05$) in bulk phase when compared with SS. All the other materials caused similar *A. calcoaceticus* CFU reductions when analyzing the bulk phase (> 3.80 log CFU/cm^2) ($P > 0.05$).

Concerning *S. maltophilia* single species biofilms (Figure 6-1F), it was possible to observe that all the copper materials had similar impact on non-adhered bacteria (24 h), being the numbers of CFU in bulk phase lower on copper materials than on SS ($P > 0.05$). The 96% copper alloy was the one causing the highest CFU reduction (5.59 log CFU/cm^2) in the bulk phase for the 48 h analysis. The other copper alloys caused similar CFU reductions (between 4.11 and 5 log CFU/cm^2) ($P > 0.05$).

Figure 6-1G and Figure 6-1H show culturability of *A. calcoaceticus* and *S. maltophilia* in the bulk phase when dual species biofilms were present, respectively. All the copper materials were able to reduce significantly the culturability of non-adhered *A. calcoaceticus* (Figure 6-1G) and *S. maltophilia* (Figure 6-1H) for 24 h analysis ($P < 0.05$). No *A. calcoaceticus* were found for the 48 h analysis and for all the copper materials tested. The culturability of *S. maltophilia* (48 h – Figure 6-1H) released was significantly reduced when copper surface materials were used ($P < 0.05$). The highest reductions (> 5.3 log CFU/cm^2) were observed for the 83, 96 and 100% copper alloys.

6.1.3 Formation of reactive oxygen species

The ROS generation by *A. calcoaceticus* and *S. maltophilia* biofilms was monitored using DCFH-DA. This molecule reacts with ROS present inside cells and is converted into DCF (a fluorescent by-product). Therefore, fluorescence was evaluated to monitor ROS formation. It is possible to observe higher formation of ROS in 24 h-old *S. maltophilia* biofilms than in *A. calcoaceticus* (Figure 6-3) ($P < 0.05$). The formation of ROS inside *S. maltophilia* increased when exposed to materials with higher copper content (87, 96 and 100% Cu) ($P < 0.05$). Regarding *A. calcoaceticus* biofilms, the highest ROS formation was observed for the alloy with lowest copper content (57% Cu) ($P < 0.05$).

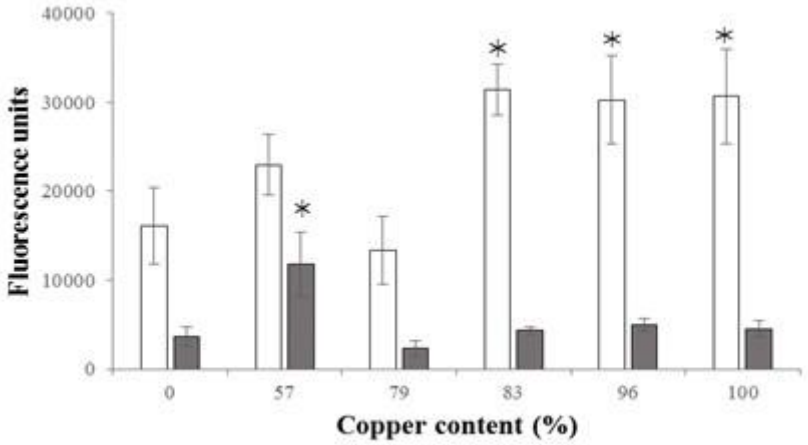

Figure 6-3. Reactive oxygen species (ROS) formation in *A. calcoacteicus* (■) and *S. maltophilia* (□) from biofilms formed on the surface materials with different copper contents. * - Differences statistically significant ($P < 0.05$) in comparison with SS (0% copper).

6.1.4 Copper materials corrosion and leaching

The corrosion rate of all copper materials was found to be faster during the first month of contact with STW (Figure 6-4). Afterwards, the corrosion rate stabilized for all the materials, except for elemental copper that presented an increase in corrosion levels after 90 d in STW (Figure 6-4A).

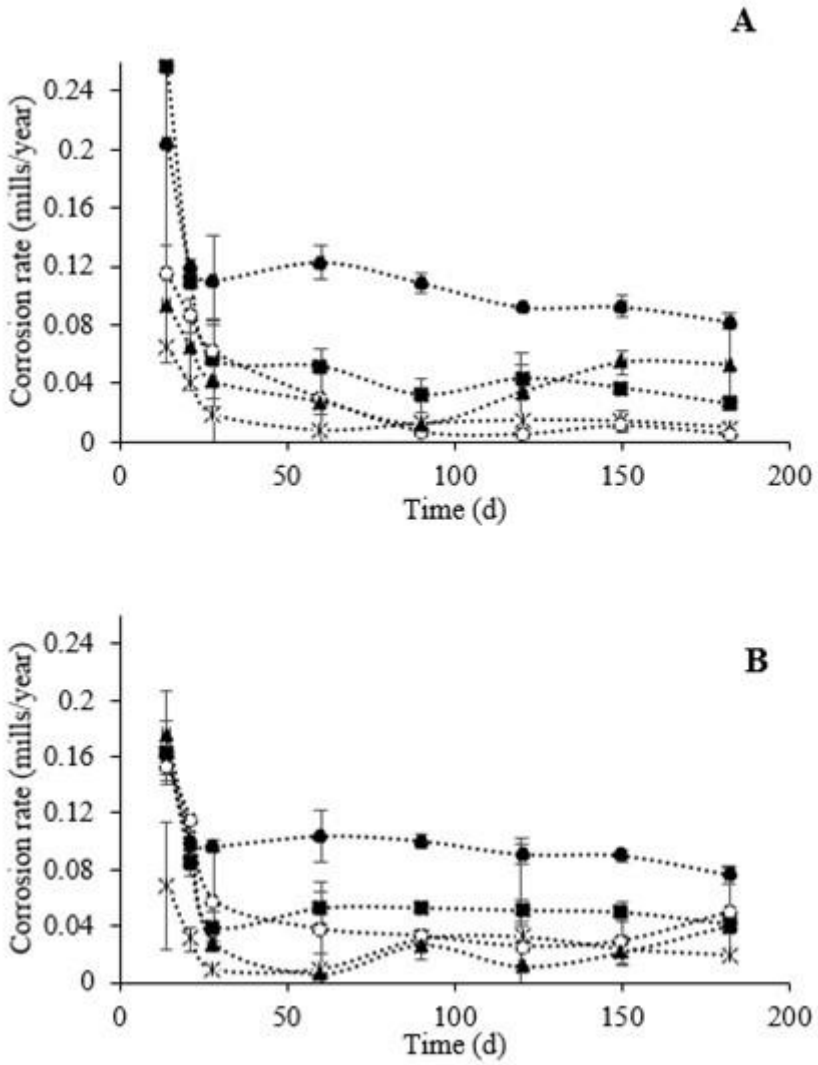

Figure 6-4. Corrosion rate of copper materials in STW (A) and in STW with 1 mg/L of free chlorine (B) for 182 days. ● – 57% copper alloy; * - 79% copper alloy, ■ –83% copper allot; ○- 96% copper alloy; ▲- elemental copper (100% copper). The assay was performed with three replicates.

In the absence of chlorine, all the materials demonstrated similar behaviour in terms of copper leaching (Figure 6-5): copper concentrations did not exceed the values recommended by WHO/EU Directive 98/83/EC (2 mg/L) and the EPA Standard (1.3 mg/L). No copper was detected in bulk water for alloy with 57% of copper ($[Cu^{+}/Cu^{2+}]$ < 0.3 mg/L). The presence of chlorine did not change significantly ($P > 0.05$) the leaching from materials with 57, 79, 96 and 100%, being the maximum for each material. On the other hand, the exposure of the 83% copper alloy to chlorine in STW increased

significantly copper leaching (Figure 6-5B). After 90 d of contact with chlorinated water, the copper concentration in stagnated water increased to values higher than those recommended by the WHO/EU Directive 98/83/EC (2 mg/L) and the EPA Standard (1.3 mg/L).

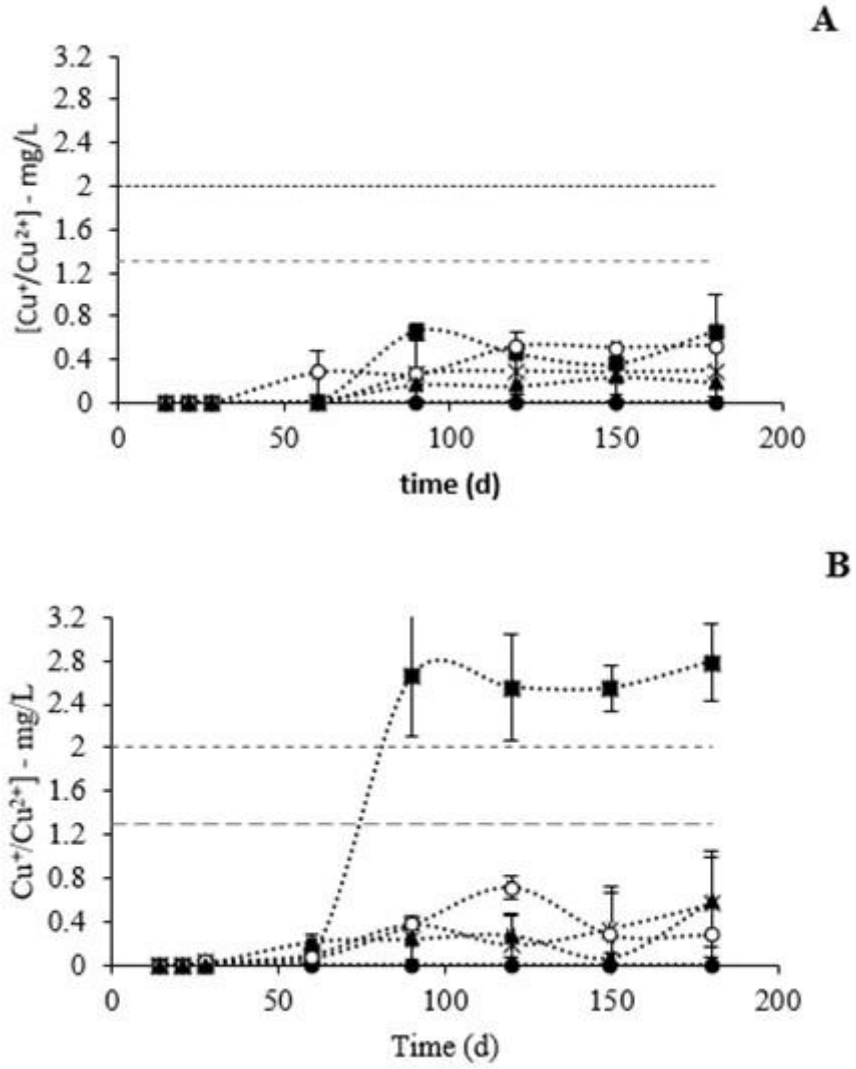

Figure 6-5. Copper leaching to STW in the absence (A) and presence of 1 mg/L of free chlorine (B) for 182 days. ● –57% copper alloy; * - 79% copper alloy, ■ – 83% copper alloy; ○- 96% copper alloy, ▲- elemental copper (100% copper). --- Maximum copper concentration in DW as recommended by WHO/EU Directive 98/83/EC (2 mg/L) – – – Maximum copper concentration in DW as recommended by EPA Standard (1.3 mg/L). The detection limit of the analytical method used was 0.3 mg/L. The assay was performed with three replicates.

6.2 Discussion

The selection of pipe materials is a critical aspect for the chemical and microbiological stability of plumbing systems. Several characteristics should be ensured to guarantee a safe plumbing system: controlled microbial proliferation - including biofilm development, no leaching of products to the drinking water, low installation and material costs, and high life-time (Lee 2013). Copper is a metal used in plumbing systems for decades and its antimicrobial properties have been described as advantageous (Armanious and Johannsen 2012). Nevertheless, copper expensive cost was one of the main reasons to increase the use of plastic materials in plumbing systems (Connell *et al*. 2013, Kelley *et al*. 2014). Copper leaching to drinking water and its possible negative effects on water quality and public health also contributed to the misuse of this material (Armanious and Johannsen 2012, Vargas *et al*. 2017). Nevertheless, the choice between copper or plastic pipes is still controversial (Lee 2015). Although plastic pipes have lower installation costs, they usually have a shorter life-time and can be responsible for the leaching of some chemicals to the drinking water, changing the water characteristics and causing some concern to consumers' health, including potential carcinogenic effects and the favoring of pathogen growth (Connell *et al*. 2016, Kelley *et al*. 2014, Lund *et al*. 2011). The present work aims to screen alloys with different copper contents (0, 57, 79, 83, 96 and 100%) in order to understand the possibility of using less expensive copper materials in plumbing systems to reduce biofilm development, guaranteeing the microbial and chemical quality of the delivered water. The corrosion rate of each material was also evaluated as well as copper leaching to bulk water.

The present results demonstrated that copper materials were consistently more efficient in controlling biofilms of *A. calcoaceticus* than of *S. maltophilia*. In fact, this effect was

not evident in the bulk phase. No *A. calcoaceticus* CFU were detected after single species biofilm development on copper materials. This result corroborates the study of Różańska *et al*. (2018) where no CFU of *Acinetobacter* species were detected on copper (100% Cu) after 5 h contact. Moreover, significant reductions were observed when *Acinetobacter* species were in contact with 63 and 94% copper surfaces for 5 h. *S. maltophilia* single species biofilms were more tolerant to copper and CFU were detected in 24 h-old biofilms formed on all the surface material tested. Previous studies already described some strains of *S. maltophilia* tolerant to copper and able to bioremediate this metal (Brooke 2012, Mukherjee and Roy 2016, Tang *et al*. 2016). Moreover, *S. maltophilia* biofilms were found to be more tolerant to antimicrobials than *A. calcoaceticus* (Gomes *et al*. 2018a, Gomes *et al*. 2016).

It is important to highlight that copper materials reduced significantly the culturability for all the scenarios tested, even when using the alloy with the lowest copper content. However, the control efficiency of *S. maltophilia* in single and dual species biofilms was slightly increased when the contact time with copper surfaces increased for 48 h. Moreover, no relationship was observed between CFU reduction and surface copper content. Morrier *et al*. (1998) also found that antimicrobial activity of dental amalgams was not related to copper content. For instance, in the present study it was observed that in particular cases alloys with lower copper content demonstrated better results than those with higher content. As example, no CFU were detected for 48 h-old *S. maltophilia* biofilms formed on 57 and 79% copper alloys. However, complete inactivation of *S. maltophilia* was not achieved using the higher copper content materials (83, 96 and 100%). These different behaviours may not be exclusively associated to the copper content, which reinforces the importance to evaluate the possibility of using and/or developing alloys with reduced copper content for application in plumbing systems. The

presence of other metals such as zinc and lead (Morrier *et al*. 1998, Yasuyuki *et al*. 2010) in the alloy with 57% copper may also have an important impact on biofilm control. It is important to note that the referred alloy has high percentage of zinc (39%). The alloy containing 79% copper is composed by 10% aluminum, which may improve copper action. A recent work described the importance of dissolved aluminum in the amplification of the toxicity of transition metals, such as copper, which helps to understand the higher CFU reductions when alloys containing both metals were used (Londono *et al*. 2017). Moreover, the hypothesis of aluminum interaction with bacterial membrane phospholipids cannot be disregarded (Piña and Cervantes 1996).

The antimicrobial action of copper surfaces has been investigated and several effects on bacteria integrity were described such as damage of membrane, copper intake (due to dissolution of copper ions from surfaces), damage caused by oxidative stress, cell death and DNA degradation (Grass *et al*. 2011, Santo *et al*. 2011). In the present work, the copper action was evaluated using biofilms developed in synthetic drinking water. Most of the previous works on copper mode of action focused the contact killing effects on dry surfaces or used planktonic bacteria (Bleichert *et al*. 2014, Parra *et al*. 2018, Santo *et al*. 2011, Warnes *et al*. 2012). Copper leaching to STW, membrane damage assessment by propidium iodide staining and the oxidative stress effects were evaluated in order to understand how copper alloys exerted antimicrobial action. In general, higher membrane damage was found in *A. calcoaceticus* compared to *S. maltophilia* and membrane damage was not related to surface copper content. The formation of ROS was higher in *S. maltophilia* than in *A. calcoaceticus*, regardless the surface material used. However, *S. maltophilia* was the bacteria more tolerant to copper. It is possible that *S. maltophilia* biofilm bacteria expressed defense mechanism to survive ROS formation. In fact, previous works described efflux systems, such as SmeYZ and SmeU1VWU2X, able to

improve the *S. maltophilia* tolerance to oxidative stress (Lin *et al*. 2015, Wu *et al*. 2018). The antimicrobial action also seems to be independent from the copper leached to water. However, no relationship was observed between surfaces copper content and the concentration of copper in the bulk water. Based on the results from analysis of bacteria in the bulk-phase and in biofilms, it is possible to observe that the action of copper is more effective on the surface than in the bulk water. Complete inactivation of non-adhered bacteria was not obtained with any material tested. This proposes that the antimicrobial action of the copper materials was not driven by metal leaching to the bulk water. This hypothesis is reinforced by results from copper leaching in the absence of chlorine. The concentration of copper was below the maximum levels recommended by EPA (2016) and WHO (2003b) (1.3 mg/L and 2 mg/L, respectively) in unchlorinated STW. Higher copper concentrations were detected in STW exposed to the 83% copper alloy. In chlorinated systems, the copper leaching from this alloy is of concern as it exceeds the maximum concentration recommended by WHO and EPA (EPA 2016, WHO 2003b). Therefore, the results proposed that the alloy with 83% copper content should not be considered for application in chlorinated plumbing systems.

Although the evaluation of culturability is the standard method commonly applied to control the microbiological quality of drinking water (WHO 2002), viability tests should also be evaluated to infer on the biofilm prevention efficacy of the materials tested. It is known that culturability analysis do not provide accurate information about the microbiological quality of drinking water. Under adverse environments, bacteria may acquire a “dormant state”, the VBNC bacteria that survive under adverse conditions and are metabolically active but unable to growth on solid growth media and form colonies. These VBNC bacteria may recover its culturability and proliferate in water when in the presence favorable conditions, compromising water quality. Bacterial regrowth in DW is

also a common problem, imposing the use of residual chlorine along DWDS. Therefore, VBNC and bacterial regrowth were also assessed in this work. Biofilm regrowth was assessed by studying bacterial ability to recover its culturability when colonized coupons were in the presence of R2A broth for 24 h. The alloy containing 96% copper was the most promising material avoiding regrowth. The exception was *S. maltophilia* in dual species biofilms that was able to recover on this alloy. Nevertheless, this result seems not be related to the number of VBNC, as viable and VBNC bacteria were detected in all the conditions tested. Viable counts and VBNC detected in the alloy with 96% copper did not recover its culturability, contrary to the observed with other alloys. This proposes that the extent of injury of VBNC bacteria in the presence of 96% copper alloy is harder to be overcome, even when exposed to high nutrient conditions. Dwidjosiswojo *et al*. (2011) and Bedard *et al*. (2014) found that VBNC of *P. aeruginosa* exposed to copper ions recovered culturability when copper chelators were used. Dwidjosiswojo *et al*. (2011) also demonstrated that copper in plumbing systems was responsible for the induction of a VBNC state in *P. aeruginosa.* Nevertheless, their study demonstrated that copper at concentrations similar to those detected in plumbing systems was unable to damage the bacterial membrane. Gião *et al*. (2015) also found that copper increased the number of *Legionella pneumophila* in VBNC state. The efficiency of copper alloys seems not to be dependent on copper leaching to bulk water or even on the ROS production but depends on the contact time, as it was observed by the reduction of CFU of *S. maltophilia* single and dual species biofilms after 48 h in contact with copper materials.

6.3 Conclusions

In conclusion, all the copper surface materials reduced culturability and viability of *A. calcoaceticus* and *S. maltophilia* from single and dual species biofilms. The most

promising results were obtained using the 96% copper alloy. This had antimicrobial effects and avoided biofilm regrowth. Additionally, it presented low corrosion rate and leaching, even in the presence of chlorine. Bacterial membrane damage and the formation of ROS was verified for all the selected copper alloys. It is important to highlight that alloys containing lower copper content were also efficient in impairing biofilm formation, with results comparable to elemental copper. These alloys are potentially relevant for plumbing systems, helping to reduce installation costs due to the lower copper content (Annex C - copper material characteristics and prices). The exception was the 83% copper alloy that was prone to prohibitive leaching in the presence of chlorine. The impact of copper alloys in DW biofilm control intensifies the need for further research about the combined effect of different metal alloys in biofilm development and DW quality.

Chapter 7

Influence of surface copper content on biofilm control using chlorine and mechanical stress

In this chapter, the action of materials with different copper content (0, 57, 96 and 100%) was evaluated on biofilm formation. The effects of surface material was further evaluated on biofilm control by chlorination and mechanical stress. A strain of *S. maltophilia*, isolated from drinking water, was used as model microorganism and biofilms were developed in a rotating cylinder reactor to provide a realism-based approach. Biofilms were characterized and exposed to three control strategies: 10 mg/l of free chlorine for 10 min; an increased shear stress (equivalent to 1.5 m/s of fluid velocity); and the combination of both treatments.

7.1 Results

7.1.1 Biofilm formation on copper materials

Biofilms formed on four different materials were characterized in terms of mass, water content, extracellular proteins and polysaccharides, and biofilm cell density (Table 7-1). The biofilms were composed mostly by water (> 96%). Dry mass and water content were similar for all the biofilms, regardless the surface material under which they were formed ($P > 0.05$). The biofilms formed on copper materials had lower wet mass than those formed on SS (0% copper), being the differences more significant when using the 57% copper alloy ($P < 0.05$).

Regarding the EPS content it was observed that biofilms formed on copper materials had lower protein levels than those formed on SS ($P > 0.05$). The use of elemental copper surfaces also allowed biofilm formation with low content of extracellular polysaccharides. No significant differences were observed when comparing the content of extracellular polysaccharides of biofilms formed on SS and on 57 and 96% copper alloys ($P > 0.05$). However, it is possible to observe differences on biofilm visual aspect after 7 d of formation on the different materials (Figure 7-1). Biofilm cell density and viability was found to be similar, regardless the surface material used ($P > 0.05$). In terms of biofilm culturability, a decrease was observed with the increase of surface copper content. Materials with 100 and 96% of copper caused reductions of 2 and 1.4 log CFU/cm^2, respectively ($P < 0.05$).

Table 7-1. Characterization of biofilms formed on selected materials with different copper content.

	Material/Copper content (%)			
	0 (SS)	**57**	**96**	**100**
Wet mass (mg/cm^2)	120 ±26	78.0 ±4.5	90.0 ±14	92.0 ±23
Dry mass (mg/cm^2)	4.50 ±1.6	2.71 ±0.76	2.61 ±0.10	3.24 ±1.3
Water content (%)	96.8 ±1.3	96.5 ±2.89	96.6 ±0.51	97.2 ±0.50
Extracellular proteins (mg/g biofilm)	16.4 ±3.4	5.08 ±1.50*	7.41 ±2.9*	4.62 ±1.6
Extracellular polysaccharides (mg/g biofilm)	143 ±33	142 ±26	113 ±58	n.d
Biofilm culturability (log CFU/cm^2)	7.04 ± 0.67	6.53 ± 0.73	5.55 ± 0.75*	5.00 ± 0.93*
Biofilm viability (log viable cells/cm^2)	7.08 ± 0.36	6.77 ± 0.54	6.77 ± 0.85	6.14 ± 1.1
Biofilm cellular density (log cells/cm^2)	7.78 ± 0.22	7.84 ± 0.06	7.78 ± 0.27	7.59 ± 0.41

* $P < 0.05$ – statistically significant different from control (0% copper)

- n.d. – non-detected (polysaccharides below the quantification limit – 5 mg/L)

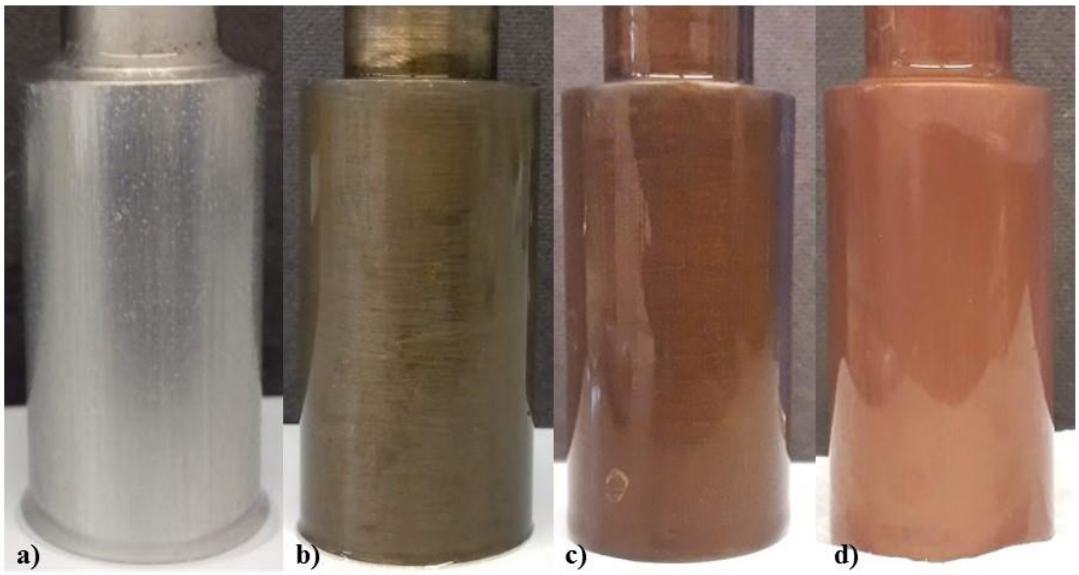

Figure 7-1. Cylinders covered by 7 d old biofilms formed by *S. maltophilia* in the RCR. (a) - Stainless steel (0% of copper content), (b) - 57% copper alloy, (c) - 96% copper alloy, (d) - elemental copper (100% of copper content**).**

7.1.2 Biofilm control by chlorine and shear stress

7.1.2.1 Chemical treatment with chlorine

Biofilm culturability after treatment with 10 mg/L of free chlorine for 10 minutes was lower (Figure 7-2) when using 100 and 96% copper surfaces ($P < 0.05$). No significant differences were observed on the culturability of biofilms formed on 57% copper alloy compared to stainless steel ($P > 0.05$). However, CFU reduction due to NaOCl exposure (in comparison to the non-NaOCl treated biofilms formed on the same material) was only significant when using 96% copper alloy ($P < 0.05$).

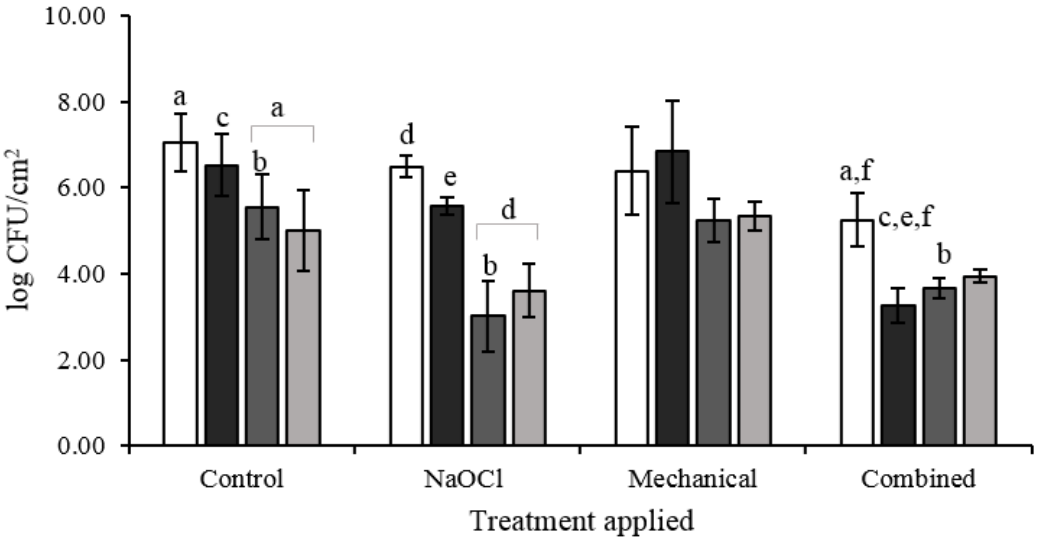

Figure 7-2. Culturability of biofilms formed on surface materials with different copper content before and after the exposure to chemical or/and mechanical treatments. Control – biofilms not exposed to any treatment, NaOCl – biofilms exposed to 10 mg/L of free chlorine for 10 min, Mechanical – biofilms exposed to 10 Pa (1.5 m/s of fluid velocity) for 30 s, Combined – biofilms exposed to both chemical and mechanical treatments. □- Material with no copper in its composition (0% copper); ■- Material with 57% copper content; ■ – Material with 96% copper content; ■- Elemental copper (100% copper). a, b, c, d, e represent comparisons with statistically significant differences with a confidence level ≥ 95%.

Also, viability of *S. maltophilia* biofilms formed on the 96% copper alloy followed the same trend observed for biofilm culturability (Figure 7-3), *i.e. S. maltophilia* viability was lower on 96% copper alloy than on stainless steel as well as than in the non-treated biofilms formed on 96% copper alloy ($P < 0.05$). Treatment with chlorine did not affect significantly *S. maltophilia* viability and culturability when biofilms were formed on SS or on 100 and 57% copper surfaces ($P > 0.05$) - compared to non-NaOCl treated biofilms formed on the same materials.

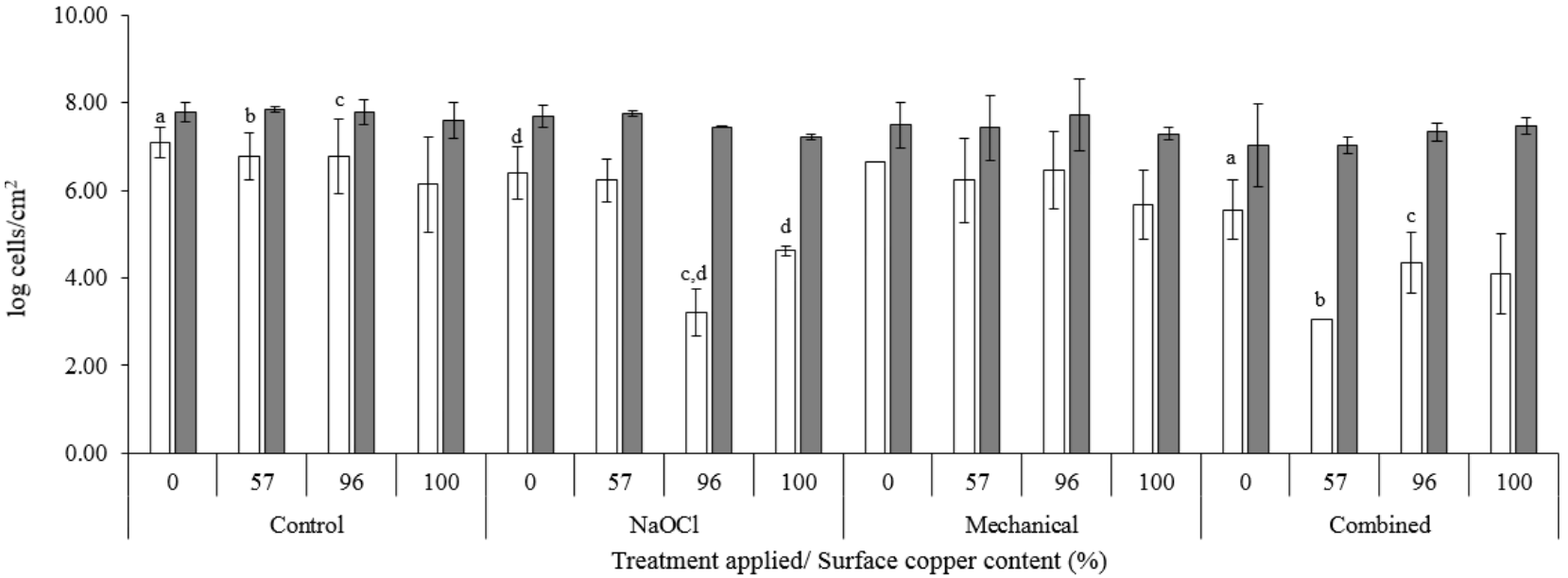

Figure 7-3. Viability (□) and cellular density (■ - total cells) of biofilms formed on surface materials with different copper content before and after exposure to chemical or/and mechanical treatments. Control – biofilms non-exposed to any treatment; NaOCl – biofilms exposed to 10 mg/L of free chlorine for 10 min; Mechanical – biofilms exposed to 10 Pa (1.5 m/s of fluid velocity) for 30 s; Combined – biofilms exposed to both chemical and mechanical treatments. a, b, c, d represent comparisons with statistically significant differences with a confidence level ≥ 95%.

7.1.2.2 Mechanical treatment by exposure to high shear stress

The exposure to 10 Pa shear stress, corresponding to a fluid velocity of 1.5 m/s for 30 s caused no significant biofilm removal ($P > 0.05$). This treatment had no effects on *S. maltophilia* biofilm culturability (Figure 7-2) and viability (Figure 7-3), regardless the surface material tested ($P > 0.05$).

7.1.2.3 Combined treatment – exposure to chlorine and high shear stress

The combination of chemical and mechanical treatments did not reduce biofilm culturability (Figure 7-2) when those were formed on elemental copper (100%) and compared to the behaviour of biofilms non-exposed to chemical and mechanical treatments ($P > 0.05$). Culturability reduction was observed when biofilms formed on alloys containing 96, 57 and 0% of cooper were exposed to both treatments in comparison to non-treated biofilms ($P < 0.05$). However, CFU reduction caused by the combination of treatments was only higher than the reduction caused by chlorine treatment alone for biofilms formed on 57% copper alloy ($P < 0.05$). Nevertheless, it was also possible to observe viability reduction caused by the combined treatments against biofilms formed on SS (Figure 7-3) ($P < 0.05$). However, higher reduction on *S. maltophilia* viability was obtained in biofilms formed on 96 and 57% copper alloys ($P < 0.05$).

7.1.2.4 Bulk phase analysis after biofilm treatment

Biofilm released cells were quantified (total and viable) to understand the state of those cells potentially reaching the consumers tap. The total number of cells in the bulk water (Figure 7-4) was not dependent on the surface material or treatment applied ($P > 0.05$). Nevertheless, significant differences were observed in terms of viable cells released from the chemically and/or mechanically treated biofilms (Figure 7-4). The number of viable

cells in the bulk phase after chlorine treatment was lower when materials with 100 and 57% copper content were used (4.1 and 4.4 log viable cells/cm^2, respectively) compared to SS (5.4 log viable cells/cm^2) ($P < 0.05$).

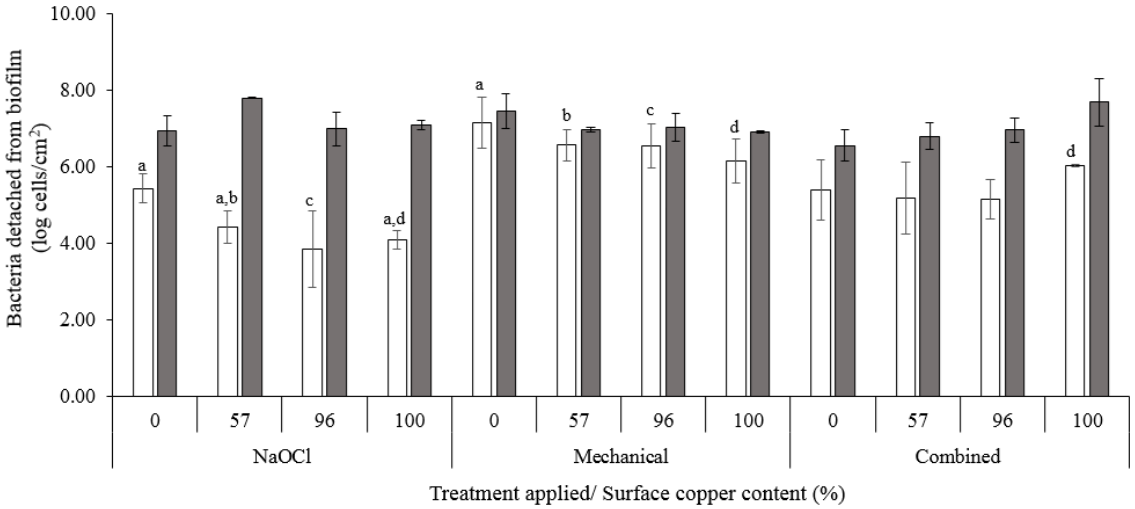

Figure 7-4. Cells removed from the cylinder`s surface after each treatment. Control – biofilms non-exposed to any treatment; NaOCl – biofilms exposed to 10 mg/L of free chlorine for 10 min; Mechanical – biofilms exposed to 10 Pa (1.5 m/s of fluid velocity) for 30 s; Combined – biofilms exposed to both chemical and mechanical treatments. □ - viable cells; ■ - Total cells. a, b, c, d represent comparisons with statistically significant differences with a confidence level ≥ 95%.

The viability of *S. maltophilia* released from biofilms formed on 96% copper alloy was not statistically different from that observed for biofilms formed on SS ($P > 0.05$). The use of copper materials did not affect the number of viable cells detected in the bulk water after mechanical treatment ($P > 0.05$). The comparison between the effects of chemical and mechanical treatments on the numbers of viable cells in the bulk phase shows higher numbers after mechanical treatment ($P < 0.05$). The combination of treatments did not reduce the viability of released bacteria in comparison to NaOCl alone ($P > 0.05$). In general, higher numbers of viable bacteria are released after mechanical treatment (for all the materials) and after the combination of chemical and mechanical treatments - in biofilms formed on elemental copper - than for the chemical treatment ($P < 0.05$). For biofilms formed on surface materials with 0, 57 and 96 % of copper, the viability of

bacteria released from biofilms treated with the combination of mechanical and chemical stresses was similar to the viability of bacteria released after the chemical treatment ($P > 0.05$).

7.2 Discussion

Biofilm formation in DWDS typically changes the aesthetic characteristics of the delivered water, accelerates pipe corrosion, and is a potential causative agent of waterborne diseases due to the presence of pathogens (Simões and Simões 2013). Therefore, the existence of efficient strategies to control biofilm development in DWDS is of utmost importance. Current disinfection strategies are unable to prevent or eradicate biofilms (Simões and Simões 2013). The use of materials with antimicrobial properties requires further research as they may play a crucial role in preventing biofilm set-up. Copper has been applied in plumbing systems and it is losing attractiveness for plastic pipes, mainly due to its expensive cost. However, copper has known antimicrobial characteristics that may have significant impact in biofilm control. The use of copper alloys, with reduced copper content, could help to reduce the costs from material acquisition while having the potential advantage of exerting antimicrobial activity. However, validation on the biofilm control action of copper alloys needs to be evaluated. In the present work, materials with distinct copper content were tested in a RCR to develop biofilms under conditions found in DWDS, particularly the use of curved surfaces, typical of pipes, and the hydrodynamic conditions under which biofilms were formed. Mimicking the conditions found in DWDS has significant importance to obtain results of relevance for a practical application. In fact, several works describing disinfection strategies were performed using bacteria in planktonic state that are inadequate to represent the behaviour of these found adhered on surfaces (Forbes *et al*.

2019, Khan *et al*. 2017, Köhler *et al*. 2018, Mir *et al*. 1997, Samir *et al*. 2019). Several other works studied DW biofilms in reactors inadequate to promote biofilm formation simulating the conditions found in DWDS, particularly the annular reactor (Chang and Craik 2012), the rotating disc reactor (Murga *et al*. 2001, Pelleïeux *et al*. 2012), the CDC biofilm reactor (Abe *et al*. 2011, Armbruster *et al*. 2012, Park and Hu 2010).

The present study discloses that elemental copper and copper alloys influenced biofilm formation. The 57% copper alloy reduced the dry mass of biofilms compared to those formed on SS. Despite of the use of 57% copper alloy did not influence biofilm cell density, it reduced extracellular proteins content in biofilms. The EPS content was also influenced by the elemental copper material - biofilms had lower content of extracellular polysaccharides. Previous studies reported that copper nanoparticles or copper ions may influence EPS formation. For example, Chari *et al*. (2017) observed that exposure to copper nanoparticles (CuNP) was responsible for the reduction of EPS production by aquaculture pathogens ***(****Vibrio alginolyticus****,*** *Vibrio parahaemolyticus and Aeromonas hydrophila).* Contradicting the present results, Katner *et al*. (2018) found that exposure to increasing Cu(II) concentrations was associated with an increase in EPS production by anaerobic ammonia-oxidizing bacteria. These authors also observed higher influence of copper in proteins production than in polysaccharides. Miao *et al*. (2017) observed an increase in EPS production by wastewater activated sludge after 24 h contact with copper nanoparticles. Tabrez Khan *et al*. (2013) tested the antibiofilm activity of copper nanoparticles and observed a decrease in EPS production after exposure to CuNP at 50 mg/mL for 16 h. Therefore, the effects from copper exposure on EPS production is non-consensual. However, no previous data exists on the influence of alloys with diverse copper content on biofilm EPS production.

The use of a residual disinfectant concentration along the DWDS is an important strategy to minimize biofilm regrowth and avoid waterborne diseases (Al-Jasser 2007). Chlorine is one of the most commonly used disinfectants in DWDS and a concentration between 0.2 and 1 mg/L should be kept at the delivery point (WHO 2011b). In plumbing systems, chlorine concentration decreases with time and the control of free chlorine concentration is found critical (Zheng *et al*. 2015). The chlorine decay along DWDS and plumbing systems depends on several factors: temperature, pipe material and age, biofilm and corrosion products, flow regime, organic matter content and initial chlorine concentration (Kim and Kim 2017). The results from the present study propose that copper materials are an attractive complementary strategy to overcome the problems from chlorine decay. The number of CFU from biofilms formed on materials with 96 and 100% copper was lower than these of biofilms formed on SS and treated with chlorine. In fact, the CFU of non-treated biofilms formed on 100% copper were similar to those observed in biofilms formed on SS after treatment with the combination of chlorine and shear stress. The use of 96 and 100% copper materials demonstrated to be more effective in *S. maltophilia* biofilm control than the use of chlorine against biofilms formed on SS. This corroborates the findings of Zhou *et al*. (2009) where biofilm CFU on copper surfaces in the absence of chlorine was lower than the CFU levels of biofilms formed on SS exposed to 0.6 mg/L chlorine.

A shock treatment that could be applied in situations of critical DW contamination was also simulated (free chlorine at 10 mg/L for 10 minutes and an increased shear stress - 1.5 m/s - for 30 s) (EPA 2010, Van Nevel *et al*. 2017). The results demonstrated that 10 mg/L of chlorine was inefficient in *S. maltophilia* biofilm control. The higher biofilm CFU reduction caused by chlorine was observed on 96% copper alloy. However, it is important to highlight that chlorine did not reduce *S. maltophilia* CFU in biofilms grown

on 0, 57 and 100%. Other studies also reported the modest effects of chlorine in biofilm control. Buse *et al*. (2019) achieved log reductions of 2, 3 or 4 in *L. pneumophilia* biofilms for concentration × time (Ct) values of 13, 51 and 88 mg.min/L, respectively. In the present study, the Ct value was 100 mg.min/L (10 mg/L for 10 min) and the reduction of *S. maltophilia* CFU was lower than that presented by Buse *et al*. (2019), for all the materials tested. Gomes *et al*. (2018a) also found that chlorine was inefficient against *S. maltophilia* biofilms, obtaining 35% removal after exposure to extreme chlorine conditions (175 mg/L of NaOCl for 30 min – Ct of 5250 mg.min/L).

Pipe flushing and the use of high chlorine doses are important strategies commonly used to control critical contaminations. However, these strategies may be responsible for the detachment of small portions of biofilm into the transported water that can further reach the consumer's tap. Therefore, it is important to characterize the bacteria in the bulk phase in order to evaluate if significant numbers of viable bacteria would be dispersed as result of treatment failure. Flushing was found to be not effective in the control of biofilms formed on the diverse surface materials tested. Fish *et al*. (2017) also tested flushing of DW biofilms using increasing shear stresses (0.42, 1.75 and 2.91 Pa) and found no significant biofilm removal. Despite no significant reduction of biofilm cellular density were observed during flushing treatment, it is important to notice that relevant abundance of viable bacteria were detected in the bulk water (6.1 to 7.1 log cells/cm^2). El-Chakhtoura *et al*. (2018) recently described the increase of CFU abundance and Shannon diversity in transported water after flushing treatment.

The number of viable cells in transported water after chlorine treatment seems to be dependent on the materials used for biofilm formation. The use of copper materials reduced the number of viable bacteria in the bulk phase compared to SS. This proposes that copper materials can be an adequate choice for plumbing systems. Their use reduced

the levels of viable bacteria detached from biofilms to the bulk phase, minimizing the microbiological risks from exposure to contaminated water. It is important to emphasize that the recommended shock treatments - Ct value of 100 mg.min/L (EPA 2010) - for critical levels of microbiological contamination only caused CFU reductions of 0.5 (SS); 1.4 (57% copper alloy); 2.5 (96% copper alloy); 0.9 (100% copper) log CFU/cm^2.

The combination of hydrodynamic stress and chlorination had no advantage in comparison to chlorination alone. The only exception was found for biofilms formed on the 57% copper alloy where high viability reduction was observed from the combined treatment. Lemos *et al*. (2015b) also found that the combination of chemical and mechanical treatments was not effective on biofilm removal when biofilms were formed at 0.12 Pa and 0.17 Pa (hydrodynamic stress similar to that tested in the present work).

7.3 Conclusions

The formation of mature biofilms (7 d-old) was not significantly influenced by the use of copper surfaces in comparison to SS. In fact, many biofilm characteristics, particularly the cell density, was not affected by the surface type. However, the benefits from the use of copper surfaces rely on the efficiency to disinfect. Copper materials demonstrated better performance in biofilm prevention/control than chlorine as higher CFU reduction were obtained using copper materials than from the use of chlorine against biofilms formed on SS. The application of shock treatments (10 mg/L of free chlorine for 10 min and/or high shear stress – 1.5 m/s for 30 s) was not effective in biofilm inactivation or removal. Biofilm disinfection was dependent on the material used for biofilm formation. Chlorine treatment did not reduce biofilm culturability when formed on surface materials with 0, 57, and 100 % copper, culturability reduction due to chlorine treatment was only observed in *S. maltophilia* biofilms formed on 96% copper alloy. Flushing also was not

effective in biofilm control and the combination of both treatments (free chlorine at 10 mg/L for 10 min and fluid velocity of 1.5 m/s for 30 s) only had positive impact in the control of biofilms formed on 57% copper alloy. Therefore, the strategies (residual chlorine and flushing) commonly used to control critical levels of contaminations in DWDS were not effective in biofilm control. However, the results demonstrated that copper materials reduced the number of viable *S. maltophilia* in the transported water.

Chapter 8

Impact of copper leachates on virulence, cytotoxicity and genotoxicity

Possible implications from copper presence in DWDS for public health are presented along this chapter, in particular changes in *S. maltophilia* virulence, and cytotoxicity and genotoxicity for HT29 cell line.

8.1 Results

8.1.1 Copper effect on *S. maltophilia* virulence factors production

The exposure of *S. maltophilia* to copper solutions or the contact with copper materials may have a slight impact on bacterial motility and production of virulence factors (Table 8-1 and Table 8-2).The exposure to different copper concentrations (0.8, 1.3, 2.0 and 2.8 mg/L) had no significant impact on *S. maltophilia* motility ($P > 0.05$). Nevertheless, the exposure to the 96% copper alloy caused a significant reduction in swimming and twitching halos ($P < 0.05$). No significant variation on swimming, swarming or twitching motilities of *S. maltophilia* was verified after exposure to materials with 0, 57, 79 and 100% of copper content (Table 8-1).

Table 8-1. *S. maltophilia* motility after exposure to copper solutions and copper materials for 24 h. Mean of halo diameter ± SD (mm).

Motility type	[Copper] (mg/L)					Surface copper content (%)					
	0	0.8	1.3	2	2.8	0	57	79	83	96	100
Swimming	11.4 ± 3.7	10.8 ± 2.5	11.9 ± 3.9	11.5 ± 3.9	10.0 ± 1.9	14.1 ± 5.2	9.5 ± 1.5	11.3 ± 3.1	9.8 ± 1.7	8.0 ± 3.5*	10.4 ± 4.2
Swarming	4.0 ± 0.9	4.3 ± 1.0	4.2 ± 0.4	4.2 ± 1.0	4.5 ± 1.0	4.7 ± 1.0	3.8 ± 0.9	3.8 ± 1.2	3.8 ± 1.2	3.2 ± 0.8	3.8 ± 0.6
Twitching	5.3 ± 0.8	4.9 ± 0.8	5.1 ± 0.9	5.1 ± 0.9	5.1 ± 0.9	5.8 ± 1.5	4.6 ± 1.0	5.4 ± 1.5	4.9 ± 0.8	3.8 ± 0.4*	4.8 ± 0.6

* - statistically significant differences from the respective control (surface with 0% of copper content) ($P < 0.05$)

Table 8-2. Production of virulence factors by S. maltophilia (protease, gelatinases and siderophores production) after the exposure to copper solutions and copper materials for 24 h. Mean of halo diameter ± SD (mm).

Virulence factors	[Copper] (mg/L)					Surface copper content (%)					
	0	0.8	1.3	2	2.8	0	57	79	83	96	100
Proteases	21.3 ± 8.2	32.2 ± 3.8	35.5 ± 5.2*	30.5 ± 4.2	28.8 ± 7.4	19.7 ± 8.8	18.0 ± 6.3	19.7 ± 6.1	10.3 ± 9.3	n.g	12.2 ± 5.8
Gelatinase	13.7 ± 1.0	13.2 ± 1.0	12.9 ± 1.8	11.2 ± 1.0	9.6 ± 0.9*	11.9 ± 1.5	10.4 ± 0.8	10.8 ± 1.1	11.6 ± 1.3	n.g	8.0 ± 1.9*
Siderophores	2.4 ± 0.5	2.5 ± 0.4	2.7 ± 0.4	2.7 ± 0.5	2.8 ± 0.3	2.7 ± 0.4	2.1 ± 0.4	2.4 ± 0.5	2.6 ± 0.3	2.3 ± 0.3	2.4 ± 0.7

* - statistically significant differences from the respective control (0 mg/L of copper or surface with 0% of copper content); n.g. No growth observed ($P < 0.05$)

Protease production was stimulated by the exposure to copper solutions at different concentrations (Figure 8-1). Statistically significant increase of protease production was observed after *S. maltophilia* exposure to 1.3 mg/L of copper ($P < 0.05$). Oppositely, the contact with copper materials decreased protease production, although not statistically significant ($P > 0.05$). *S. maltophilia* was not able to growth on PCA with skim milk agar medium after being exposed to 96% copper alloy for 24 h.

The exposure for 24 h to 2.8 mg/L of copper reduced significantly the production of gelatinases by *S. maltophilia* ($P < 0.05$). Nevertheless, no significant change of gelatinase production was observed when *S. maltophilia* was exposed to 0.8, 1.3 and 2.0 mg/L of copper ($P > 0.05$). The exposure to 57, 79 and 83 % copper alloys had not significant impact on gelatinase production ($P > 0.05$). Nevertheless, the exposure to elemental copper reduced *S. maltophilia* gelatinase production. Similarly to the observed during the protease assay, *S. maltophilia* was not able to growth on gelatin agar medium after exposure to 96 % copper alloy.

On the other hand, the exposure to copper solutions and materials had not significant impact on *S. maltophilia* siderophores production ($P > 0.05$).

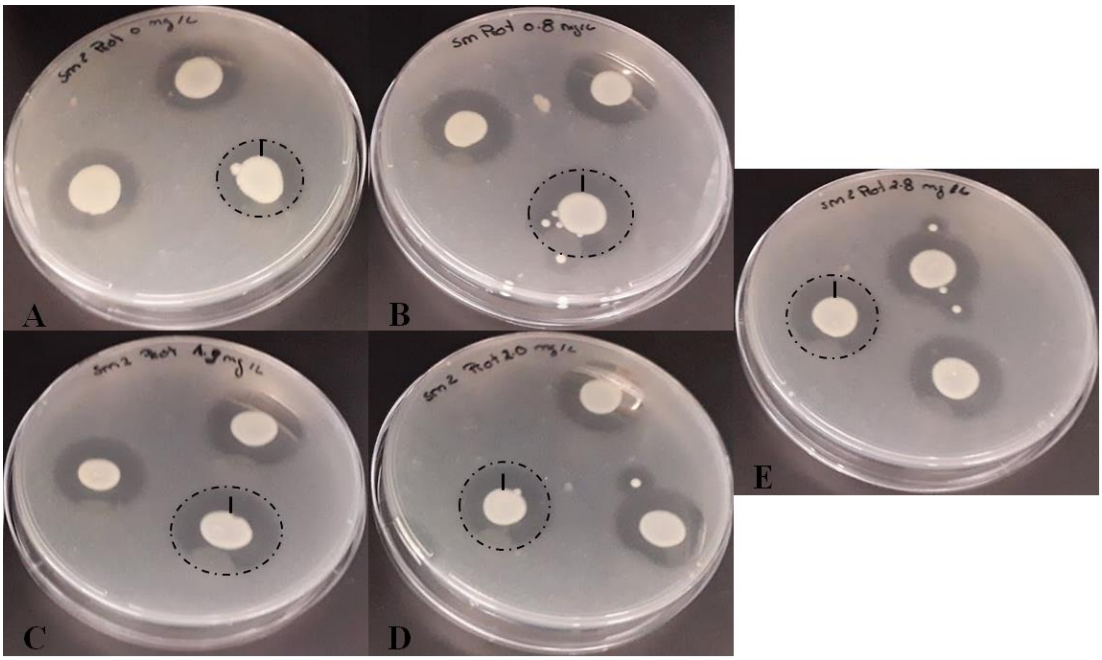

Figure 8-1. *S. maltophilia* growth on skim milk PCA plates for determination of protease production. Clearance halo indicates protease production. A - *S. maltophilia* non-exposed to copper solution. B - *S. maltophilia* exposed to 0.8 mg/L of copper. C - *S. maltophilia* exposed to 1.3 mg/L of copper. D - *S. maltophilia* exposed to 2.0 mg/L of copper. E - *S. maltophilia* exposed to 2.8 mg/L of copper. ___ segments with equal length for easier comparison of halo dimension. ----- Halo delimitation for easier comparison of halo dimension.

8.1.2 Effects of copper leachates on HT29 cells viability

It is important to understand if the leachates resulting from the contact of DW with copper alloys may constitute some risk for consumer's health. The use of copper materials in unchlorinated systems had not a significant impact on HT29 cell line viability (92.1 to 98.4% of cells remained viable – Figure 8-2.A). Nevertheless, the presence of chlorine in DWDS may be responsible for the production of more toxic leachates. The exposure to leachates resulting from alloys with 57, 79, 83 and 96% of copper exposed to 1 mg/L chlorine caused a significant decrease in HT29 viability ($P < 0.05$). The most toxic effects were obtained after exposure to leachates from alloys with 83 and 79% of copper exposed

to chlorine. Leachates resulting from elemental copper exposure to chlorinated water were not toxic for HT29 ($P > 0.05$).

In order to understand if the toxic effects were caused by copper leaching or by the leaching of other metals, HT29 cells were also exposed to solutions with different copper concentrations: 0.8 mg/L (minimum copper concentration detected in leachates -Figure 6-5), 2.8 mg/L (maximum copper concentration detected in leachates –Figure 6-5) and 1.3 and 2.0 mg/L (maximum recommended copper concentration in DW - according EPA and WHO). The results demonstrated that the toxic effect of copper was not directly correlated with the solution concentration (Figure 8-2.B). Higher toxic effects were obtained after exposure to 1.3 mg/L of copper (64.9% of cells remained viable), followed by the exposure to 2.8 mg/L of copper (81.5% of cells remained viable) ($P < 0.05$).

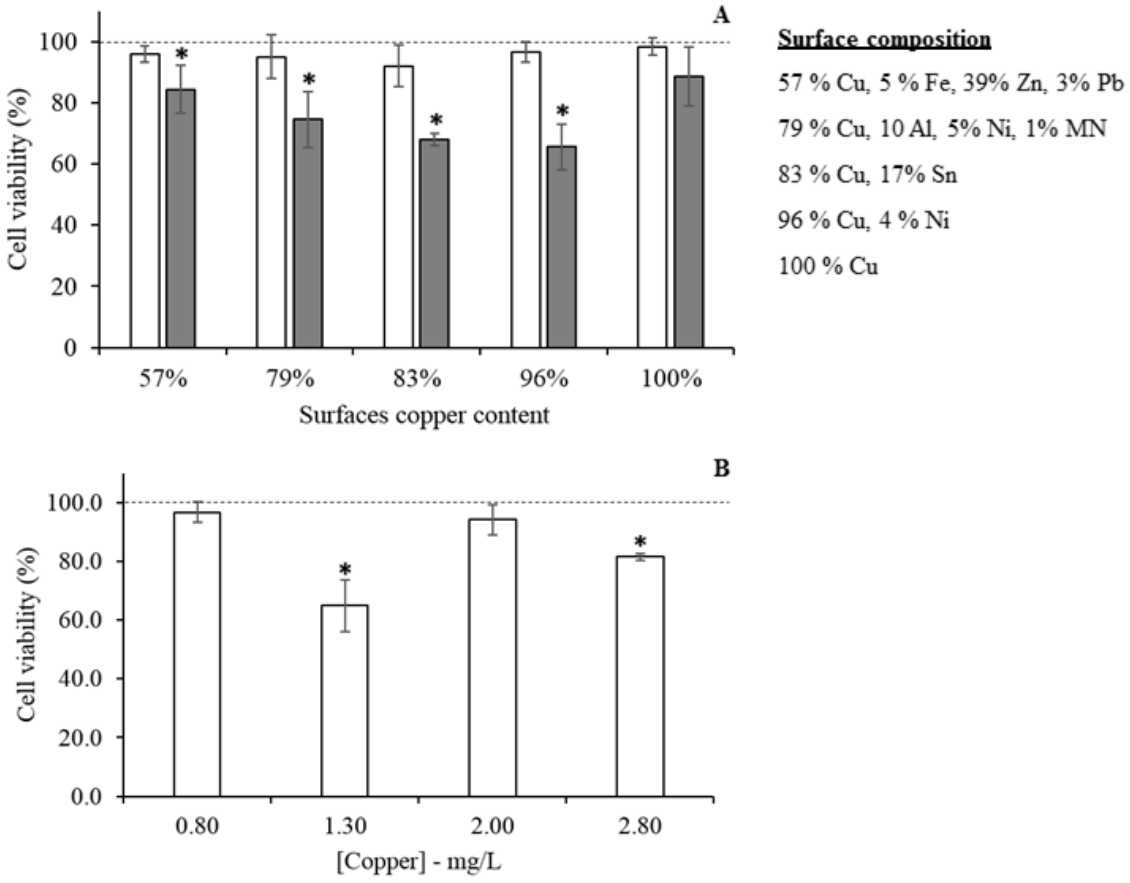

Figure 8-2. A – HT29 cells viability after exposure to leachates from materials with different copper content non-exposed to chlorine (□); and exposed to chlorinated STW (1 mg/L of free chlorine) (■). B - HT29 cells viability after exposure to solutions with different copper concentrations. (---) Non–exposed HT29 cells (control). * - Results statistically different from the control.

8.1.3 Genotoxic effects of copper leachates on HT29 cells

The effects of exposure to copper leachates or copper solutions on DNA damage of HT29 cells were assessed through the alkaline comet assay (Figure 8-3). Leachates resulting from the exposure of materials containing 79, 83, 96 and 100% of copper exposed to unchlorinated water significantly increased DNA damage of HT29 cells ($P < 0.05$). Nevertheless, leachates from 57% copper alloy exposed to unchlorinated water had not significant genotoxic effects to HT29 cells ($P > 0.05$).

Higher variations in DNA damage were observed in leachates resulting from materials exposed to chlorinated water, as represented by higher standard deviations. Therefore,

statistically significant increase of DNA damage was only observed with leachates of 96% copper resultant from chlorine exposure ($P < 0.05$). For all the other leachates formed in the presence of residual chlorine, no significant differences were observed regarding DNA damage of HT29 cells. The exposure to elemental copper at 1.3 mg/L also induced DNA damage ($P < 0.05$), yet copper at 0.8, 2.0 and 2.8 mg/L no significant genotoxic effects were observed ($P > 0.05$).

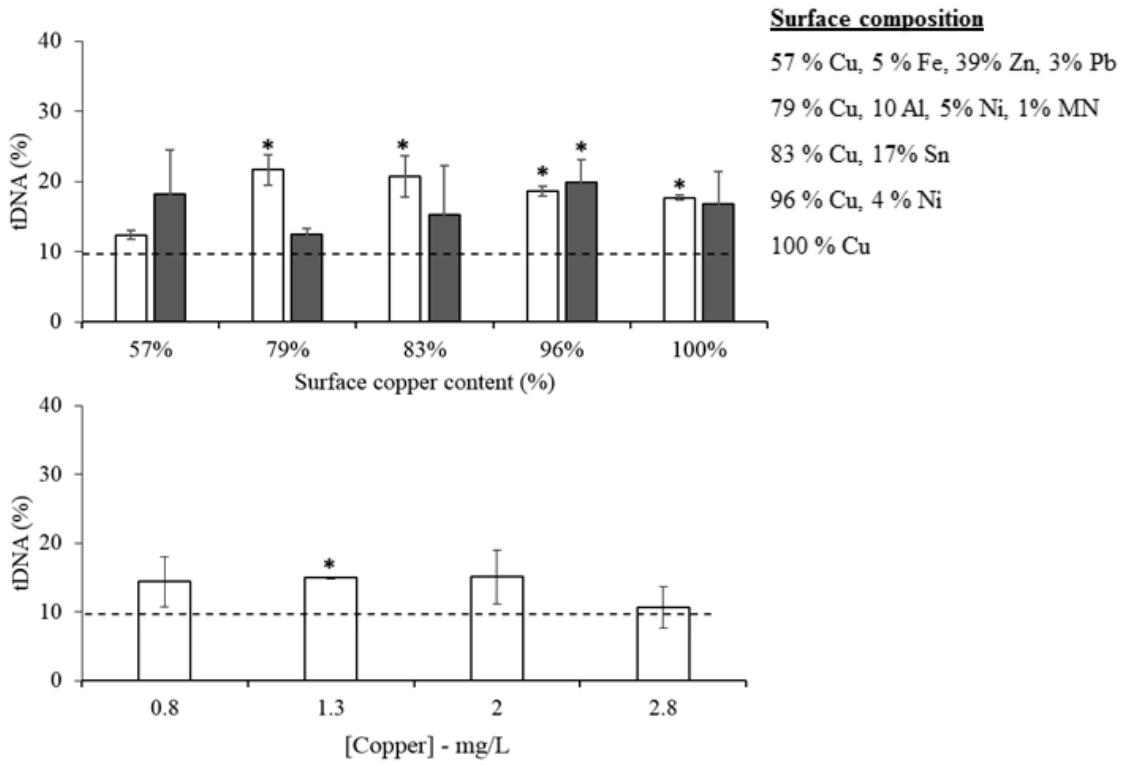

Figure 8-3.A - DNA damage (expressed as % of DNA intensity in comet tail) of HT29 cells after exposure to leachates from materials with different copper content non-exposed to chlorine (□); and exposed to chlorinated STW (1 mg/L of free chlorine) (■). B - DNA damage of HT29 cells after exposure to solutions with different copper concentrations. (---) Non-exposed HT29 cells(control) *- Results statistically different from the control ($P < 0.05$).

8.2 Discussion

The use of copper materials in DWDS may have an important role in the control of biofilms formation and in decreasing the consumers' exposure to pathogens in viable state (Chapter 7). Nevertheless, it is important to understand the effects from copper exposure on the bacteria able to grow along the DWDS and may reach the consumers' tap. *S. maltophilia* has several characteristics that confer pathogenicity, such as the secretion of extracellular enzymes (proteases, lecithinases, gelatinases, lipase,s hyaluronidases, heparinases, DNAses, etc), siderophores production, biofilm formation ability and motility (Brooke 2012, Thomas *et al*. 2014). The present results demonstrated that *S. maltophilia* isolated from DW was motile (demonstrated swimming, swarming and twitching motilities) and was also able to produce proteases, gelatinases and siderophores. Extracellular proteases play a crucial role in the invasion of host cells, damaging host connective tissues (*i.e.* collagen and fibronectin) and evading host defense (Brooke 2012, Thomas *et al*. 2014). Gelatinases have important action in biofilm formation, however, they also facilitate host cell invasion due to the degradation of several host tissues (*i.e.* collagen, fibrinogen, fibrin, endothelin-1, bradykinin, LL-37, and complement components C3 and C3a) (Thurlow *et al*. 2010, Zeng *et al*. 2005). As referred in Chapter 5 siderophores are molecules produced by microorganisms whose main function is to chelate the ferric iron (Fe^{3+}) from different environments making it available for the producer microorganism. These molecules are produced during infectious processes to acquire ferric iron from hosts (Wilson *et al*. 2016). Bacterial motility is also an important aspect to pay attention as it may encourage biofilm formation and the interaction of pathogens with host cells (Trifonova and Strateva 2018). Therefore, it is of utmost importance to understand if copper exposure can affect *S. maltophilia* production of

virulence factors and, consequently, be a concern for the consumers' health. *S. maltophilia* motility was not affected by the exposure to ionic copper; nevertheless, the contact with 96% copper alloy may decrease *S. maltophilia* swimming and twitching motilities. Cruz *et al*. (2014) also observed a decrease in twitching motility of *Xilella fastidiosa* associated with the presence of a copper solution. The inhibition of swimming and twitching motilities by 96% copper alloy may justify the high performance of this alloy on biofilm control and prevention of biofilm regrowth. Although the antimicrobial action of the copper alloy may also influence results due to a possible decrease in the abundance of culturable bacteria which will influence the halo diameter formed on agar plates. The results demonstrated that the production of extracellular proteases may be stimulated after *S. maltophilia* exposure to copper solutions. Oppositely, the exposure to copper materials had not the same effect, possibly because the availability of copper ions is lower. The stimulation of protease production was observed in contact with copper concentrations similar or below the maximum recommended values by EPA and WHO (EPA 2016, WHO 2003b). This may constitute an additional concern when using copper materials for DW transport. Doddapaneni *et al*. (2009) also observed that the presence of copper may enhance the activity of proteases produced by *Bacillus cereus*, suggesting that these proteins may be metalloproteases, depending on the presence of copper to be active. Nascimento and Martins (2004) observed that copper may inhibit the activity of a protease produced by a thermophilic *Bacillus* sp. strain.

Copper solutions and materials had not a huge impact on gelatinases production. A slight decrease on the activity of produced gelatinases was observed after exposure to copper at 2.8 mg/L and to elemental copper surface. Therefore, the activity of these enzymes will not be stimulated in *S. maltophilia* colonizing copper DWDS.

Copper is an essential element required for the activity of many metalloenzymes in the human body, however, this metal can also be toxic (Gupta and Gupta 2011). Acute copper toxicity in humans is rare, however, the intake of 0.1 to 0.2 mg Cu/kg body weight can lead to gastrointestinal disturbances in more sensitive population (Gupta and Gupta 2011). Since the use of copper materials in DWDS may increase the consumers' exposure to copper ions and to other metals leached from alloys, in the present study a situation of copper intake was simulated *in vitro* to evaluate possible cytotoxic and genotoxic effects. HT29 cells (from human colorectal adenocarcinoma) were exposed to leachates resulting from copper alloys in contact with chlorinated and unchlorinated water. Copper solutions at concentrations similar to those quantified in leachates (0.8 and 2.8 mg/L) and to the maximum recommended by regulatory agencies (1.3 and 2.0 mg/L, EPA and WHO, respectively) were used in order to understand if the effects observed in HT29 cells exposed to leachates were caused by copper or by the combination of different materials leached from alloys. Leachates resulting from copper alloys exposed to unchlorinated water did not exhibit cytotoxic activity on HT29 cells, while leachates resulting from copper materials exposed to chlorine at 1 mg/L caused a slight reduction of HT29 cells viability. Nevertheless, copper concentrations in leachates from 57, 79, 96 and 100% copper materials was below the maximum recommended value (1.3 mg/L). Therefore, their toxic effects may be due to the presence of other metals in solution or due to the combined effect of different metals. The 96 % copper alloy is also constituted by 4% of nickel. The presence of this metal may also be responsible for the decrease on cell viability and the increase of DNA damage. Milheiro *et al*. (2016) observed cytotoxic effects of copper and nickel at 10 ppm in a mouse fibroblasts cell line. Zarei *et al*. (2018) found that nickel may cause toxic effects to human blood lymphocytes. Nickel ions may increase cell death, DNA fragmentation and ROS formation in human leukemia HL-60

cells (Jia and Chen 2008). The alloy with 83% copper is also composed by 17 % of tin (Sn). Cytotoxic and genotoxic effects of respective leachates seems not related to the combined effect of both metals. In fact, Milheiro *et al*. (2016) did not observe cytotoxic effects of Sn in mouse fibroblasts cell line 929. The toxic effect of 83% copper alloy seems to be related to the high leaching of copper in the presence of chlorine (2.8 mg/L of copper were quantified in leachates, overcoming the maximum recommended values – Chapter 6). Regarding the 79% copper alloy, it is also composed by 10% aluminum and 5% nickel. Therefore, these metals may also be present in respective leachates. Kamalov *et al*. (2011) evaluated the cytotoxic effect of environmental relevant concentrations of aluminum in murine thymocytes and lymphocytes. Concentrations of 10, 20 and 30 µM of aluminum chloride caused injuries in murine thymocytes and lymphocites (Kamalov *et al*. 2011). The present study demonstrated that the use of 57% copper alloys in DWDS will have less impact for consumers' health than all the other materials. Lower toxicity of this alloy was predictable attending that no copper was detected in its leachates (Chapter 6). Since 57% copper alloy has also a 39% of zinc and 3% of lead content, some toxic effect could be resultant from metal leaching. In a previous study, nanoparticles of copper and zinc (60% of Cu and 40% of Zn) at proportions similar to those present in 57% copper alloy had cytotoxic and genotoxic effects on human lung epithelial cells (Kumbicak *et al*. 2014). The differences between the results obtained in the present work and those obtained by Kumbicak *et al*. (2014) may possibly be due to the high surface area of nanoparticles, the concentration tested and the low presence of these metals in the produced leachates.

Regarding the effects of copper solutions, special attention should be given to the effects of the exposure to 1.3 mg/L of copper that demonstrated some cytotoxic and genotoxic effects (ca. 30 % of cell viability reduction and increase of 5% in DNA intensity comet

tail). Keenan *et al*. (2018) evaluated the effects of copper sulfate (32 mg/L) and other sources of organic copper on intestinal cells (HT29 and Caco2 cells) and observed an increase in the production of ROS. The formation of ROS is one of the main causes of copper cellular toxicity. ROS may be responsible for oxidative damage to proteins, lipids and DNA (Keenan *et al*. 2018). Therefore, the oxidative stress caused by the exposure to copper may also justify the DNA damage observed when HT29 were exposed to leachates of copper materials with 100, 96, 83 and 79% copper, and also to copper at 1.3 mg/L. It is important to notice that the leachates from 57% copper alloy did not cause significant DNA damage to HT29 cells. The present results did not demonstrate a correlation between the concentration of copper in solution with its cytotoxicity and genotoxicity. These fact may be caused by the complexation of copper with aminoacids present in RPMI 1640 medium, which may alter the availability of copper in solution (Wu *et al*. 2010). Nevertheless, it is also important to take into account that cancer cells may have high levels of copper transporter proteins which will regulate the needs for copper and consequently copper cytotoxic effects (Cui *et al*. 2017). In the presence of high copper concentrations, the human copper transporter protein (hCtr 1) is inhibited and low µM of copper may be enough to stimulate the transporter (Petris *et al*. 2003). Furthermore, Xiao *et al*. (2016) observed that for concentrations higher than 12 mg/L, a direct correlation was observed between cytotoxicity and copper concentration for the HT29 cell line.

The overall results demonstrate that copper use in DWDS and copper leaching may alter the virulence of *S. maltophilia.* Copper leaching may stimulate the protease production by *S. maltophilia*, however, it may decrease the production of gelatinase and *S. maltophilia* swimming and twitching motilities. The leachates from copper materials demonstrated higher cytotoxic effects when copper materials were used in chlorinated STW. Nevertheless, all the leachates caused DNA damaged of HT29 cells, with the

exception of 57% copper alloy - demonstrated lower cytotoxic activity. It is also important to notice that these effects may be caused by the leaching of different metals that were not quantified, such as aluminum, nickel or zinc. The presence of copper at 1.3 mg/L, the maximum recommended value by EPA for DW, caused significant reduction of cell viability and an increase on HT29 cells DNA damage.

8.3 Conclusions

The presence of copper in DW or the use of copper materials had no worrying impact in *S. maltophilia* virulence factors, with exception of protease production. Protease production was stimulated by the exposure to copper solutions at different concentrations.

Chlorine in DWDS may be responsible for the increase of leachates causing cytotoxic effects. Copper leachates significantly increased the DNA damage in HT29 cells, even when copper materials were not exposed to chlorine. Nevertheless, the use of 57% copper alloy in chlorinated or unchlorinated DWDS had low impact on HT29 cells and on *S. maltophilia* virulence.

Concluding remarks and future work

The major conclusions of the presented work are described in this chapter as well as some recommendations of future work that would be important to complement the present study.

General conclusions

The presence of ECs in DWDS can constitute a cause of concern for consumers and DW companies regarding their effects on the behaviour of the DW-colonizing microbiota. This study provides new information about the consequences of ECs presence in DWDS for the selected bacteria isolated from DW. The exposure to some ECs (DCF, IBP, TY and CA+CBZ+IBP at 100 × [DW]) for 26 d altered *S. maltophilia* ability to form biofilms and their tolerance to NaOCl (CA+CBZ at [DW] and CA+CBZ+IBP at 100 × [DW]). The simultaneous presence of different compounds in DW can potentially hinder the disinfection of biofilms in DWDS. As CA was the common compound in all the combinations of ECS tested that altered *S. maltophilia* behaviour, the impact from the exposure to this EC was studied in more detail and for longer periods (12 weeks). The presence of trace concentrations of CA did not affect planktonic *S. maltophilia* tolerance to chlorine, neither its ability to form biofilm or to produce virulence factors. Nevertheless, the presence of CA in DW was responsible for the formation of biofilms with higher tolerance to chlorine disinfection. Of particular concern was the increase of *S. maltophilia* tolerance to erythromycin after being exposed to CA at 17000 ng/L. Oppositely, CA exposure decreased the ability of *S. maltophilia* to internalize human colonrectal cells (HT29 cell line).

Regarding the effects of copper materials on DW bacteria it is important to highlight that alloys containing lower copper content were also efficient in impairing biofilm formation, with results comparable to elemental copper. All the copper materials reduced culturability and viability of *A. calcoaceticus* and *S. maltophilia* from single and dual species biofilms. The most promising results were obtained using the 96% copper alloy that had antimicrobial activity but also avoided biofilm regrowth. All the alloys were resistant to corrosion and leaching, even in the presence of residual chlorine. The

exception was the 83% copper alloy that was prone to prohibitive leaching in the presence of chlorine.

The use of a RCR allowed the formation of mature biofilms for 7 days in conditions that mimic real DWDS and the application of convention DWDS treatment strategies. Copper materials demonstrated better performance in biofilm prevention/control than chlorine as higher CFU reduction were obtained using copper materials than from the use of chlorine against biofilms formed on SS. The application of shock treatments (10 mg/L of free chlorine for 10 min and/or high shear stress – 1.5 m/s for 30 s) was not effective in biofilm inactivation or removal. This work also allowed to conclude that biofilm disinfection was dependent on the material used for biofilm formation. Culturability reduction due to chlorine treatment was only observed in S. *maltophilia* biofilms formed on 96% copper alloy. Flushing also was not effective in biofilm control and the combination of both treatments (free chlorine at 10 mg/L for 10 min and fluid velocity of 1.5 m/s for 30 s) only had positive impact in the control of biofilms formed on the 57% copper alloy. Therefore, the strategies (residual chlorine and flushing) commonly used to control critical levels of contaminations in DWDS were not effective in biofilm control. However, the analysis of bulk water to understand the influence of copper on bacteria released into transported water during conventional treatments allowed to conclude that copper materials in DWDs have an important role reducing the number of viable *S. maltophilia* in the transported water. These results may have significant impact on the choice of pipe materials for plumbing systems since copper may be important to reduce the consumers' exposure to possible pathogens through the transported water.

It is also important to evaluate possible consequences from copper use on consumers´ health. Copper leachates have not a worrying impact on *S. maltophilia* virulence factors. However, the use of copper in DWDS may increase the production of proteases by

S. maltophilia. The presence of chlorine in DWDS lead to the production of more toxic leachates that caused reductions of HT29 cells viability and increased DNA damage. Nevertheless, the use of 57% copper alloy in chlorinated or unchlorinated DWDS had low impact on HT29 cells and on *S. maltophilia* virulence. Therefore, this alloy has apparently reduced public health impact in terms of cytotoxicity and genotoxicity. It is important to highlight that the antimicrobial properties and the toxicity of alloys can be the result of the combined effect of different metal elements. Therefore, the impact of copper alloys in DW biofilm control clearly proposes the need for further research on the combined effect of different metals in biofilm development and DW quality.

Future work

In order to improve the understanding of the impact of ECs and copper on DW microbiome, there are several aspects requiring further research.

In the present study only one bacterial strain was evaluated and relatively significant changes were observed on its behaviour when exposed to ECs. It would be important to evaluate the effects of ECs in other bacterial strains and in real DW biofilms. The present work, even if pioneer, focused on the impact of ECs in some bacterial phenotypic alterations. However, it would be interesting to assess the impact of ECs on ARG expression and in the abundance of ARB. The study of ECs` impact on bacterial proteome and production of extracellular proteins and on biofilm structure and EPS composition would also be important aspects to understand their effects on bacterial behavior.

A study about the presence and frequency of detection of ECs in Portuguese tap water would be important for the selection of different ECs used to implement a more realistic approach to the Portuguese scenario. The presence of ECs able to adsorb on organic matter should be studied - quantifying these ECs on the biofilm matrix and predicting

situations of accumulation and increased exposure to ECs would facilitate the understanding on the potential biomagnification of effects from such increased concentrations.

In the literature review, the reaction of chlorine with ECs is reported as ECs removal strategy. This reaction should be studied in more detail, in order to understand how ECs may hinder the disinfection power of chlorine and what are the DBPS formed and its consequences for the microbiome.

The ability of *S. maltophilia* to invade HT29 cells was evaluated. Nevertheless, the evaluation of the ability to invade different tissues before and after exposure to ECs or copper materials, using adequate animal models, would also be interesting.

In order to better conclude about the importance of copper materials in DW biofilm control, these should be formed by natural DW microbiome and studied for longer periods. Based on the best results, it would be interesting to study the effects of the metal content and diversity in biofilm prevention. For example, it would be important to understand the effects of copper and aluminum alloys or copper and zinc alloys, attending the good performance obtained by the use of both metallic combinations. The study of the effects of copper on biofilm cohesiveness and consequent resistance using atomic force microscopy would be an intersting topic that will complete this work.

The study of the effects of water flow, water composition and temperature on new copper materials corrosion, leaching and ability to control biofilms would also give relevant information about the use of copper alloys in plumbing systems.

The use of copper materials may be important to retard biofilm formation, prevent consumers' exposure to pathogens and to overcome chlorine decay. However, the impact of copper on bacterial behaviour in the presence of ECs was not evaluated. This would

be an important topic as well as the role of copper materials and leachates on the acquisition of antimicrobial resistance elements.

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Annex A - Synthetic tap water characterization

Synthetic tap water (STW) was used in the experiments in order to simulate real DW conditions. The characteristics of this solution are presented in Table A-1.

Table A-1. Synthetic tap water characterization.

Parameters	Measured values
pH	8.5 ± 0.2
Conductivity (µS/cm)	222 ± 12
Temperature (ºC)	23.8 ± 0.2
Ryznar index	8

Annex B - Comparison of different solvents to prepare ECs solutions

In order to select an appropriate solvent for the preparation of ECs solutions, four different solvents were tested (dimethyl sulfoxide – DMSO, ethanol – EtOH, acetone – ACET and propilenoglycol – PG). *S. maltophilia* biofilms were formed for 26 days in the presence of 1% (v/v) of each and the effects on biofilm viability are presented in Figure B-1.

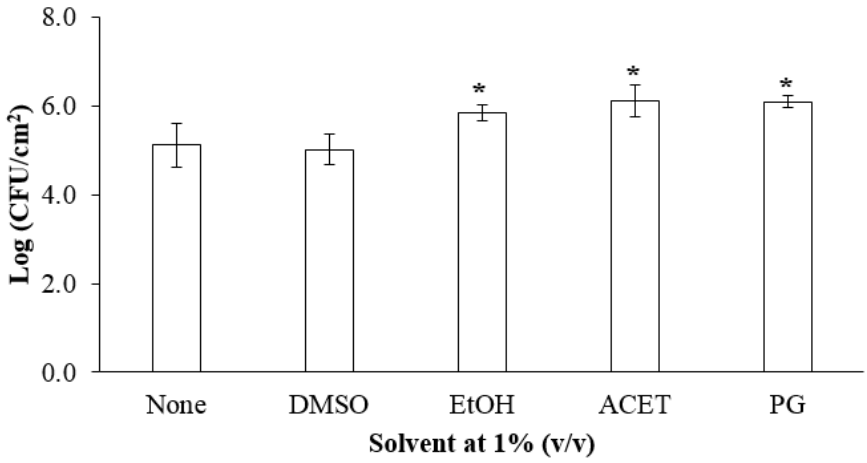

Figure B-1. *S. maltophilia* culturability in biofilms formed in the presence of different solvents at 1 % (v/v) for 26 d.

Results demonstrated that the abundance of culturable *S. maltophilia* increased in the presence of EtOH, ACET and PG ($P < 0.05$) when compared with biofilms formed in the absence of any solvent (synthetic tap water – STW). Nevertheless, biofilms formed in the presence of DMSO had similar numbers of culturable *S. maltophilia* to that of biofilms formed only in the presence of STW.

Annex C - Characterization of copper materials

Materials price

Copper alloys were used attending the possibility to found a less expensive strategy to retard biofilm formation in DWDS than the use of elemental copper. The price of each alloy will be influenced by the metals constituting the alloy. Table C-1 presents the price of each alloy at the time they were purchased (November 2015). At June 2019 metal prices are the described: copper for 5183.95 €/ton, Ni for10797.74 €/ton, Sn for 16913.65 €/ton, Zn for 2407.91 €/ton and Al for 1579.26 €/ton. Therefore, it is possible to understand that the alloy containing 57% of copper and 39% of zinc is the cheaper alternative. However, the price of the 96, 83 and 79% copper alloys are not directly related to the price of each metal.

Table C-1. Price of each alloy at the time they were purchased (November 2015).

	Materials composition						
Material (US Standard)	Cu	Fe	Ni	Sn	Al	Zn	Price (€/kg)
C11000	100	-	-				12.4
C18000	96	-	4				22
C90800	**83**	**-**	**-**	**17**			**6**
C95500	79	-	5		10		13.15
C38500	**57**	**5**	**-**			**39**	**10.30**

Surface characterization

The surface characterization of each material was performed by sessile drop contact angle measurement according Lemos *et al*. (2015a). The main characteristics of each materials are also described in Table C-2. No relationship was observed between surfaces characteristics and the bacterial adhesion.

Table C-2. Surface energy parameters (Lifshitz-van der Waals component- γ^{LW}; electron acceptor component – γ^{+} and electron donor component – γ^{-}) and hydrophobicity ΔG_{iwi} for the different materials selected.

Material/ Copper content	**Surface energy parameters (mJ m^{-2})**				**ΔG_{iwi}**
	γ^{LW}	**γ^{AB}**	**γ^{+}**	**γ^{-}**	**(mJ m^{-2})**
0%	35.862	0	0	11.91	-35.77
57%	35.97	0	0	15.4	-26.27
79%	32.49	0	0	60	52.35
83%	32	2.67	0.042	42.71	26.83
96%	35.07	0	0	26.62	-0.92
100%	37.42	0	0	10.03	-42.24

Annex D - Cytotoxicity and genotoxicity of selected ECs

Exposure to emerging contaminants

HT29 cells were exposed to emerging contaminants (CA, CBZ, GAL and IBP) for 5 days. CA was selected due to the observed effects on *S. maltophilia* behaviour. CBZ is a recalcitrant EC, hard to be removed from the environment, therefore its concentration in water is increasing the importance of understanding possible effects on consumers. IBP was selected as member of the most used pharmaceuticals worldwide, the non-antinflammatory drugs. GAL was selected since it is a musk fragrance, commonly detected in the environment and that had already been detected in human tissue, blood and breast milk (Hu *et al*. 2010, Moon *et al*. 2012, Reiner *et al*. 2007). These ECs were prepared at two concentrations ([DW] and 100 × [DW]) as described in 3.4.1. The exposure initiated when HT29 were seeded in plates. The final concentration of DMSO in cell culture was of 1%. Negative and positive controls were performed, RPMI 1640 and DMSO at 1% in RPMI 1640 were used as negative control and ethanol at 70% was used as positive control. Culture media was replaced by fresh RPMI 1640 containing the tested ECs at selected concentrations after 3 days of incubation.

Cytotoxicity and genotoxicity were assessed as described in Sections 3.84 and 3.8.5.

Figure D.1 represents the cytotoxicity results, regarding the absorbance measured during MTT assay. DMSO slightly reduced the viability of cells. Nevertheless, as DMSO was used as solvent this will be the negative control for comparison of the activity of ECS. No statistically significant differences were observed in HT29 viability when were exposed to ECs. Therefore, no cytotoxic effects were observed.

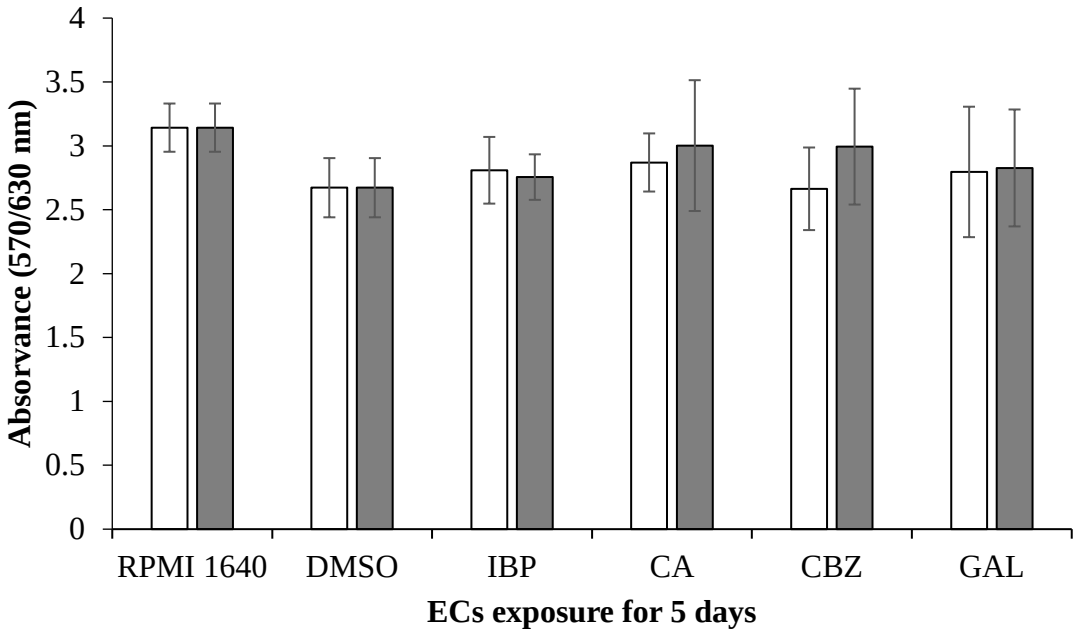

Figure D-1. Cytotoxicity of ECs in HT29 cells after 5 d of exposure. Absorvance measurement at 570 nm with reference to 630 nm, from MTT assay. □ –Exposure to ECs at [DW] for 5 days, ■ – Exposure to ECs at 100 × [DW] for 5 days.

Nevertheless, no conclusion could be obtained attending the genotoxic effects of the selected ECs since the negative control (DMSO) caused significant DNA damage (≈ 30 % of DNA in tail) in HT29 cells which could influence the interpretation of results. Other solvent should be selected to perform these experiments.