

INTEGRATED MASTER IN BIOENGINEERING

The effects of emerging contaminants on the behaviour of drinking water-isolated bacteria

Dissertation for Master's degree in Bioengineering
Specialization in Biological Engineering

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Work Outputs

Preliminary results of the present dissertation have been published on the following:

1. Pinto, I. C., Gomes, I.B., Simões, M., The effects of emerging contaminants on the behaviour of *Acinetobacter calcoaceticus* isolated from drinking water. Book of Abstracts of the 14th Meeting of Young Researchers at the U.PORTO, 2021.
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Abstract

Anthropogenic activities have led to the continuous emission of toxicants into aquatic environments. However, even after treatment, they persist in treated water and make their way into the consumer's tap. Emerging contaminants (EC) are frequently detected at trace level concentrations (ng/L e µg/L) and still to this day, little is known about their ecotoxicological effect in drinking water distribution system (DWDS) microbiota. Given the issues associated with the formation of biofilms in DWDS, this work studied the effects of a 7-day exposure of *Acinetobacter calcoaceticus* biofilms to four ECs, at environmental concentrations, each representing a different kind of commonly found class of pharmaceuticals on drinking water (DW): caffeine – CAF (stimulant and psychoactive drug), carbamazepine – CBZ (anticonvulsant and neuroactive steroid), ciprofloxacin – CIP (antibiotic) and ibuprofen – IBP (non-steroidal anti-inflammatory drug). The exposure assays were performed either individually or combined (MIX). Effects evaluated aim to predict and better understand possible effects of selected ECs on DWDS biofilms, namely: their composition (EPS and cellular density) and bacteria phenotypical characteristics (ability to form biofilms, susceptibility to antibiotics (CIP, levofloxacin (LEV), tetracycline (TET) and trimethoprim-sulfamethoxazole (TMP-SMX)), tolerance to DWDS disinfection methods (NaOCl), motor capabilities, auto and coaggregation ability, and growth rate). The results suggest that exposure to ECs affected biofilm structure and bacterial behaviour. IBP exposure decreased *A. calcoaceticus* biofilm formation ability by 11%, while MIX exposure increased by 16%. In regards to antibiotics susceptibility, CAF exposure decreased *A. calcoaceticus* susceptibility to TPM-SMX, while CBZ and CIP exposure caused an increase on the susceptibility to TMP-SMX. Furthermore, CIP exposure decreased the susceptibility to CIP and LEV, and IBP exposure decreased the susceptibility to LEV and TET. The ranges of minimum bactericidal concentration (MBC) of NaOCl have also increased (for CIP and IBP exposure) or decrease (exposure to CBZ). Higher *A. calcoaceticus* growth rates were registered after CIP exposure. Lastly, biofilm structure studies revealed that biofilms exposed to MIX contain higher cellular density. In conclusion, the net results show that significant changes in *A. calcoaceticus* behaviour were observed, and that these pose a threat to DWDS and DW consumers, regarding the aspects evaluated. Specifically, IBP and CIP seem to be ECs of concern due to its impact on bacteria tolerance to antibiotics and NaOCl. This work also emphasizes the importance of studying the effects of EC exposure, and how this kind of studies lay the groundworks for knowledge enrichment regarding bacterial behaviour in biofilms and their impact in DWDS.

Keywords: *Acinetobacter calcoaceticus*, Antimicrobial resistance, Biofilms, Drinking water bacteria, Growth rate, Motility, Pharmaceutical contaminants

Resumo

As atividades antropogénicas levam à contínua emissão de tóxicos para ambientes aquáticos. Mesmo após tratamento, eles persistem na água tratada e chegam às torneiras dos consumidores. Contaminantes emergentes (EC) são compostos frequentemente detetados a concentrações residuais (ng/L e µg/L) e, atualmente, pouco se sabe sobre o seu efeito ecotoxicológico na microbiota de sistemas de distribuição de água potável (DWDS). Face à problemática de formação de biofilmes em DWDS, este trabalho avalia e averigua os efeitos causados por exposição de biofilmes de *Acinetobacter calcoaceticus* a concentrações residuais de quatro ECs representativos de diferentes classes de fármacos detetados em água potável (DW): cafeína – CAF (estimulante psicoativo), carbamazepina – CBZ (fármaco neuroativo), ciprofloxacina – CIP (antibiótico) e ibuprofeno – IBP (anti-inflamatório não esteroide), quer individualmente ou em mistura (MIX). Os efeitos avaliados visam prever e compreender possíveis efeitos destes ECs sobre biofilmes de DWDS, em particular: sua composição (EPS e densidade celular) e comportamento microbiano (capacidade de formar biofilmes, suscetibilidade a antibióticos (CIP, levofloxacina (LEV), tetraciclina (TET) e trimetoprima-sulfametoxazol (TMP-SMX)), tolerância a métodos de desinfecção em DWDS (NaOCl), capacidades motoras, capacidade de auto e coagregação, e taxa de crescimento). Os resultados sugerem que a exposição de biofilmes a ECs afetou a estrutura do biofilme e comportamento bacteriano. A exposição a IBP diminuiu a capacidade de formação de biofilme de *A. calcoaceticus* por 11%, enquanto a exposição a MIX induziu um aumento de 16%. Em relação à suscetibilidade aos antibióticos, a exposição a CAF diminuiu a suscetibilidade de *A. calcoaceticus* a TPM-SMX, enquanto que a exposição a CBZ e CIP causaram um aumento. Além disso, a exposição CIP diminuiu a suscetibilidade CIP e LEV, e a exposição IBP diminuiu a suscetibilidade LEV e TET. As faixas de concentração bactericida mínima (MBC) de NaOCl também aumentaram (para exposição a CIP e IBP) ou diminuíram (para exposição a CBZ). Maiores taxas de crescimento de *A. calcoaceticus* foram registadas após a exposição CIP. Por fim, os estudos da estrutura do biofilme revelaram que os biofilmes resultantes da exposição a MIX contêm maior densidade celular. Em geral, os resultados demonstram que ocorreram mudanças significativas no comportamento de *A. calcoaceticus*, e que estes representam uma ameaça para os membros e componentes de DWDS, quanto aos aspetos avaliados. Em particular, IBP e CIP são ECs de relevância, face ao impacto apresentaram na tolerância a antibióticos e NaOCl. Este estudo também enfatiza a importância de estudar os efeitos da exposição a EC e como estudos deste tipo estabelecem as bases para o enriquecimento do conhecimento sobre o comportamento bacteriano em biofilmes e seu impacto em DWDS.

Palavras-chave: *Acinetobacter calcoaceticus*, Biofilmes, Bactérias da água potável, Contaminante farmacêuticos, Mobilidade, Resistência a agentes antimicrobianos, Taxa de crescimento

Declaration

I, the undersigned, hereby declare on my honour that the work described within this document is original and any non-original work or sources from which I have quoted or drawn reference from have been properly cited.

A handwritten signature in black ink, reading 'Isabel Chio Pinto'.

February 18th, 2022

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Glossary

Abbreviations

AHTN	Tonalide	MH	Muller-hinton
ANTP	Antipyrine	MLs	Macrolides
ARB	Antimicrobial resistant bacteria	MPB	Methylparaben
ARG	Antimicrobial resistant genes	MIX	Mixture
ATE	Atenolol	NSAID	Non-steroidal anti-inflammatory drugs
BSA	Bovine serum albumin	OP	Opportunistic pathogens
CA	Clofibric acid	PHAC	Pharmaceutically active compounds
CAF	Caffeine	PI	Propidium iodide
CAP	Chloramphenicol	QLs	Quinolones
CBZ	Carbamazepine	SAs	Sulfonamides
CIP	Ciprofloxacin	SDZ	Sulfadiazine
DCF	Diclofenac	SMX	Sulfamethoxazole
DMSO	Dimethyl sulfoxide	STW	Synthetic tap water
DW	Drinking water	TET	Tetracycline
DWDS	Drinking water distribution systems	TMP	Trimethoprim
DWTP	Drinking water treatment plants	TON	Tonalide
EC	Emerging contaminant	TY	Tylosin
EPS	Extracellular polymeric substances	WHO	World health organization
ETM	Erythromycin	WTP	Water treatment plant
GAL	Galaxolide	WWTP	Waste water treatment plant
GFZ	Gemfibrozil		
HCTZ	Hydrochlorothiazide		
IBP	Ibuprofen		
LEV	Levofloxacin		
met	Metoprolol		
MGE	Mobile genetic elements		

Symbols and equation variables

Abs_t	Absorbance at t time	
Ab_{St0}	Absorbance at t=0	
A_{coupon}	Surface area of a single coupon	
A_{image}	Equivalent area covered by the image	
$A_{nucleopore\ filter}$	Area of the Nucleopore filter	
μ	Specific growth rate	h^{-1}
N_{cells}	Number of cells per unit area	
N		
t_d	Duplication time	h
$V_{inocule}$	Volume of biofilm sample	
V_{sample}	Sample volume	

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Chapter 1. Introduction

1.1. Project presentation and motivations

Emerging contaminants (ECs) are occurring pollutants (chemicals or microorganisms), found in groundwater, surface water, wastewater, drinking water (DW), food sources, among others, which have been described to be responsible for adverse ecological and/or human health effects ^[1]. Current water policies only focus on few legacy pollutants, however, they vary in numbers and properties ^[2]. And, too often ECs are evaluated in ecotoxicological studies at high concentrations (therapeutic doses or higher values). Thus, the results most likely prove that they are able to induce mutations or have toxic effects in tested organisms ^[3]. On the contrary, it is also important to study the effects at environmentally relevant concentrations, or trace concentrations of ECs to be more precise, as chronic exposures can lead to unpredictable and undesirable outcomes ^[4], in both target and non-target organisms ^[5]. This subject is still much underexplored ^[6]. Furthermore, in the context of drinking water distribution systems (DWDS), the formation of biofilms is responsible for many water quality, safety and operational problems ^[3]. DWDS are structurally, operationally, physiochemically and microbiologically diverse systems, and biofilm formation is an inevitable phenomenon in an environment particularly rich in ecological niches where microorganisms can thrive and proliferate ^[7]. If ECs persistence and subsequent exposure were to trigger adverse effects in the DWDS microbial community (as it has been observed in other aquatic environments and diverse organisms), water quality could be compromised. Therefore, it is crucial to recognize ECs presence in DWDS as a possible threat to society. **Figure 1** illustrates how biofilm formation inside DWDS and its exposure to occurring ECs motivates this study, to understand the extent of their presence and effect.

The results of these ecotoxicological tests have great importance in DWDS and environmental management as they should later call for reconsideration of disinfection methods (frequency or effectiveness of disinfection) and assessment of risk and toxicological potential ^[5]. Furthermore, assessment of the adverse biological effects of ECs, both individually and in mixture (with additive, synergistic or antagonistic effects) ^[8-10], with chronic or acute exposures, may lead to the need to re-establish preventive actions (either by implementing restrictions on emissions, increased effectiveness of EC screening methods in DW, or by implementing more effective

measures to eliminate ECs and organisms in aquatic systems) ^[11, 12], to ensure the quality of the service of transport and of the water distributed.

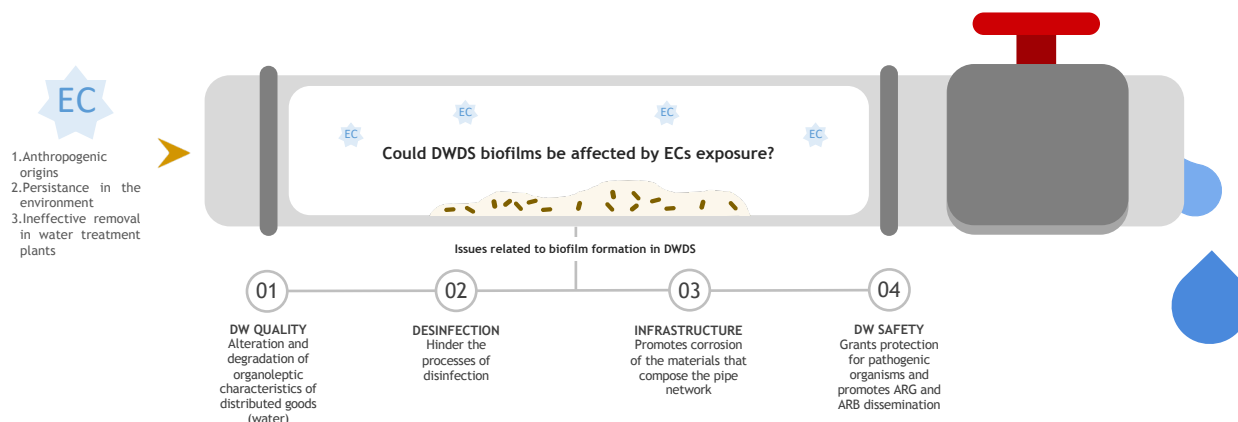


Figure 1. Graphical motivation chart. Bacterial growth in DWDS is a concerning topic to consumers and DWDS entities as it could decrease water quality, cause damage to DWDS (regarding integrity and operational performance) as well as jeopardize water safety. If ECs were to exert ecotoxicological effects on DWDS microbial community, what could this mean to DWDS?

1.2. Main objectives

The main goal of this study is to provide insight into the effects of selected ECs, at residual concentrations (ng/L and µg/L), on biofilms of the bacterial strain *Acinetobacter calcoaceticus*, isolated from DW. This organism was chosen as a representative of DWDS microbial community ^[13], particularly for the role it plays in coaggregation processes and acts as a bridging bacteria in biofilm development ^[14]. Furthermore, the four selected ECs represent distinctive kinds/class of pharmaceuticals, commonly found on DW ^[15, 16]: caffeine (stimulant and psychoactive drug - CAF), carbamazepine (anticonvulsant and neuroactive steroid - CBZ), ciprofloxacin (antibiotic - CIP) and ibuprofen (non-steroidal anti-inflammatory drug - IBP). Most are known to have a persistent and ubiquitous character in the environment (CBZ ^[17], CIP ^[18] and IBP ^[19]). Moreover, CAF ^[20] and CBZ ^[21] are common markers of anthropogenic activity or wastewaters in aquatic systems.

The effects evaluated by this study aim to predict and get a better understanding of the possible effects of the selected ECs on biofilms of DWDS, namely: their composition and microbial behaviour (ability to form biofilms, susceptibility to antibiotics, tolerance to DWDS disinfection methods (NaOCl), motility, auto and coaggregation ability, and growth rate).

Microtiter plates were selected as a platform for biofilm formation, as they have been proven to be excellent platforms for primordial assessment of EC exposure effects ^[22-24], despite its

limitations. This contributes to the development of standardized methods for studying the effects of ECs on bacterial communities, to ensure their reproducibility, similarity to real systems or predictability of effects, and whether the effects of exposure are relevant and how they can be assessed. Therefore, the experimental apparatus for exposure consisted of 48 well microtiter plates, where biofilm growth was promoted in PVC coupons, representative of piping material.

1.3. Thesis organization

This dissertation includes six chapters. The present chapter, **Chapter 1**, is an introductory chapter in which the motivation and main goals of this study are described, as well as the outline of the dissertation.

Chapter 2 provides a literature review regarding the topic, including a discussion of DWDS concerns (primarily on biofilm formation and control) and, occurrence and effects of ECs. This chapter also presents a brief discussion and review regarding existing approaches to biofilm exposure and support/platforms used for these studies, as well as the lack of information on the matter and discrepancy in the number of research in different aquatic environments comparing to DWDSs.

Later on **Chapter 3**, experimental work carried out, to study the effects of ECs on sessile *A. calcoaceticus* behaviour, is described. More specifically: their biofilm structure and characteristics, growth, susceptibility to antibiotics and chlorine as a DWDS disinfectant, auto and coaggregation capabilities and bacterial motility.

In **Chapter 4** and **Chapter 5**, the results are presented and discussed, respectively, offering an interpretation to significant changes on bacterial behaviour and possible repercussions in DW quality and safety.

Finally, general conclusions and overview of this work, as well as insight for future works, are summarized on **Chapter 6**.

Work outline

Chapter 2. Literature review

2.1. Drinking Water Distribution Systems

Water is one of the most abundant elements on Earth. In a liquid state, it is found in numerous bodies of water, such as: oceans, rivers, groundwater (sub-surface water reservoirs, *i.e.*, aquifers), among others. Still, it can be classified into saltwater (estimated 97.5% of the existing water on Earth) and freshwater (estimated 2.5%) [25], with fresh water being the most used. It is noted that 0.26% of fresh water is distributed by surface water bodies such as rivers, lakes or reservoirs, and 29.9% exists as groundwater [25]. Both are potential sources of drinking water (DW) [26]. Commonly, this water has to be treated before commercialization or distribution, however, in some cases it is consumed directly from their source (untreated water from private wells, for example).

DWDS are complex infrastructures designed to transport DW [27]. This lengthy network of water mains consists of pipes, pumps, valves, storage tanks, reservoirs and hydraulic appurtenances [7, 28], connecting drinking water treatment plants (DWTP)/water source to the point-of-use (consumer). Overall, these systems are rich in ecological niches that compose its distinctive micro-environment. However, it is the microbial growth within DWDS that compromises water quality and water authorities throughout the globe's mission to deliver safe DW to all communities.

2.1.1. Drinking water needs and concerns

DW has many purposes and plays an important role in various activities associated with domestic, hygienic, agricultural and/or industrial lifestyles, and is indispensable for any form of life (as a physiological need). Ensuring the supply and quality of DW has a huge impact on the socio-economic development and health of communities [29]. Sanitization and access to safe DW is a human right (rightfully so, according to the United Nations Resolution 64/292) and, although different countries have different DW standards, water fit for consumption must meet common basic quality requirements, regarding organoleptic, aesthetic, chemical and biological properties. Furthermore, the World Health Organization (WHO) states that drinking water should be clear, colourless, odourless, tasteless, and free of pathogenic bacteria/organisms and other substances that may jeopardize public safety and health [30]. However, not all waters satisfy these standards, and water scarcity and quality degradation are significant societal concerns.

Water has a very dynamic profile, but its hydrological cycle has been affected as a consequence of anthropogenic influences (*e.g.*, deforestation, overexploitation of water resources, and agricultural and industrial pollution). Furthermore, there are numerous articles that report the occurrence of contaminants in the environment, and potential ecological repercussions are thoughts that often follow such statements. Studies have proven that no water environment is free of pollutants, regardless of its source ^[31] or sampling time ^[32]. Furthermore, the aggravation of contamination of various ecosystems is a scenario that is expected to increase in the coming years, due to the increase in global population, urbanization, and the continued use of contaminants, particularly, in densely populated areas ^[3]. Climate change also poses a threat to water quality and quantity, as extreme weather conditions (*e.g.*, rainstorms, flooding or droughts) will likely lead to DW production relying on alternative water sources and advanced treatment technologies ^[33].

In Europe, the European Union Drinking Water Directive, 98/83/EC provides criteria and standards for water quality, applied with the intent of protecting people from adverse health effects derived from contamination of water destined for human consumption. This directive was eventually transposed into the Portuguese Legislation, in law decree no. 243/2001. However, even though finished treated water meets DW quality standards enforced by water authorities (meaning the microorganism concentrations are below detection limits or set values), this does not ensure sterility and there is a risk of quality deterioration throughout its distribution process.

2.1.2. Microbiology in drinking water distribution systems and biofilm formation related issues

DWDS are bacteriologically heterogenous, and water flowing through DWDS piping network is, therefore, not sterile, containing a variety of organisms, such as heterotrophic bacteria, fungi, viruses, protozoa, and algae ^[3, 34, 35]. This microbial flora makes up the DWDS microbiome, living in bulk water, in suspended solids, sediments or attached to surfaces ^[36]. The sources of biological matter in transported DW are: raw water, treated water, reservoirs, cross-connections and perturbations in the system (*e.g.*, during installation and repair periods) ^[37]. Furthermore, DW microbiome changes dynamically (spatial and temporally) through its delivery course in DWDS ^[7]. DWDS provide a micro-environment where bacteria may thrive, with numbers ranging from 10^3 to 10^6 cells/mL ^[38]. Among a wide variety of microorganisms found in DW, some more concerning may be pointed out: *Escherichia coli*, *Legionella* spp., *Mycobacterium* spp.,

Salmonella spp., *Acinetobacter* spp., *Burkholderia* spp., *Stenotrophomonas* spp., *Pseudomonas* spp. [29, 39-42]. Additionally, certain bacteria present in DW are opportunistic pathogens (OPs) [34], in some cases classified as emerging OPs, due to the fact that they are responsible for waterborne infectious diseases, whose incidence in populations has increased over the years [43] (e.g., *Mycobacterium avium*, *Legionella pneumophila*, and *Pseudomonas aeruginosa* [33]).

It is possible to conclude from the previous statements that, despite the attempt to provide biological stability of DW, bacterial growth occurs naturally on the interior surfaces of DWDS pipes, in conditions where nutrient availability is reduced. Hence, the colonisation and formation of biofilms inside DWDS is inevitable, despite the existence and application of different disinfection processes [44]. This phenomenon is described as biofouling [45]. Biofilms are complex microbial communities, immobilized in a polymeric matrix and adhered to an inert or living solid surface [46]. The polymeric matrix is composed of extracellular polymeric substances (EPS) secreted by the cells [47], consisting of polysaccharides and other biomolecules such as proteins, lipids, and nucleic acids [48]. Other substances such as inorganic particles, corrosion particles, sand, viruses, protozoa, fungi and algae can also be contained in DWDS biofilms [44, 49].

Biofilm development in DWDS, similar to other aquatic environments, is a dynamic process. Its life cycle comprises a series of stages, including: (1) surface preconditioning and initial attachment of planktonic cells (reversible attachment); (2) colonization (irreversible attachment); (3) proliferation; (4) biofilm maturation; (5) active dispersal (fragment detachment into bulk water and return to planktonic growth) [50, 51]; (6) recolonization elsewhere within DWDS piping network. In detail, biofilm formation starts with the formation of conditioning films, composed of macromolecules adsorbed to the surface (such as polysaccharides, DNA, lipids, proteins, and humic acids) [52] and with free-living bacterial cells attachment to conditioning films [53]. Conditioning films are crucial for biofilm formation as they change the physicochemical properties of the surface in nutrient-depleted environments [54] and promote initial cell attachment [55]. Transport of cells to substratum surface may be mediated by Brownian motion, sedimentation, diffusion, chemotaxis, convection and the intrinsic motility of the cells [36, 56]. Later, cell adhesion is promoted by weak interactions involving, van der Waals forces, electrostatic forces and hydrophobic interactions, mass transport, surface roughness, growth medium and surface microtopography [57]. Primordial biofilm formation is also mediated by surface attachment structures, such as type IV pili and flagella appendages [58]. Following the previous step,

microcolony formation takes place along with the excretion of EPS and quorum-sensing molecules ^[53] (actively involved in microbial communication processes ^[59], and biofilm maturation and maintenance, whose regulatory pathways are activated by high cell densities ^[60]). This process ensures that bacteria is now irreversibly attached to the surface ^[36] and consequently, continued growth leads to mature biofilms development. As the biofilm grows, further colonization by microbes that may lack biofilm-forming abilities occurs, which benefit from the protective nature of biofilms and access to nutrients ^[36]. Finally, active dispersal is the last step, completing the biofilm formation cycle. The detached biofilm fragment later starts biofilm formation in new uncolonized sites. Detachment usually occurs by erosion or sloughing (shear forces), abrasion (by suspended particulate matter in flowing water), grazing by predators, and cell-mediated dispersal ^[36]. Other environmental cues and conditions can also trigger active dispersal, including changes in nutrient availability, oxygen depletion, and nitric oxide build-up ^[61].

Biofilms differ in their functional and structural properties (composition and architecture, both spatial and temporal) ^[49, 62], and numerous micro-environments exist within a matured biofilm ^[36]. Moreover, biofilm detachment processes can influence biofilm structure, composition, biomass density, and thickness ^[36, 63, 64]. For instance, biofilms dominated by sloughing detachment have rougher surface morphology and patchy spatial distributions, while biofilms with dominant erosion mechanisms have smoother surface morphology, with a more continuous spatial distribution ^[36]. Hydrodynamical conditions ^[65-67] and water chlorination ^[68] also appear to have an effect on biofilm morphology.

DWDS provide high surface area, and microbial adhesion to surfaces promotes biofilm formation, which may depend on a myriad of biotic and abiotic factors, these include: (1) hydrodynamic conditions (*e.g.*, flow rate (associated with transport and interferes with bacterial adhesion) and network design); (2) water source and treatments used at DWTP; (3) cellular properties (cell surface, fimbriae, flagella, production of EPS); (4) adhesion substrate (*e.g.*, material, hydrophobicity, condition status, and roughness); (5) nutrients availability (organic or inorganic) and water composition; (6) environmental factors (*e.g.*, temperature, oxygen and pH); (7) type and concentration of residual disinfectant; (8) predator presence; (9) bacterial concentration and biodiversity; (10) sediment accumulation ^[38, 39, 44, 49, 69, 70]. As a result, detachment of biofilm fragments, through shearing/erosion ^[45], is one of the factors that affects water quality ^[69]. The fragment that breaks off moves to a non-populated area and forms a new

microbial community ^[44], through sloughing and seeding ^[33]. This makes prevention of the spread of microbial growth in DWDS an arduous job, as those fragments are a source of suspended bacteria ^[44]. **Figure 2** summarizes and highlights crucial elements and events that affect DWDS microbiome, ultimately leading to water quality deterioration.

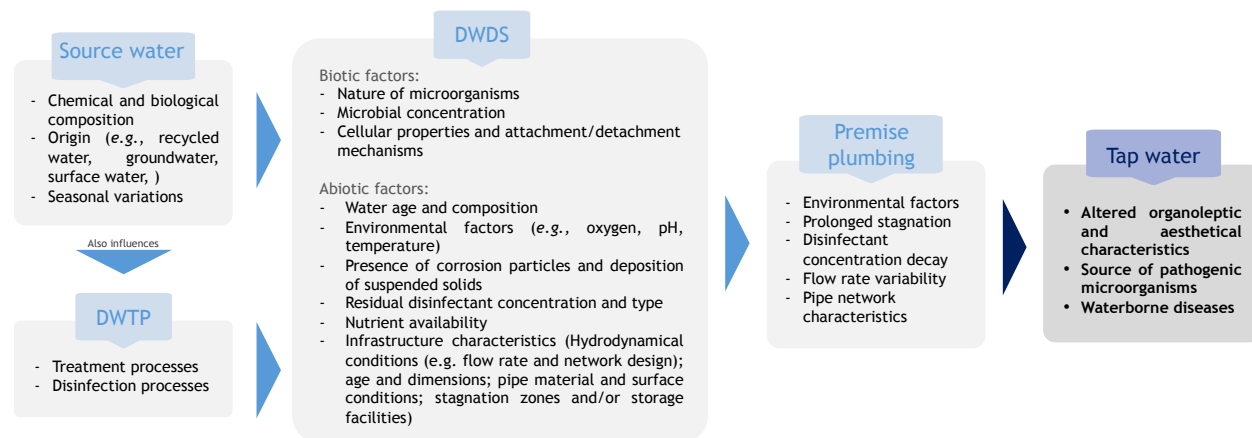


Figure 2. Key factors that affect DWDS microbiome and biofilm formation, leading to DW quality deterioration. Source water and DWTP represent upstream microbiome influencing factors. Right of DWDS box, premise plumbing represents downstream influencing factors to final product quality.

It is estimated that 95% of the total water biomass adheres to pipe surfaces (sessile state), while the remaining 5% are in a planktonic state ^[71]. In fact, bacteria in biofilms in aquatic environments preferentially opt for a surface-associated state, much like in living tissues or medical devices ^[49, 72], as they display a higher tolerance to environmental adversities when compared to their vulnerable suspended counterparts ^[73]. Nevertheless, bacteria can switch between their planktonic form and sessile form ^[60]. Furthermore, biofilms can reside inside DWDS from days to decades ^[50]. The advantage of residing in biofilms is based on the presence of EPS, which allows for water retention (more than 90% of the mass of biofilms corresponds to water) ^[74], and helps to maintain embedded cells hydrated. EPS also facilitate the retention of nutrients ^[75], protect organisms from stressors (disinfectants, heat stress, oxidative stress and UV radiation, for example) and allowing them to maintain a viable state even under the most adverse conditions ^[44, 47, 72].

Given the preference of bacterial communities to live in this conformation, this topic has been heavily debated, as biofilms create a reservoir for pathogenic microorganisms that persist in DWDS and may be responsible for the incidence of water-borne diseases ^[44] and proliferation of other water quality-compromising bacteria (*i.e.*, generating visual turbidity and alter organoleptic

properties of DW), the increase of hydraulic drag (compromising DW transport and distribution capacity), promotion of biocorrosion, decrease efficiency of disinfection processes [36, 40, 76] and facilitate antibiotic resistance transfer [33]. Unquestionably, there is an interlinked relationship between water quality, DWDS microbiome and DWDS infrastructure integrity [36].

2.1.3. Drinking water quality and microbial control

Ensuring DW quality is crucial as its quality affects human and animal health (*i.e.*, livestock activities) [77]. The composition of water in a DWDS may depend on its origin and residence time in these systems [69]. To guarantee biological quality of water by mediating the accumulation of biofilm means adopting actions and control measures, both in DWTP and within DWDS [69]. These measures are pivotal to prevent water quality degradation and outbreaks of infectious diseases related to DW. DW, in the DWTP, before being made available for consumption, is submitted to several combinations of treatments that represent barriers to contaminants in DW. Disinfection strategies or processes used in DWTP may also differ, depending on the type and quality of the water source [78], as groundwater does not normally require the same level of pre-treatment as surface water [78]. The DW treatment sequence generally consists of: disinfection, coagulation, flocculation, sedimentation, filtration and post-disinfection [79].

To further control biofilm formation inside DWDS, physical removal, biocides application, mechanical treatment (such as pipe flushing), manipulation of operational conditions, pipe surface modification methods and biofilm disruption by nonbiocidal detachment-promoting agents, may be applied [36, 44, 53, 80]. The disinfection strategies most used to control biofilm formation within DWDS are: chlorine, chloramines, chlorine dioxide, ozone and UV radiation [44, 78]. The use of secondary disinfectants (chlorine and combined chlorine in the form of chloramines), at residual concentrations, coupled with limiting nutrient concentrations are popular practices used to achieve such requirements [81]. Another approach that contributes to microbial control is the limitation of water residence time [36].

2.2. Emerging contaminants

Climate change, population growth and urbanization constantly put pressure on water resources. There is a rising demand to satisfy human, societal and economic needs, which contributes to the regular launch of new chemical substances to the market [82]. Due to higher diversity, design, production and consumption rates of chemicals, their release and dispersal into the environment has skyrocketed in just over the last half-century [83]. These substances, with identified harmful health and environmental impact [84] are commonly designated as emerging contaminants (ECs). ECs are organic or inorganic [8, 85] compounds of anthropogenic or natural origin [11], frequently detected in the environment [86], at residual concentrations, (ng/L and µg/L) [8, 11, 87]. However, in most cases, their emission is not yet regulated [6, 8, 9], as well as they are not included in routine screening or monitoring programs [3]. The designation of “emerging” was attributed not exclusively due to the fact that they are novel compounds, in fact, many of them occur naturally. There is still a lack of knowledge regarding ECs presence and consequent effects, which have sparked some interest concerning the adverse eco-toxicological impact in aquatic systems and/or in humans [11, 88], hence, the concept of “emergence”. ECs are also often mentioned in literature by other nomenclatures, such as micropollutants (common nomenclature: MP) [11, 89], contaminants of emerging concern (common nomenclature: CEC) [90], contaminants of emerging interest (common nomenclature: CEI) [91], emerging organic contaminants (common nomenclature: EOC) [92, 93], organic micropollutants (common nomenclature: OMP) [92, 93], or emerging pollutants (common nomenclature: EP) [85].

2.2.1. Occurrence, sources and fate of emerging contaminants

The classes of ECs frequently detected in the environment are: pharmaceuticals, cosmetics and personal care products, hormones, pesticides, herbicides, surfactants, domestic and industrial chemicals, antibiotic resistant genes and bacteria (ARG and ARB, respectively), endocrine disruptors, psychotropic substances, microplastics, among others [11, 85, 86, 94-99]. In this work, emphasis was laid on pharmaceuticals. Pharmaceutical compounds are synthetic or natural chemicals found in prescription or over-the-counter medications destined for human or animal use [97, 100]. They are known to be chemically stable active substances intended to trigger biological responses [92, 100] in target organisms. Yet, this stability impairs its degradation in the environment, which also causes repercussions of its activity on non-target organisms, further exacerbating the

concern of water safety. Pharmaceutical contaminants are otherwise referred to in several studies as biologically active pharmaceutical compounds (PhACs) [4, 101], or active pharmaceutical ingredients (APIs) [102]. Additionally, pharmaceutical products can be classified according to their properties and therapeutic use [103], *e.g.*, analgesics, antibiotics and antimicrobials, antihistamines, antidepressants, hormonal compounds, stimulants, among many other classes [97, 103].

There are currently over 20,000 approved prescription drug products on the market [104], and the extensive and abusive use of PhACs substantially contributed to their presence in the environment. PhACs have been detected in wastewater, DWTP and waste water treatment plants (WWTPs), seawater, sediments, surface waters and groundwater [100, 105-107]. The study of ECs occurrence, fate and effects in the environment has become a trend in the scientific community. Over the years, the number of studies regarding ECs occurrence has boomed. However, there is a noticeable contrast in research activity of the occurrence of ECs in DW and groundwater compared to wastewater and surface water environments. **Figure 3** evidences the discrepancy in search results of various aquatic systems, as well as the promising increase in the number of reports.

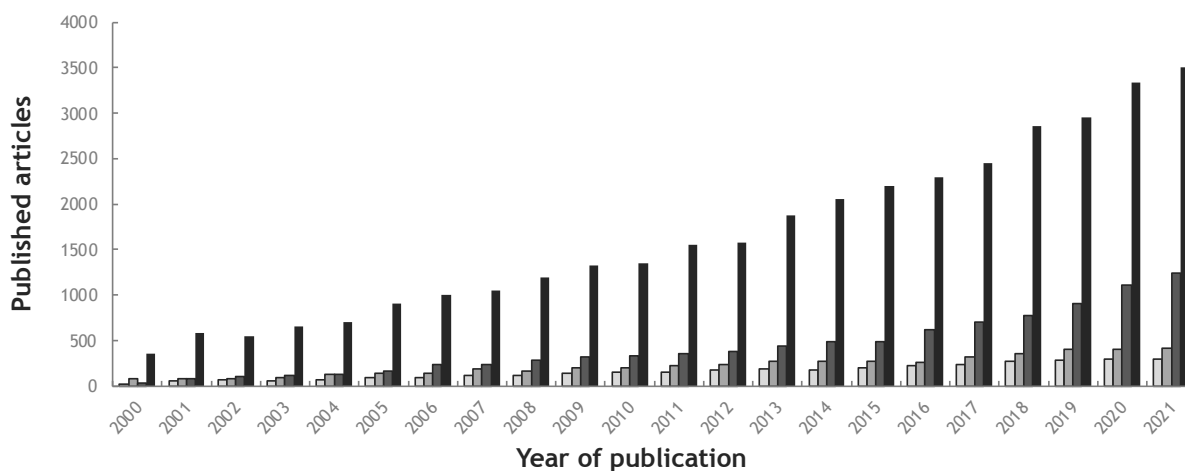


Figure 3. Publications reporting the occurrence of ECs in different water systems from 2000-2021. Results were achieved through an advance search in PubMed database. The methodological approach was by selecting title and abstracts as search fields and by using key search words, such as: Pharmaceuticals + (occurrence OR presence) + groundwater/wastewater/drinking water/surface waters (River OR Streams OR Wetlands)) (■ – groundwater; ■ – DW or tap water; ■ – wastewater ; ■ – surface water).

In general, the main point sources of emission of PhACs in the environment are untreated and treated waste water, hospital waste, livestock activities, aquaculture, human and animal excretion and improper disposal of expired medical prescriptions [108-110]. Other non-point sources

are: runoff from waste water sludge discharge and stormwater runoff^[110]. Currently, the equipment and processes used in DWTP and WWTP do not fully, specifically (target) and effectively eliminate most ECs present in their influents [9, 87, 100, 111]. As pharmacological drugs contain complex structures and varied biological and physicochemical properties^[92, 100], their stability and resistance to biodegradation or water solubility influences the effectiveness of removal in Water Treatment Plants (WTPs) [4, 87, 108, 112]. Since groundwater and surface water, are the main resources of water for consumption, they end up becoming sources of ECs in DW [4, 100, 105]. **Figure 4**, illustrates the sequence of events stated above, demonstrating how ECs reach water bodies and make their way into DW.

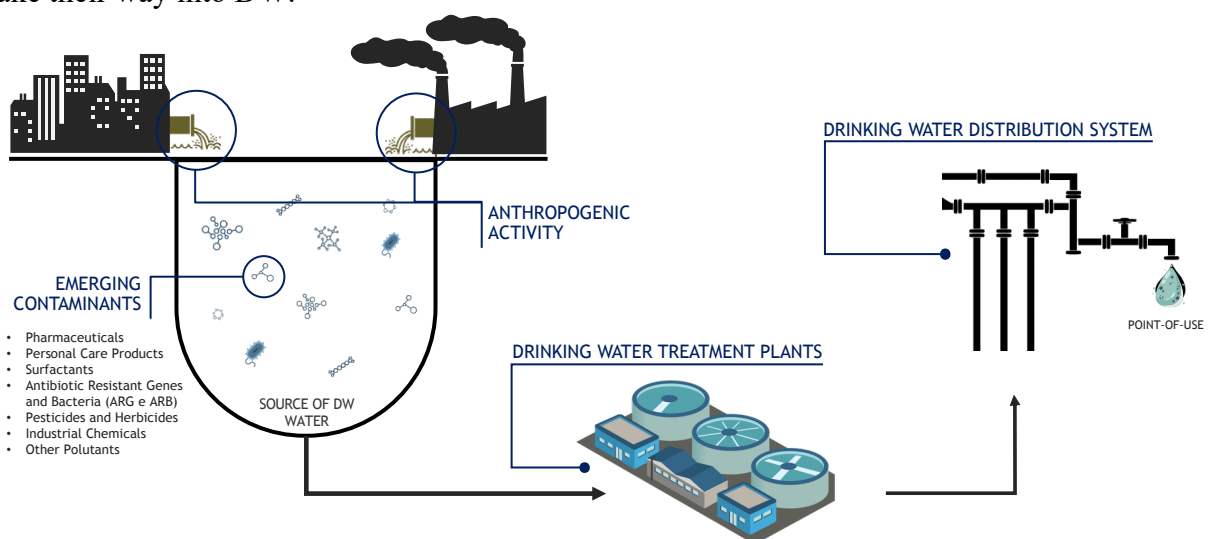


Figure 4. Overview of ECs sources and transport to point-of-use. *Anthropogenic activities (coming from human and animal consumption and excretion; hospital waste; industrial discharge; untreated or inefficiently treated wastewater; direct disposal; landfill leaching; agricultural activities; runoff^[113, 114]) are the main sources of emission of these pollutants, that are not currently monitored and are usually detected at trace level concentrations in the environment. Contaminated water resources are the source of EC presence in DW, as the equipment used in WTPs do not remove these contaminants, and resulted treated water is redirected to DWDS. Finally, the consumer is exposed to ECs.*

Additionally, concentrations in different water resources may vary seasonally and culturally, depending on the region^[11, 86], as well as in WWTP influents and effluents, due to factors such as: production, sale and consumption rate of these compounds (both by humans and animals); on metabolism and rate of excretion^[11]; and their persistence (governed by biodegradation, photolysis, and other abiotic transformations, such as hydrolysis^[115]). All these factors contribute to significant variations in concentration between compounds. Special attention should also be paid to other factors that contribute to their concentration increase in the environment, such as

bioaccumulation and biomagnification ^[3]. Also, it is not necessary for ECs to be persistent in the environment, since the rates of transformation and removal are offset by their constant introduction into aquatic systems ^[116]. Additionally, other environmental events also contribute to this variation, such as: rainfall (greater flow of water into water resources and dilution of ECs) and low temperatures (reduce the biodegradation activity) ^[4]. In the case of DWDS, another factor that can influence and reduce concentrations in DW is the reaction between ECs and disinfecting agents (chlorine, chloramine, chlorine dioxide) ^[117]. On **Appendix I, Table A I - Table A IV**, show relevant information regarding selected ECs occurrence and factors that affect their presence in DW resources (possible sources and fate) are summarized.

2.2.2. Impact of pharmaceuticals in environmental biofilms and relevancy

The modern era environmental movement was ignited by Rachel Carson's book "Silent Spring", published in 1962 ^[118]. Although it sparked controversy and considerable opposition in that time, Carson exposed the problematic effects of the indiscriminate use of agricultural chemicals, pesticides, and other modern chemicals, in the following years of World War II ^[119, 120]. Her work pioneered today's ecotoxicological studies ^[121]. Recently, a global and emerging concern of the scientific community is reflected in the possible effect of continuous exposure to residual concentrations of ECs on multiple organisms and microenvironments, particularly in biofilms, which are of interest for this study. Many studies describe the effects of diverse ECs exposure on fluvial, marine, wetlands, soil and WWTPs microbiota ^[3].

However, there are still many knowledge gaps in this topic of research that cause numerous hardships and limitations in assessing the environmental risks of ECs, particularly, the extrapolation of laboratory results with real systems ^[122]. These challenges include the complexity of EC mixtures, exposure duration, sublethal concentrations, integration of multiple environmental stressors, and diversity and interactions between aquatic systems flora and fauna (trophic interactions) ^[122]. Moreover, it is important to mention that many PhACs undergo a series of transformations resulting in the formation of metabolites or by-products ^[123], while some of their metabolites are biologically active, affecting non-target aquatic organisms ^[124] and are more toxic than parent molecule ^[125].

Romero et al. ^[126] discussed the impact of multiple stressors (temperature, hydrological stress, and pesticide exposure) in stream biofilm bacterial communities, proving that multiple-stressor

scenarios could possibly lead to unexpected shifts in community composition and associated functions of fluvial bacterial communities. Granted, the focus of this study is PhACs. Subirats et al. ^[127] concluded that the combination of nutrients and ECs (CIP, erythromycin, sulfamethoxazole, diclofenac, and methylparaben) have a synergistic effect on bacterial composition, abundance and antibiotic resistance profile (increase of *sull* and *intI1* ARGs) of artificial stream biofilms. On the other hand, long-term exposures to atenolol, CBZ, diclofenac, erythromycin, gemfibrozil, hydrochlorothiazide, IBP, metoprolol and sulfamethoxazole have induced adverse effects on biofilms (such as: changes in structure, metabolism, and bioaccumulation of PhACs), with flow intermittency modulating these effects ^[128].

Alternatively, Corcoll et al. ^[9] proved that the presence of nonsteroidal anti-inflammatory drugs (NSAIDs) (mixtures of IBP and diclofenac, preventer of WWTP effluents) and their effect on fluvial microbiota goes beyond the acquisition of tolerance or resistance, suggesting that chronic pollution results in altered microbial metabolism and structures of algal communities. As for individual and mixture ecotoxicological tests, Rosi-Marshall et al. ^[129] determined that PhACs (specifically CAF, cimetidine, CIP, diphenhydramine and mixtures (CAF + cimetidine + CIP + diphenhydramine + metformin + ranitidine)) can alter biofilm bacterial community composition, suppress algal growth and/or microbial respiration in biofilms.

Proia et al. ^[130] found that IBP and acetaminophen (paracetamol) exposure negatively impacted photosynthesis and caused a decrease of the green algae/cyanobacteria ratio, while diclofenac was associated with higher phosphatase activity, whose effects on biofilm structure and function may have negative repercussions in river ecosystem functioning. Furthermore, Lawrence et al. ^[131] studied the effect of CAF, CBZ, furosemide and IBP on fluvial biofilms, resulting in the suppression of cyanobacteria. Similarly, Aubertheau et al. ^[132] also observed a reduction in cyanobacteria in river biofilms due to water contamination by WWTPs discharges (namely, WWTPs that use activated sludge followed by a reed-planted bed filter). The extinction of cyanobacteria (responsible for the production of O₂ and CO₂ fixation) could lead to catastrophic consequences for the maintenance of the global activity of the microbiosphere ^[133]. Since benthic river biofilms are the basis of the balance of aquatic ecosystems, any negative impact resulting from the presence of PhACs can affect biofilm-dependent trophic cycles ^[129].

Instinctively, there is a growing concern about the possible impact on public health resulting from phenotypic and genotypic alterations of microorganisms present in DW biofilms due to EC

exposure ^[86]. As previously mentioned, research is still exceedingly limited, particularly in the DW scenario. Only five studies, known to date, were found to have sought to assess the effect of ECs on the DWDS surface associated biofilms and whose objective was to determine the extent of EC exposure impacts ^[22-24, 134, 135]. Although the concentrations of ECs found in DW are low, it is necessary to study the problems associated with prolonged exposures to determine potential changes induced by exposure to ECs in DWDS micro-ecosystems, by quantifying their ecotoxicological repercussions and, consequently, effects on quality, chemical and microbiological treatment of distributed DW. In consequence of significant changes, adverse effects may add concern to the existing problems related to the formation of biofilms in DWDS.

Gomes et al. ^[24] determined that pre-exposure (for 26 days) of *Stenotrophomonas maltophilia* biofilms to mixtures of clofibric acid (170 ng/L) + CBZ (258 ng/L) increased resistance to removal by NaOCl. Consequently, Gomes et al. ^[23] studied the isolated effect of clofibric acid (for 12 weeks, at 170 ng/L) on *S. maltophilia* biofilms, resulting in the formation of biofilms with higher tolerance to chlorine disinfection and antibiotic erythromycin, that directly affect DWDS. Alternatively, Gomes et al. ^[22], determined that the inactivation of *Burkholderia cepacia*, after exposure to CAF (119 ng/L) and trimethoprine-sulfamethoxazole (TMP-SMX) (1.7 and 8.2 ng/L, respectively), was hindered. The effects of sulfadiazine and CIP on DWDS biofilms was also studied by Wang et al. ^[134]. Their results suggest that exposure of DWDS biofilms to studied antibiotics resulted in an increase in 16S rRNA, ARGs and *intI*, as well as an increase enzymatic activities and production of EPS which, in turn, promotes bacterial aggregation and adsorption on DWDS surfaces, and dissemination of ARGs. Lastly, Zhang et al. ^[135] found that tetracycline promotes the growth of bacteria in biofilms, and antibiotic (tetracycline, sulfadiazine and chloramphenicol) exposure had enhanced chlorine and antibiotic resistance, and richness of biofilm communities. These results show how unpredictable microbial behaviour can be and emphasize the importance of studies of the same nature, and alert to the possible repercussions of the presence of ECs in DWDS. Any new information on the impact of ECs in microbial communities of DW may be of utmost importance to further prioritize these compounds in DWTPs and WWTPs.

In regards to antibiotic PhACs, the great popularity of antibiotics and their success in treating infections leads to these compounds being used excessively and inappropriately in non-clinical settings ^[136], accompanied by an increase of antibiotic release into the environment. Antibiotics

not only exert toxic effects, but also selective pressure on microbial communities [12, 93, 137, 138], contributing to the continued selection of ARBs that contain ARGs (repressing susceptible species), thus accounting to a threat to public health [93] and as an environmental issue. ARG-bearing bacteria have been described in various aquatic environments, including DW, surface water, wastewater, and sediments [138-140]. Furthermore, biofilms are known to be hotspots for ARBs and ARGs proliferation [44, 141] (due to their high cell density, proximity, and accumulation of mobile genetic elements (MGE) [142]). Therefore, the need to assess antibiotic impact on biofilm communities development of resistance is justified. Moreover, biofilm detachment contributes to the possible transport of ARB in DWDS [143].

Furthermore, it is known that sub-inhibitory concentrations are major contributors to resistance promotion [144, 145]. However, little is known about the influence of non-antibiotic PhACs on the spread of antibiotic resistance in the environment [146]. Contrary to what has been previously mentioned, there are also non-antibiotic ECs that exert selective pressure and whose presence is related to increased resistance of microorganisms in biofilms to disinfection processes, yet, this topic is rather unmentioned [147]. In a study by Lu et al. [147], it was evaluated whether exposure to environmental concentrations of the compound triclosan (antimicrobial EC) (0.2 to 20 µg/L) contributes to the spread of ARGs. Alternatively, Wang et al. [148] studied the effects of polycyclic aromatic hydrocarbons (naphthalene and phenanthrene, 10 mg/L) on the spread of antibiotic resistance in coastal microbial communities. Their results indicated that exposure resulted in the spread of ARGs and the presence of naphthalene and phenanthrene induces a significant increase in *intI1*, *sulI* and *aadA2*. Additionally, certain metals (Cu(II), Ag (I), Cr(VI) and Zn(II), at subinhibitory concentrations) have the ability to select and stimulate antibiotic resistance (by promoting ARG transfer between *E. coli* strains), as revealed by Zhang et al. [149]. Similarly, the study by Wang et al. [146], CBZ (at concentrations of 0.05 mg/L) exposure resulted in an increase in ROS (reactive oxygen species) levels, SOS response, membrane permeability and in the generation of *pilus*, which contribute for greater efficiency in resistance transfer. These results prove that, even at environmentally relevant concentrations, multiclass ECs and PhACs, antibiotics or non-antibiotics, have the ability to promote the dissemination of ARGs and the acquisition of resistance by exposed microorganisms.

2.3. Support for studying emerging contaminants effect on microbial behaviour

Biofilms are very useful tools to assess the impact of anthropogenic activities ^[132], and have been widely used as bioindicators of the ecological status of aquatic systems. Thus, there is a need to develop exposure systems, where biofilm formation is supported for studying the possible outcomes of EC presence in aquatic environments. As a requirement, these systems should mimic the native environment (both in terms of environmental conditions and microbiome) as an ecotoxicological test principle. So, the results can be correlated with real environments.

Aquatic ecosystems differ in their composition (nutritive, microbiological and contaminant loads), as well as in substrates for biofilm formation ^[49]. In rivers, the availability and diversity of surfaces to which bacteria can adhere and proliferate is superior compared to that of a DWDS. River microbiota can be found on surfaces such as rocks or stones (epilithic), in gravels or sands (epipsammic), aquatic plants, clays, and submerged woods ^[150]. While in DWDS, even though pipes and fittings materials vary within a system ^[36], the predominant surface available is piping surfaces ^[151].

The biofilm composition is variable depending on the environment where they are inserted in. Therefore, this aspect should also be taken into consideration when choosing the exposure model used to assess the ecotoxicological effects of ECs. Moreover, another extremely relevant factor to be considered is the variability of conditions among systems. More specifically, the response to different stress factors depends and varies from organism to organism (which also differ in ecosystems) and the concentrations of ECs found in that environment, that exert different actions on tested subjects. These limitations hamper the ability to predict impacts in DWDS.

Some exposure models used in this type of studies are artificial in nature, meaning that the conditions of exposure to ECs, (exposure time, nutrient load, flow dynamics, environmental or climatic conditions, and other stress factors, according to the region of impact), are simulated and carried out in controlled and monitored systems, with the objective of mimicking natural environments, in order to obtain reproducible results and predict what really happens in these ecosystems. However, these tests could also be done directly on its native environment. On **Table 1**, examples of biofilm exposure to ECs from different studies, that further eludes to different attempts to achieve exposure in many aquatic environments, are shown.

Table 1. Examples of platforms for biofilm exposure to ECs in 3 different aquatic environments.

Aquatic environment	Type of Exposure	Exposure system	Test organism	Colonization substrata	ECs/class of ECs	Reference
DWDS	Artificial	Microtiter plates	<i>Burkholderia cepacia</i>	PVC coupon	AHTN, ANTP, CA, CAF, CBZ, DCF, GAL, IBP, TMP-SMX, TY	[22]
			<i>Stenotrophomonas maltophilia</i>		ANTP, CA, CBZ, DCF, GAL, IBP, TON, TY	[24]
					CA	[23]
		Glass fiber-reinforced plastic bottles	Native DWDS microbiota (North of China)	Cast iron coupon	CIP, SDZ	[134]
		Glass column reactor filled with glass beads		Glass beads	TET, SDZ, CAP	[135]
River	Field study		Native fluvial microbiota of Segre River (NE Iberian Peninsula)	Flat brick with attached ceramic tiles	NSAIDs	[9]
	Artificial	Indoor streams facility (methacrylate channels)	Epilithic and epipsammic biofilms from an unpolluted area of Llémena River	sterile sand particles	CIP, ETM, SMX, DCF, and MPB	[127, 152]
			Microbiota from semi-polluted area of Llémena River	Ceramic tiles	ATE, CBZ, DCF, ETM, GFZ, HCTZ, IBP, MET, SMX	[128]
Marine		Glass jars	Native fluvial microbiota Llobregat River (Spain)	Glass slides	MLs, QLs, SAs, TCs, chemotherapeutic	[153]
	Field study		Native coastal microbiota of Toulon Bay (France)	polycarbonate plates	Trace metals	[154]
	Artificial	Cylindrical plastic tanks	Benthic biofilms	Carbon steel disks	hydrocarbons and chemical dispersants	[155]

AHTN – Tonalide, ANTP – Antypirine, ATE – atenolol, CAF – Cafféine, CBZ – Carbamazepine, CA – Clofibric acid, CAP – chloramphenicol, DCF – Diclofenac, ETM – erythromycin, GAL – galaxolide, GFZ – gemfibrozil, HCTZ – hydrochlorothiazide, IBP – Ibuprofen, MET – metoprolol, MLs – macrolides, MPB – methylparaben, QLs – quinolones, SAs - sulfonamides, SDZ – sulfadiazine; TCs – tetracyclines; TET – tetracycline, TMP – SMX – Trimethoprim – sulfamethoxazole, TON – tonalide, TY – Tylosin.

As previously mentioned, DWDS are diverse in terms of source water used, (which varies in its chemical and biological compositions) treatments applied at DWTP, and in DWDS length and characteristics (*e.g.*, pipe materials, pipe age, hydraulic regimes, water age and their variations

within the system). This diversity in DWDS poses as an adversity for studying and characterizing DWDS biofilms. Furthermore, ecotoxicological tests are not restricted to testing only one parameter *i.e.*, the impact of ECs may also be dependent on other environmental conditions. So, the impact of various variables can be tested and simulated simultaneously, such as hydrodynamics, presence or absence of nutrients or disinfectants, types of surfaces or types of organisms (individual studies or in mixture) [128, 152].

The study of DW biofilms in real DWDS is a hard task, since it may imply operational restrictions to DWDS, but also the high variability of operational conditions (water parameters, disinfection, hydrodynamics, temperature, among others) will hinder the reproducibility of obtained results between different experiments. So, to evaluate the impact of ECs in DWDS biofilms, it will be crucial to promote biofilm growth at first. One of the greatest constraints encountered in studying DWDS biofilms is selecting an appropriate experimental device or system that replicates conditions found in these plumbing networks [70]. However, this can be achieved through bench top devices, that allows the operator to reproduce DWDS conditions to its desire. The devices used for this purpose are: annular reactors, concentric cylinder reactors, Propella[®] reactor, Rotatorque[™] reactor, flow cell system, rotating disk reactor, Robins device (Robins device), Pendersen device (Penderson device), Calgary device (Calgary biofilm device), among other attempts to model DWDS [70, 76, 156-162] and enable the creation of microcosms for biofilm studies. Static biofilm formation assays are mostly performed on microplates, which by their nature allow to simulate low shear forces in reservoirs, pipes in homes (which have a varying hydrodynamic pattern throughout the day) [163], valves and corners (areas also associated with abundant organic matter accumulation) [164]. Furthermore, microplates are used as a standardized system for biofilm formation of bacteria isolated from different environments and providing information and reproducible results [165]. However, despite their convenience and simplicity, microplates are very restricted in terms of hydrodynamic simulation, since flow conditions cannot be accurately replicated. Likewise, simulation of biofilm growth in DWDS can be performed in containers [134, 166], supplied with DW.

In comparison to bench top devices, *in situ* studies are as close as one can get to achieve realistic DWDS conditions. Even so, every method has its own limitations or advantages and disadvantages. Furthermore, the goals of the study and the expected outcomes, as well as all the requirements to achieve them have to be taken into account when deciding the most appropriate

method to be used. For example, if biofilm sampling is of importance, a method that readily provides or facilitates biofilm sample extraction is a more appropriate choice. Another problem associated with *in situ* DWDS biofilm formation studies is that the interior of the pipe network is not easily accessible due to the systems closed infrastructure. Thus, acquiring biological samples representative of the spatial, temporal and physicochemical variation of real DWDS is challenging [76]. Also, contrary to lab-scale executed experiments, in *in situ* experiments, biofilm sample extraction could interfere with operational DWDS and their water supply needs. However, monitorization of operational and environmental conditions can be overcome, made possible by the use of online sensors [167], offering real-time monitoring of hydraulic and basic water quality parameters (pressure, flow rate, pH, oxidation-reduction potential, conductivity, and fluorescent dissolved organic matter) [168] without disturbing DWDS environment.

One method of sampling biofilms in DWDS (*in situ*) is called pipe cut-out sampling [169-171], which requires cutting off the water circulation, runoff/drainage and then collecting the biofilm by scraping or swabbing. However, this is a laborious method which may lead to contamination of the system. Moreover, scraping and swabbing are very invasive techniques of biofilm collecting, and may interfere with further analysis [172]. Similarly, but in an artificial and smaller scale platform, Fu, et. al, (2021) [173] used a pilot platform, where water deviated from a DWTP flows through a PE pipe, without disturbing actual DWDS network. For biofilm sampling, pipe sections were cut and biofilm samples were scrapped from the pipe surface. Furthermore, for a more realistic approach of biofilm formation, full scale experimental pipe loop facilities [174] is pointed-out as an alternative. Even so, the use of larger scale facilities also means higher installation and operational costs, and higher availability of space. Additionally, the requirements for maintenance and qualified works may be another limitation.

Whitall, *in situ* sampling can be accomplished with some devices, such as bioprobes, that allow biofilm growth in its surface and following extraction for biofilm studies, directly from operational DWDS [70, 168]. Biofilm sampling devices have also been described by Douterelo et al. [175], where the device is installed within a pipe section and contains removable, curved, sampling coupons with flat inserts, which allow extraction and the study of biofilms.

Chapter 3. Materials and methods

3.1. Emerging contaminants

Four emerging contaminants were selected for this study, each representing a different class of pharmaceuticals: a central nervous system stimulant (Caffeine – CAF (Fluka, Steinheim, Germany)); an anticonvulsant (Carbamazepine – CBZ (Acros Organics, New Jersey, USA)); an antibiotic, more specifically, a fluoroquinolone (Ciprofloxacin – CIP (Fluka, Steinheim, Germany)) and a non-steroidal anti-inflammatory drug (NSAID) (Ibuprofen – IBP (Alfa Aesar, Karlsruhe, Germany)). The exposure assays were conducted at residual concentrations (ng/L), thus reflecting the conditions of DW (Table 2). Furthermore, since exposure to complex mixtures of ECs is of significant concern, the combination of previously mentioned ECs was also tested (MIX), in which the concentration of each EC corresponds to the concentration of the individual contaminant.

Table 2. Selected emerging contaminants: concentrations found in DWDS and other relevant information.

EC	Abbreviation	Class of contaminant	Registered concentration (ng/L)	Reference
Caffeine	CAF	central nervous system stimulant	158.7	[4]
Carbamazepine	CBZ	anticonvulsant	586	[90]
Ciprofloxacin	CIP	antibiotic	679.7	[176]
Ibuprofen	IBP	non-steroidal anti-inflammatory drug	223.6	[4]

Stock solutions of CBZ and IBP were prepared using dimethyl sulfoxide (DMSO) (Fisher Scientific, Leicestershire, UK). CAF and CIP stock solutions were prepared in sterile distilled water. Stock solutions were stored at -20°C, until further used. To prepare these solutions, 10 mg of EC were dissolved in 10 mL of the corresponding solvent. When preparing CIP solution, 1 or 2 drops of hydrochloric acid (HCl) at 39 % (v/v) were added. Subsequently, the solutions were diluted in sterile distilled water, to a concentration a hundred times higher than the final concentration used. The final concentration of DMSO is considered residual, varying between 0.0002 and 0.0005 % (v/v).

3.2. Microorganism and culture media

Acinetobacter calcoaceticus, previously isolated from drinking water [13], was selected as a representative bacteria of DW. Furthermore, much like some other bacteria existing in DWDS (*i.e.*, *Methylobacterium sp.*) [177], *A. calcoaceticus* is a bridging bacteria [14]. *A. calcoaceticus* was grown

overnight in batch cultures of 25 mL of R2A broth: 0.5 g/L peptone (Oxoid, Hampshire, England); 0.5 g/L glucose (CHEM-LAB, Zedelgem, Belgium); 0.1 g/L of hepta-hydrated magnesium sulphate (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 of casein hydrolysate (Oxoid, Hampshire, England); 0.5 g/L of soluble starch (Sigma) and 0.4 g/L of di-potassium phosphate trihydrate (Applichem Panreac, Darmstadt, Germany), at 25 °C and under 120 rpm agitation.

3.3. Biofilm exposure to Emerging Contaminants

3.3.1. Biofilm formation substrate

The materials that make up the pipes network are variable and can be composed by polyvinyl chloride (PVC), polypropylene, steel and stainless steel, concrete, cast iron, copper, among others [27, 69]. In this work, PVC was chosen as a representative material of DWDS pipes. PVC is the most used material that composes DWDS pipelines and its usage has increased over the years, due to their low manufacturing and installation costs, and lesser probability of corrosion in comparison to inorganic materials [173]. Schwartz et al. [178] has suggested that plastic materials such as PVC pipes appear to be frequently colonized by γ -proteobacteria (of which species of the genus *Acinetobacter* belong to [179]).

Before colonizing the surface of PVC coupons (0.9×1 cm), the substrate for biofilm formation, it is necessary to sterilize it in order to eliminate any contamination. The material sterilization process was adapted from the method performed by Gomes et al. [24]. Briefly, the coupons were washed with commercial detergent (Continente, Portugal) and distilled water for 30 minutes (in a beaker with stirring). Following this, the coupons were then rinsed with distilled water to remove all the remaining detergent, and later immersed in 70% alcohol for 30 minutes. Then, each face of the washed coupons was exposed to UV radiation for 30 minutes before being placed on the microplate. Once the radiation sterilization was completed, the 14 coupons (for 7 different tests: control (CONT), DMSO solvent control (SOLV), CAF, CBZ, CIP, IBP and MIX, carried out in duplicate) were inserted on the microtiter plates wells. Finally, the coupons, now installed on the microtiter plate, were again exposed to UV radiation for 30 minutes.

3.3.2. Biofilm formation and exposure to emerging contaminants

The experimental set-up for biofilm exposure to ECs had 2 stages: biofilm formation or PVC coupon colonization, and biofilm exposure to ECs. Thus, to promote biofilm formation on the surface of the sterilized coupons (from **section 3.3.1**), bacterial suspensions were prepared. Bacterial cells, obtained on **section 3.2**, were harvested by centrifugation (Eppendorf centrifuge 5810R, Germany) at 3700 g for 15 min and resuspended in fresh R2A medium. The cell density was then adjusted to 10^7 cells/mL, to reflect the number of heterotrophic bacteria in DWDS [38]. To 4.950 mL of this suspension, 50 μ L of the previously prepared EC solution (**section 3.1**), R2A medium (control condition) or DMSO solution (solvent control) were added. The microtiter plate was incubated at 25°C and 120 rpm for 24 hours. In order to renew the nutrients and reinforce bacterial growth, the previous cell suspension was replaced by 1 mL of fresh R2A medium, prepared with EC or DMSO solutions (4,950 ml medium + 50 μ L of EC solution, R2A or DMSO medium), as shown on **Figure 5**, and the microplate was again incubated at 25°C and 120 rpm for 12 hours.

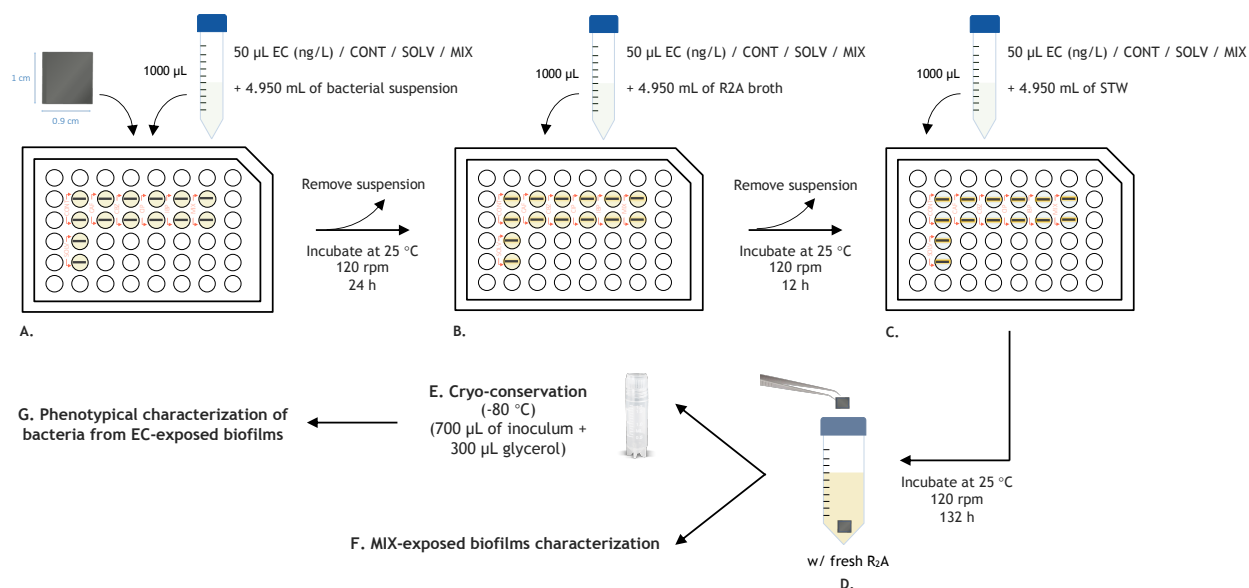


Figure 5. Experimental set-up: Biofilm formation and exposure (A - Microplate inoculation by adding bacterial suspension at adjusted concentration (25°C and 120 rpm, 24h); B - Exchange and renewal of nutrients (25 °C and 120 rpm, 12h); C - Exposure to ECs, at environmental conditions (25°C and 120 rpm, for 5 days); D - Harvesting *A. calcoaceticus* from exposed biofilms; E - cryoconservation of bacteria from ECs-exposed biofilms; F - MIC-exposed biofilm characterization; G - Phenotypical characterization of *A. calcoaceticus* from EC-exposed biofilms)).

The previous suspension was later replaced with exposure solutions (prepared with 50 μL of EC (**section 3.1**), synthetic tap water - STW (control condition) or DMSO (solvent control) solution to 4,950 mL of STW (prepared according to Gomes et al. ^[24]: 100 mg/L of NaHCO_3 (Fisher Scientific, Leicestershire, UK); 13 mg/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Merck, Darmstadt, Germany); 0.7 mg/L of K_2HPO_4 (Applichem Panreac, Darmstadt, Germany); 0.3 mg/L of KH_2PO_4 (CHEMLAB, Zedelgem, Belgium); 0.01 mg/L of $(\text{NH}_4)_2\text{SO}_4$ (Labkem, Barcelona, Spain); 0.01 mg/L of NaCl (Merck, Darmstadt, Germany); 0.001 mg/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (VWR PROLABO, Leuven, Belgium); 1 mg/L of NaNO_3 (Labkem, Barcelona, Spain); 27 mg/L of CaSO_4 (Labkem, Barcelona, Spain) and 1 mg/L of humic acids (Sigma-Aldrich, Steinheim, Germany)), requiring a washing step prior to STW replacement, to remove remaining nutrients and weakly bonded and non-adhered bacteria. This process proves relevant to the purpose of this work, as the use of STW aims to create a nutrient deficient environment mimicking the conditions found in DWDS. Microtiter plates were then incubated at 25°C and 120 rpm, for 5 additional days. Exposure to ECs for 7 days was reproduced in two independent assays, in duplicates.

At the end of the exposure period, coupons were carefully removed from the wells and inoculated into 10 mL of R2A medium (in 50 ml falcon tubes), with vortex agitation for 2 min, to promote dissociation of the cells from the PVC surface into the medium. Tubes were further incubated at 25°C and 120 rpm overnight. The resulting inoculum were stored at -80°C , to preserve the results obtained, collecting 700 μL of each inoculum and inserting them into sterile cryotubes with 300 μL of glycerol, to be later used in further studies, as detailed on **section 3.4**.

3.4. Behaviour characterization post selected emerging contaminants exposure

Aiming to study the effects of the selected ECs exposure on microbial behaviour, cryopreserved bacteria were characterized according to their ability to produce biofilms (**section 3.4.1**), their susceptibility to antibiotics (**section 3.4.2**), NaOCl minimum bactericidal concentration (**section 3.4.3**), motility (**section 3.4.4**), auto and coaggregation ability (**section 3.4.5**), and growth rate (**section 3.4.6**).

To initialize each characterization test, 50 μL of cryopreserved exposed bacteria were inoculated in 10 mL of R2A medium or Muller Hinton (MH) broth (for **section 3.4.2**), and incubated at 25°C and 120 rpm, overnight. Subsequently, they were resuspended and cell concentration was adjusted accordingly to each characterization assay.

3.4.1. *A. calcoaceticus* biofilm production: Biomass quantification

In order to assess *A. calcoaceticus* ability to form biofilms after exposure to ECs, a quantitative method was used: crystal violet staining, as described by Stepanović et al. [180]. This test was carried out three times (with five replicates), for two independent exposure assays. 200 µL of bacterial suspension with adequate cell concentration (10^7 cells/mL) were inserted in each well of the 96-well microtiter plate. The plates were then incubated at 25 °C and 120 rpm for 24 hours. Negative control was performed with R2A medium.

After incubation, bacterial suspensions were carefully discarded with a micropipette and wells were washed with 200 µL of STW to remove any non-adhered bacteria. Afterwards, 250 µL of absolute ethanol (Fisher, Leicestershire, England) was used to fix biofilms (incubating at room temperature for 15 minutes). Ethanol was later discarded, and fixed biofilms were dried at room temperature for 5 minutes. Crystal violet (Merck, Darmstadt, Germany) (1% (v/v)) was then added (200 µL) to stain biofilms for 5 minutes. Thereafter, the crystal violet was discarded and acetic acid (33% (v/v)) was used to elute leftover crystal violet after the staining process. Absorbance was measured using a microtiter plate reader (SPECTROstar Nano, BGM LABTECH), at 570 nm.

3.4.2. Susceptibility to antibiotics

A. calcoaceticus susceptibility to antibiotics was determined by disk diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. The antibiotics selected for this study are commonly used for treatment of *A. calcoaceticus* infections: CIP, levofloxacin – LEV, tetracycline – TET and trimethoprim - sulfamethoxazole - TMP-SMX [181]. The doses used for antibiotic susceptibility testing are described in **Table 3**.

Table 3. CLSI Standards for Antibigram Testing by Disc Diffusion Method.

Antibiotic	Abbreviation	CLSI guidelines (µg/disc)
Ciprofloxacin	CIP	5
Levofloxacin	LEV	5
Tetracycline	TET	20
Trimethoprim-sulfamethoxazole	TMP-SMX	1.25/23.75

An aliquot of 100 µL of bacterial suspension (6×10^8 cells/mL_{MH}, equivalent to 0.5 by McFarland standard) was spread on MH agar plates, using a sterile swab. Sterile paper discs with 10 µL of antibiotic, at proper concentration, were placed on the plate. Control tests were performed

using antibiotic solvent, DMSO. Plates were further incubated at 37°C for 24 h. Each exposure condition was tested in three times (with duplicates), for two independent exposure assays. After the incubation period, inhibition halos (mm) were measured.

3.4.3. Minimum bactericidal concentration of NaOCl

To test susceptibility to sodium hypochlorite – NaOCl, the minimum bactericidal concentration was determined according to Gomes et al. [23]. Different concentrations of NaOCl (Sigma-Aldrich, Steinheim, Germany) (1 - 10 mg free chlorine/L) are used in order to determine the minimum concentration of free chlorine necessary so that the growth of *A. calcoaceticus* does not occur in DWDS – minimum bactericidal concentration (MBC). Free chlorine concentrations were adjusted using a test kit by Hanna Instruments (using a photometer and the N,N-diethyl-p-phenylenediamine (DPD) method), as described by Gomes et al. [23]. Assays were run three times (with duplicates), for two independent exposure assays.

Tests were performed in 96-well microplates, where 20 µL of prepared NaOCl solution and 180 µL of bacterial suspension (to a cell concentration of 10^6 /mL in STW) were added to each well. STW was used to simulated the low availability of nutrients found in DWDS. The microplates were incubated at 25°C and 120 rpm for 24 h. After incubation, 180 µL of each well was discarded and 180 µL of sodium thiosulfate (0.5% w/v) (Labkem, Spain) was added to neutralize NaOCl. Subsequently, 20 µl from each well was transferred to R2A agar medium and incubated at 25°C for 48 h. The MBC of NaOCl corresponds to the lowest concentration at which no *A. calcoaceticus* growth was observed.

3.4.4. Motility assay

To determine the effect induced by exposure to ECs on *A. calcoaceticus* motility, twitching, swarming and swimming motility were tested according to Gomes et al. [165]. These tests were performed three times (with duplicates), for two independent exposure assays.

Briefly, 15 µL of each exposed bacterial suspension (10^7 cells/mL in saline solution) was placed in the centre of plates composed by 1% tryptone, 0.25% NaCl and 0.3% (swimming), 0.7% (swarming) or 1.5% (twitching) agar (w/v) [182, 183]. Plates were incubated at 25°C, and motility halos were measured after 48 hours of incubation (mm).

3.4.5. Autoaggregation and coaggregation assays

Autoaggregation and coaggregation ability of *A. calcoaceticus* was quantified using a spectrophotometric method, according to Karched et al. ^[184]. As previously mentioned, *A. calcoaceticus* is known as a bridging organism, and therefore it would be interesting to understand the extent of selected ECs effect on this matter, at tested conditions. Briefly, for autoaggregation assessment of the exposed bacteria, a volume of 1 mL of each bacterial suspension (exposed *A. calcoaceticus*, and non-exposed *Burkholderia cepacia* and *Streptomonas maltophilia*, at 10^9 cells/mL in STW) were inserted into individual cuvettes. As for coaggregation assessment, aliquots of 500 μ L of exposed *A. calcoaceticus* were combined with 500 μ L of non-exposed *B. cepacia* or *S. maltophilia*. Absorbance was read at 600 nm, in 30 min intervals for 2h (including absorbance at 0 h). As a decrease in optical density of bacterial suspensions with time can relate to autoaggregation or coaggregation, their percentage of auto and coaggregation was determined by **Equation 1**. The assays were performed three times, for one independent exposure assay.

$$\% \text{ autogregation or coaggregation} = \frac{Abs_{t_0} - Abs_t}{Abs_{t_0}} \times 100\% \quad (1)$$

Where Abs_{t_0} is the initial absorbance and Abs_t is the sample absorbance at t time, incubated at room temperature.

3.4.6. *A. calcoaceticus* growth rate

A. calcoaceticus growth rate was estimated by monitoring the optical density of bacterial suspensions (with an initial cell concentration of 10^7 cells/mL in R2A) for 24 h in intervals of 30 min, at 600 nm, using a microtiter plate reader (SPECTROstar Nano, BGM LABTECH). The assays were performed in 96 well microtiter plates, reproduced three times (with triplicates), for two independent exposure assays.

3.5. Characterization of biofilms exposed to ECs mixture

Procedures for biofilm formation and exposure to ECs mixture were similar to **section 3.3.2**. Briefly, 11 sterile coupons (prepared according to **section 3.3.1**) were installed in 48 wells microtiter plates, for each tested conditions. Bacterial suspensions (**section 3.2**), at 10^7 cells/mL of R2A broth, were then distributed and exposed to MIX at environmental concentrations, and incubated at 25°C and 120 rpm. At the end of a 24 hour period, nutrients were renewed by carefully replacing bacterial suspensions with sterile R2A broth diluted by 10 times and MIX at environmental concentrations. Microplates were left for incubation at 25°C and 120 rpm, for 6 additional days. Control conditions were performed with unexposed *A. calcoaceticus* (CONT) and solvent control, DMSO. Exposure was run in duplicates, for three independent assays.

3.5.1. Total and viable cell count

BacLight staining is a dual staining method that has been proven to be an adequate method for direct enumeration of bacteria in DW ^[185]. Therefore, an epifluorescence staining method, using the LIVE/DEAD Bacterial Viability Kit (BacLight), was applied. This kit contains two nucleic acid-binding stains: SYTO 9 and propidium iodide (PI). SYTO 9 penetrates bacterial membrane, staining all the bacteria. PI is only able to penetrate damaged cell membranes ^[185]. The combined use SYTO9 and PI allows the operator to distinguish viable/non-damaged cells (generating green fluorescence by SYTO 9 staining) from non-viable/damaged cells (generating red fluorescence by PI staining).

Briefly, colonized coupons (from **section 3.5**) were washed in STW to remove weakly and non-adhered bacteria. Then coupons were inserted in a sterile centrifuge tube containing 5 mL of STW and the bacteria detachment from coupons surface was ensured by continuous agitation for 2 min in a vortex. Afterwards, an aliquot of 1 mL of the resulting suspension was mixed with 250 µL of SYTO 9 and 50 µL of PI (Invitrogen Life Technologies, Alfacene), and left for incubation for 10 min in the dark, at room temperature. Finally, the mixture was filtered through a 0.22 µm black polycarbonate Nuclepore™ filter (Whatman, UK) and samples were examined under LEICA DM LB2 epifluorescence microscope (LEICA Microsystems), using a 515-560 nm excitation filter in combination with a 590 nm emission filter. Images were obtained with a LEICA DFC300 FX camera system (LEICA Microsystems Ltd, Heerbrugg) attached to the microscope, and a LEICA IM50 image manager, processing and archiving software. Furthermore, the number of cells was

estimated from counts of a minimum of 15 microscopic view fields per sample. Total and viable cell count was done for each independent exposure assay.

Cell enumeration was performed using the free image processing software FIJI/ImageJ (<https://imagej.net/software/fiji/>), version 2.0.0-rc-59/1.51n. Exported images from the microscope software were processed in order to obtain a better and high-quality image (intensify fluorescence and contrast, and remove background noise) and later split into separate colour channels (red, green and blue (blue colour channel proves no relevancy to Live/Dead cell counting)). Threshold was then applied, which highlights the structures the operator desires the software to count. In some instances, cells merge together after setting the threshold value, causing the software to assume a glomerate of cells as an individual cell. Therefore, the image was run through a watershed program, cutting merged cells apart with a 1 pixel thick line where the ImageJ program assumes is the division between two different cells. Finally, the Analyse Particles function was used for automated cell counting (while defining size and circularity parameters for particle counting and exclude particles located on the edges of the image). For more information on image processing and step-by-step automated counting, refer to **Appendix II**.

3.5.2. EPS analysis

In order to assess the effects of ECs exposure on *A. calcoaceticus* biofilms matrix, EPS quantification was done according to Gomes et al. ^[186].

3.5.2.1. EPS extraction

Ten 7 day old colonized coupons (previously washed with STW) were transferred into 7 mL of extraction buffer (0.76 g/L of $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$; 0.36 g/L of $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$; 0.53 g/L of NaCl and 0.08 g/L of KCl) and biofilm was collected with vortex agitation for 2 min. EPS extraction was done with 1g of Dowex® resin (Sigma Aldrich, Steinheim, Germany), in a 25 mL beaker with 400 rpm agitation at 4°C, for 4h. Concluding the extraction period, the suspension was centrifuged at 3700 g for 5 min, and supernatant and pellet (later resuspended in 7 mL of extraction buffer) were collected for further analysis.

3.5.2.2. Proteins and polysaccharides quantification

For proteins quantification, the commonly used Bradford microassay was used. The Bradford protein assay is a colorimetric assay based on a colour shift in the visible spectrum, resulting from the formation of a protein-dye complex ^[187]. As a result, proteins presence can be detected and quantified.

A standard curve was drawn from a dilution series of protein solutions (Bovine serum albumin (BSA)), with known concentrations, allowing to interpolate the protein content of an unquantified sample. 150 μ L of Bradford reagent and 150 μ L of sample or BSA were added to each well and microtiter plates were left for incubation for a period of 5 min. Absorbance was later read by a microtiter plate reader (SPECTROstar Nano, BGM LABTECH), at 595 nm. These assays were performed in 96-well microtiter plates, in triplicates for each independent exposure assay. The detection limit of the method is 0.530 mg/L.

The total polysaccharides quantification was performed through the phenol-sulfuric method ^[188]. A glucose was used as standard to prepare a calibration curve, which is needed to determine polysaccharide concentrations on EPS matrix. An aliquot of 250 μ L per sample was transferred to a glass test tube, to which 0.5 mL of phenol and 2.5 mL of sulphuric acid were added and succinctly homogenized by vortex agitation. The resulting solution was left to incubate at room temperature for 5 min and absorbance was later measured and registered, at 490 nm. Assays were performed in duplicates, for each independent exposure assay. The detection limit of the method is 0.855 mg/L.

3.6. Statistical analysis

Statistical analysis of acquired data was accomplished by applying a one-way analysis of variance (ANOVA), and comparisons between and within experimental groups were performed using the Tukey test. These calculations were based on a confidence level $\geq 95\%$ (*i.e.*, for a P value lesser than 0.05, the result is considered statistically significant). Data was processed using Microsoft Excel, version 16.53 and/or IBM® SPSS® Statistics, version 27.0.

Chapter 4. Results

4.1. Effect of ECs exposure on *A. calcoaceticus* growth rate

Table 4 presents the specific growth rate (μ - h^{-1}) and doubling time (t_d - h) of non-exposed and exposed *A. calcoaceticus*. Higher specific growth rates were found for *A. calcoaceticus* biofilms exposed to ECs. However, only CIP had a significant impact on specific growth rate and duplication time ($P < 0.05$), increasing μ by 90.5%, and t_d by 51.2%.

Table 4. Effect of EC exposure on growth parameters of A. calcoaceticus (25 °C, 500 rpm). Reported values are the mean value $\pm$ standard deviation of six independent assays, resultant from two independent exposure assays.

	CONT	Solv	CAF	CBZ	CIP	IBP	MIX
μ (h^{-1})	0.21 $\pm$ 0.08	0.27 $\pm$ 0.10	0.33 $\pm$ 0.14	0.38 $\pm$ 0.16	0.40 $\pm$ 0.11 *	0.30 $\pm$ 0.14	0.38 $\pm$ 0.06
t_d (h)	3.85 $\pm$ 1.87	2.89 $\pm$ 1.00	2.45 $\pm$ 1.01	2.21 $\pm$ 1.13	1.88 $\pm$ 0.54 *	3.03 $\pm$ 1.83	1.87 $\pm$ 0.27

CONT - control, Solv - DMSO solvent control, CAF - caffeine, CBZ - carbamazepine, CIP - ciprofloxacin, DMSO - Dimethyl sulfoxide, IBP - ibuprofen, MIX - mixture of ECs, μ is the specific growth rate (h^{-1}), t_d is population doubling time (h), * corresponds to results with statistically significant differences in comparison to CONT ($P < 0.05$), ** corresponds to results with statistically significant differences in comparison to Solv ($P < 0.05$)).

4.2. Effect of ECs exposure on *A. calcoaceticus* antimicrobial susceptibility

4.2.1. *A. calcoaceticus* NaOCl susceptibility

The susceptibility of *A. calcoaceticus* from ECs-exposed biofilms to free chlorine was evaluated in terms of MBC. Results are presented in **Table 5**. Resulting MBCs are presented as the minimum range of values at which no bacterial growth was observed. Complete inactivation of *A. calcoaceticus* occurred in concentrations between 1.0 and 2.0 mg/L. The exposure to DMSO seemed to have slightly increased the MBC range. Same effect was observed for CIP exposure (when compared to non-exposed biofilms) and IBP exposure (when compared to DMSO solvent control) further increased the MBC range. However, CBZ exposure decreased the MBC range.

Table 5. Minimum bactericidal concentration of NaOCl (mg/L) after exposure of A. calcoaceticus to ECs for 7 days. Values are reported as the range of MBC obtained from six independent assays, resultant from two independent exposure assays.

	CONT	Solv	CAF	CBZ	CIP	IBP	MIX
MBC (mg/L)	1.0 - 2.0	1.0 - 3.0	1.0 - 2.0	1.0 - 2.0	1.0 - 3.0	1.0 - 4.0	1.0 - 3.0

CONT - control, Solv - DMSO solvent control, CAF - caffeine, CBZ - carbamazepine, CIP - ciprofloxacin, DMSO - Dimethyl sulfoxide, IBP - ibuprofen, MIX - mixture of ECs

4.2.2. *A. calcoaceticus* antibiotic susceptibility

The CLSI standards define the breakpoints for which a particular bacterial strain is considered resistant (R) or susceptible (S) to an antibiotic. According to CLSI standards [189], inhibition halos ≥ 0.21 , ≥ 0.17 , ≥ 0.15 and ≥ 0.16 mm, for CIP, LEV, TET and TMP-SMX, respectively, indicate that *Acinetobacter* spp. is considered susceptible to each of these antibiotics. Obtained results (**Table 6**) suggest that *A. calcoaceticus* is susceptible to all antibiotics analyzed.

Results proved that a 7 days exposure to ECs (antibiotics and non-antibiotics) at residual concentrations (ng/L), had indeed affected the susceptibility of *A. calcoaceticus* from ECs-exposed biofilms to CIP, LEV, TET and TMP-SMX. Regarding the impact of non-antibiotic tested ECs (CAF, CBZ and IBP), it is possible to point out some alterations in *A. calcoaceticus* susceptibility to specific antibiotics. For instance, CAF exposure significantly ($P < 0.05$) decreased *A. calcoaceticus* susceptibility to TMP-SMX in comparison to *A. calcoaceticus* from non-exposed biofilms, while CBZ exposure resulted in a statistically significant ($P < 0.05$) increase in their susceptibility to TMP-SMX. As for the DW contaminant IBP, a decrease in LEV and TET susceptibility was observed ($P < 0.05$).

In terms of *A. calcoaceticus* behaviour after biofilm exposure to the antibiotic CIP, a statistically significant ($P < 0.05$) decrease in susceptibility was observed to LEV and CIP, as well as an increase in TMP-SMX susceptibility ($P < 0.05$).

Table 6. Inhibition halo diameter (mm) for CIP, LEV, TET and TMP-SMX. Reported values are the mean value $\pm$ standard deviation of six independent assays, resultant from two independent exposure assays.

	Inhibition halo diameter (mm)				
	CIP	LEV	TET	TMP-SMX	DMSO
CONT	27.5 $\pm$ 1.0	27.8 $\pm$ 1.4	23.4 $\pm$ 2.4	27.0 $\pm$ 0.9	0.0 $\pm$ 0.0
Solv	27.4 $\pm$ 1.9	27.8 $\pm$ 1.5	23.6 $\pm$ 2.4	27.2 $\pm$ 1.0	0.0 $\pm$ 0.0
CAF	27.6 $\pm$ 1.4	26.8 $\pm$ 1.5	24.2 $\pm$ 1.3	26.0 $\pm$ 1.1 *	0.0 $\pm$ 0.0
CBZ	26.8 $\pm$ 2.1	27.2 $\pm$ 1.8	23.2 $\pm$ 1.3	29.0 $\pm$ 0.7 **	0.0 $\pm$ 0.0
CIP	26.4 $\pm$ 1.5 *	26.2 $\pm$ 1.0 *	23.9 $\pm$ 2.7	28.0 $\pm$ 0.8 *	0.0 $\pm$ 0.0
IBP	27.6 $\pm$ 2.0	26.6 $\pm$ 1.2 **	22.0 $\pm$ 0.9 **	26.4 $\pm$ 0.9	0.0 $\pm$ 0.0
MIX	27.3 $\pm$ 1.0	27.3 $\pm$ 1.5	22.3 $\pm$ 1.4	27.3 $\pm$ 1.4	0.0 $\pm$ 0.0

CONT - control, Solv – DMSO solvent control, CAF - caffeine, CBZ - carbamazepine, CIP - ciprofloxacin, DMSO - Dimethyl sulfoxide, IBP - ibuprofen, LEV - levofloxacin, MIX - mixture of ECs, TET -tetracycline, TMP-SMX - trimethoprim – sulfamethoxazole, * corresponds to results with statistically significant differences in comparison to CONT ($P < 0.05$), ** corresponds to results with statistically significant differences in comparison to Solv ($P < 0.05$)).

4.3. *A. calcoaceticus* ability to form biofilm

A. calcoaceticus from EC-exposed biofilms were used to assess their biofilm formation ability through crystal violet staining. **Figure 6** presents the results obtained.

The exposure of *A. calcoaceticus* to the solvent (Solv) DMSO, increased biofilm formation capacity ($P < 0.05$). Therefore, the effect of ECs prepared with DMSO (CBZ, IBP and MIX) were evaluated by comparison with Solv. Statistically significant changes ($P < 0.05$) were detected after IBP and MIX exposure. IBP exposure for 7 days induced a decrease of approximately 11% in the *A. calcoaceticus* ability to form biofilm. On the contrary, MIX exposure increased biofilm formation by 16%. In other instances, even though changes in biofilm formation are visible, statistical analysis proved the insignificance of registered alterations ($P > 0.05$).

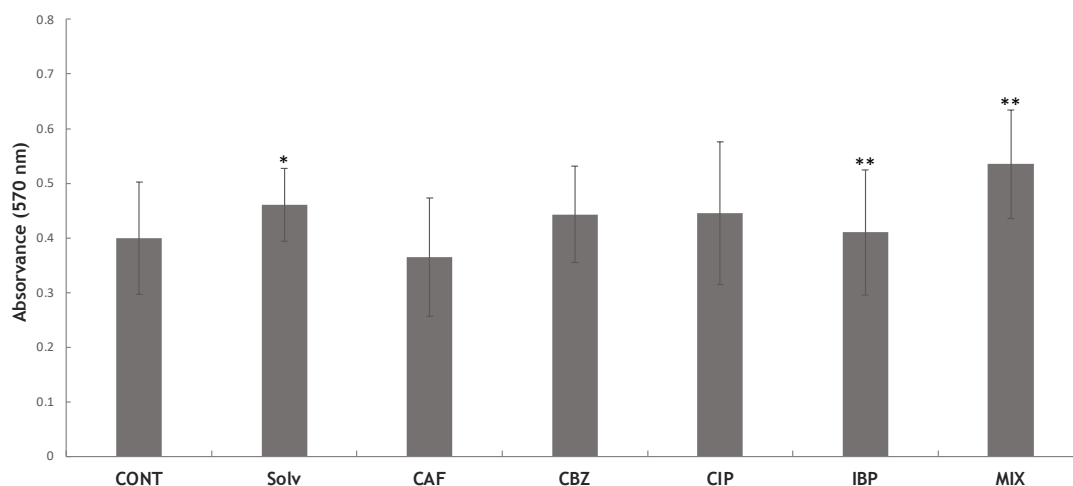


Figure 6. Biofilm formation (24h) of *A. calcoaceticus* after exposure to ECs for 7 days. Results were obtained from OD measurements at 570 nm and are presented as the average of six independent assays, resultant from two independent exposure assays (CONT - non-exposed *A. calcoaceticus*, Solv – DMSO solvent control, CAF - caffeine, CBZ - carbamazepine, CIP - ciprofloxacin, IBP - ibuprofen, MIX - ECs mixture, * corresponds to results with statistically significant differences in comparison to CONT ($P < 0.05$), ** corresponds to results with statistically significant differences in comparison to Solv ($P < 0.05$)).

4.4. Effect of ECs exposure on *A. calcoaceticus* motility

A. calcoaceticus expresses at least three motility types: swimming, swarming and twitching [13, 190-192]. Therefore, the impact of ECs exposure on the motility of *A. calcoaceticus* from exposed biofilms was evaluated and results are presented in **Table 7**.

Despite slight changes in motility expression observed on bacteria from ECs-exposed biofilms, no statistically significant alterations ($P > 0.05$) were detected.

Table 7. Assessment of *A. calcoaceticus* motility (swarming, swimming or twitching) after exposure to ECs for 7 days. Reported values are the mean value $\pm$ standard deviation of six independent assays, resultant from two independent exposure assays.

	Motility development halo diameter (0 h - 48 h) (mm)		
	Swarming	Swimming	Twitching
CONT	1.5 $\pm$ 0.3	0.5 $\pm$ 0.2	1.3 $\pm$ 0.4
Solv	1.6 $\pm$ 0.6	0.6 $\pm$ 0.3	1.4 $\pm$ 0.3
CAF	1.1 $\pm$ 0.3	0.7 $\pm$ 0.3	1.4 $\pm$ 0.6
CBZ	1.2 $\pm$ 0.5	0.7 $\pm$ 0.3	1.5 $\pm$ 0.2
CIP	1.6 $\pm$ 0.2	0.7 $\pm$ 0.3	1.6 $\pm$ 0.5
IBP	1.6 $\pm$ 0.5	0.9 $\pm$ 0.3	1.3 $\pm$ 0.5
MIX	1.6 $\pm$ 0.6	0.5 $\pm$ 0.2	1.3 $\pm$ 0.4

CONT - control, Solv - DMSO solvent control, CAF - caffeine, CBZ - carbamazepine, CIP - ciprofloxacin, DMSO - Dimethyl sulfoxide, IBP - ibuprofen, MIX - mixture of ECs

4.5. Effect of ECs exposure on *A. calcoaceticus* auto and coaggregation

A. calcoaceticus is able to form multicellular clusters, an event called autoaggregation [14]. As **Figure 7** shows, *A. calcoaceticus*, at all tested conditions, is able to autoaggregate ($P < 0.05$). On the contrary, *B. cepacia* and *S. maltophilia* do not possess this feature ($P > 0.05$). Overall, *A. calcoaceticus* autoaggregation ability did not significantly change after exposure ($P > 0.05$).

The bridging function of *A. calcoaceticus* after exposure to ECs when combined with two other DW bacteria: *B. cepacia* and *S. maltophilia*, was also assessed. Coaggregation results with *B. cepacia* and *S. maltophilia* are presented in **Figure 8** and **Figure 9**, respectively. Indeed, non-exposed *A. calcoaceticus* had the ability to coaggregate with *B. cepacia* and *S. maltophilia* as expected, and a more prominent aggregation between *A. calcoaceticus* and *S. maltophilia* was observed. Coaggregation with *B. cepacia* had visibly decreased with exposure to ECs in general (**Figure 8**), most noticeably after CBZ and CIP exposure. However, these decreases had no statistical significance ($P > 0.05$). As for *A. calcoaceticus* and *S. maltophilia* coaggregation, CBZ and CIP increased coaggregation percentage and Solv and MIX exposure decreased this ability. Yet again, these changes were ruled as statistically insignificant ($P > 0.05$).

Results

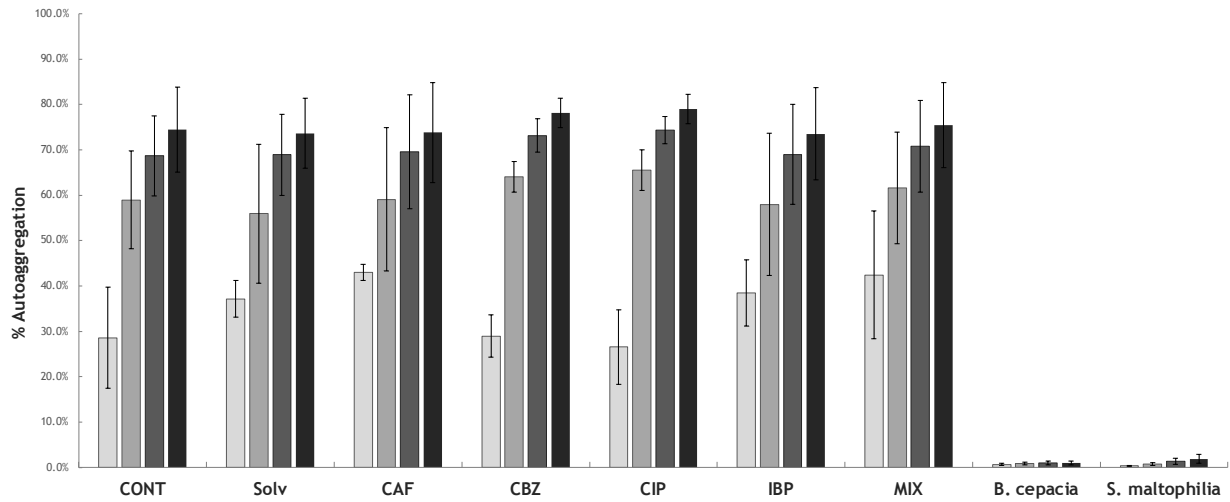


Figure 7. Autoaggregation ability of *A. calcoaceticus* from non-exposed (CONT) and exposed (Solv – DMSO solvent, CAF – caffeine, CBZ – carbamazepine, CIP – ciprofloxacin, IBP – ibuprofen, MIX – mixture of ECs) biofilms, and non-exposed *B. cepacia* and *S. maltophilia*. OD_{600} was measured every 30 min and results were converted into autoaggregation percentage. The results are the average and standard deviation of three independent exposure, resultant of one independent exposure assay¹ (■ – 30 min; ■ – 60 min; ■ – 90 min ; ■ – 120 min).

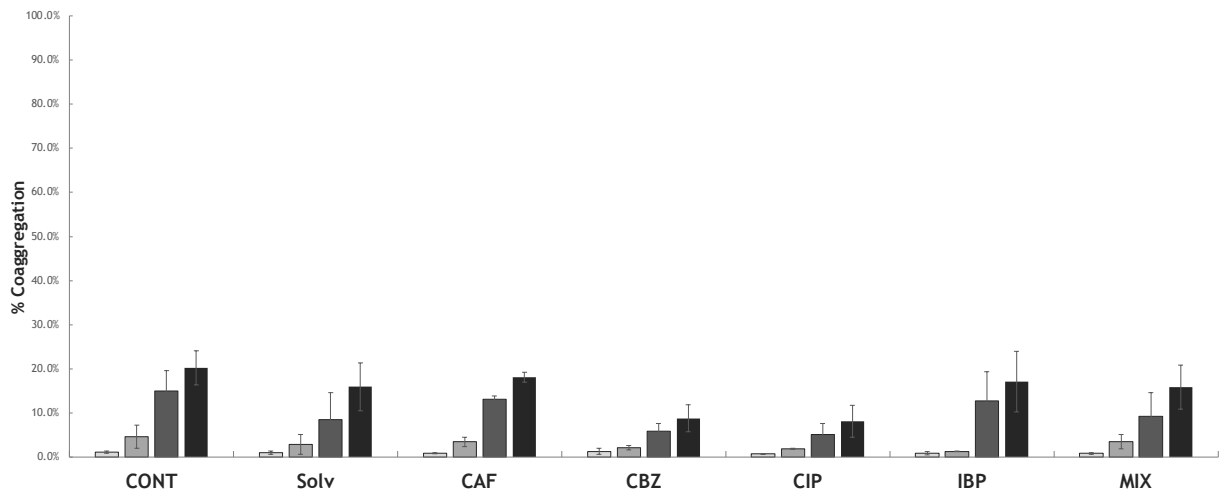


Figure 8. Coaggregation ability of *A. calcoaceticus* from non-exposed (CONT) and exposed (Solv – DMSO solvent, CAF – caffeine, CBZ – carbamazepine, CIP – ciprofloxacin, IBP – ibuprofen, MIX – mixture of ECs) biofilms paired with non-exposed *B. cepacia*. OD_{600} was measured every 30 min and result were converted into coaggregation percentage. The results are the average and standard deviation of three independent exposure, resultant of one independent exposure assay¹ (■ – 30 min; ■ – 60 min; ■ – 90 min ; ■ – 120 min).

¹ In some cases, the average was obtained from two replicates, resultant of one independent exposure assay. Therefore, more independent trials would be needed in order to obtain a solid and robust result and statistical analysis.

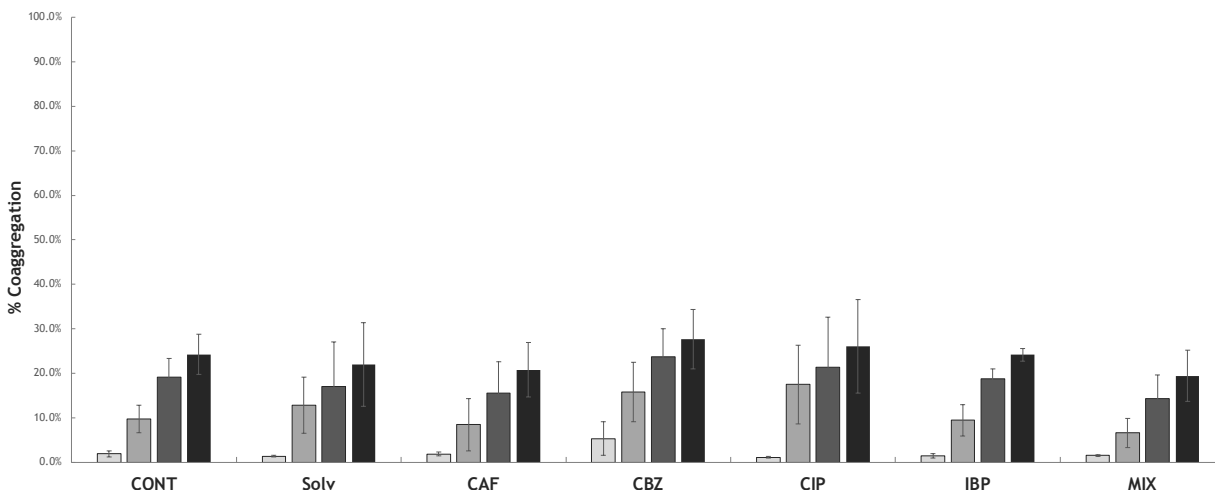


Figure 9. Coaggregation ability of *A. calcoaceticus* from non-exposed (CONT) and exposed (Solv – DMSO solvent, CAF - caffeine, CBZ - carbamazepine, CIP - ciprofloxacin, IBP - ibuprofen, MIX – mixture of ECs) biofilms paired with non-exposed *S. maltophilia*. OD_{600} was measured every 30 min and result were converted into coaggregation percentage. The results are the average and standard deviation of three independent exposure, resultant of one independent exposure assay¹ (■ – 30 min; ■ – 60 min; ■ – 90 min ; ■ – 120 min).

4.6. Effect of EC exposure on *A. calcoaceticus* biofilms

Given the impact of MIX in biofilm production (section 4.3), and its resemblance to real DWDS (complexity of EC mixtures), this condition was selected to address EC mixtures impact on *A. calcoaceticus* biofilms. Biofilms were characterized in terms of proteins and polysaccharides content (section 4.6.1) and cellular density (section 4.6.2), after exposure to the mixture of ECs for 7 days.

4.6.1. Effect of MIX exposure on EPS content

The extracellular polymeric matrix of exposed *A. calcoaceticus* biofilms were characterized in terms of protein and polysaccharides content. Results are presented on Table 8.

Regarding protein content of exposed biofilms, the lowest values are attributed to biofilms exposed to DMSO solvent, exhibiting the lowest protein contents in EPS matrix but also intracellularly. However, this change is not statistically significant ($P > 0.05$) compared to non-exposed biofilms. Concerning polysaccharide content of biofilms, DMSO solvent exposure increased intracellular and extracellular polysaccharide content. This increase in intracellular polysaccharide content was statistically significant ($P < 0.05$). However, the same did not seem to happen to extracellular polysaccharide content ($P > 0.05$). MIX exposure increased intracellular

and decreased extracellular polysaccharide substances production of *A. calcoaceticus*, however, when compared to DMSO, that difference was proven to be statistically irrelevant ($P > 0.05$)

Table 8. EPS composition of extracted biofilms, after exposure. Reported values are the mean value $\pm$ standard deviation for three independent exposure assays.

	Protein content ($\mu\text{g}/\text{cm}^2$)			Polysaccharide content ($\mu\text{g}/\text{cm}^2$)		
	CONT	Solv	MIX	CONT	Solv	MIX
Intracellular	0.74 ± 0.05	0.62 ± 0.05	0.72 ± 0.05	0.96 ± 0.21	3.24 ± 0.14 *	5.65 ± 0.15
Extracellular	0.54 ± 0.05	0.35 ± 0.05	0.61 ± 0.05	3.48 ± 0.12	5.17 ± 0.14	2.54 ± 0.12

CONT - control, Solv - DMSO solvent control, MIX - mixture of ECs, * corresponds to results with statistically significant differences in comparison to CONT ($P < 0.05$)

4.6.2. Effect of MIX exposure on cellular density of *A. calcoaceticus* biofilms

Cell density of biofilm and/or their physiological state play a role in the phenotypic drug resistance [193, 194], therefore, exposed biofilms were characterized in terms of cellular density through a Live/Dead assay. **Figure 10** presents the bacterial density on exposed biofilms.

DMSO solvent exposure affected biofilm bacterial composition ($P < 0.05$), by decreasing the number of viable and total cells contained in the biofilm. Therefore, statistical analysis was performed between the solvent control and MIX groups. As a result, biofilm exposure to MIX increased biofilm cellular (total and viable) density significantly ($P < 0.05$), even though this change is not noticeable and cell numbers are slightly lower when compared to CONT.

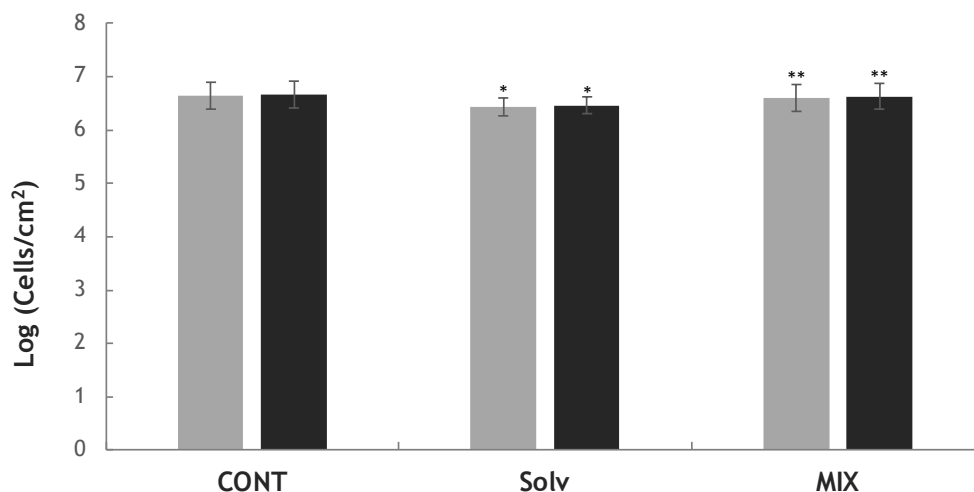


Figure 10. ECs effect on *A. calcoaceticus* biofilm cellular density. The results are means from three independent exposure assays (■ – Viable cells; ■ – Total cells, CONT - non-exposed *A. calcoaceticus*, Solv – DMSO solvent control, MIX - ECs mixture; * corresponds to results with statistically significant differences in comparison to CONT ($P < 0.05$), ** corresponds to results with statistically significant differences in comparison to Solv ($P < 0.05$)).

Results

Chapter 5. Discussion

Biofilm, an intrinsic and adaptive resistance mechanism, is essential for the survival of bacteria when faced with adverse environmental stimuli ^[195]. Although information is scarce, recent studies have revealed that exposing DW biofilms to occurring ECs can affect their balance and behaviours ^[22-24, 134, 196]. However, to date, no studies have addressed the effects of ECs on *A. calcoaceticus*. *Acinetobacter* is an emerging ^[197], opportunistic ^[198], pathogenic, gram-negative coccobacillus ^[199], that inhabits water, soils and animals, having the ability to colonize skin, wounds, and respiratory and intestinal tracts ^[200, 201]. Infections caused by *A. calcoaceticus* commonly result in pneumonia ^[44, 202]. Their residence in DW indicates that even under conditions of low concentration of nutrients and residual concentrations of disinfecting agents ^[3], this organism is able to survive in DWDS and form biofilms. Given the reports of the presence of selected ECs in DW ^[4, 85, 90, 95, 105, 176, 203, 204], biofilms in DWDS and premise plumbing are constantly exposed to residual concentrations of these pharmaceutical compounds. Therefore, it is extremely important to study its effect on bacterial behaviour.

Boe-Hansen et al. ^[205] indicated that the bulk phase growth in DW accounts for a significant part of the total aftergrowth along DWDS at nutrient deficiency conditions, however biofilms are still the major contributors. Furthermore, the environmental history and physiological state ^[206] affects bacteria growth rate. An increase and faster regrowth of suspended bacteria in the bulk water due to EC exposure could represent issues in DW quality management. Indeed, biofilm exposure to CIP affected *A. calcoaceticus* growth rate, increasing by an astounding 90.5%. The solemn purpose of studying this parameter was to assess possible negative repercussions due to EC exposure, and results suggest that biofilm exposure to CIP could potentially impact the resuspended *A. calcoaceticus* behaviour. Furthermore, growth rate is an important modulator of drug activity in biofilms ^[207]. Growth rate is related to bacterial metabolic activities ^[208, 209], which are known to influence bacterial susceptibility to antimicrobial agents ^[210]. Changes in growth rate and nutritional environment are accompanied by changes in the cell envelope composition, thereby affecting the susceptibility of the bacteria to antimicrobial agents ^[207]. Baillie et al. ^[207] revealed that planktonic *Candida albicans* cells are more resistant to antifungal agents at lower growth rates. However, in this work, CIP exposure had further increased *A. calcoaceticus* tolerance to NaOCl,

CIP and LEV, which does not correlate with expectations from increased growth rate. Exceptionally, only *A. calcoaceticus* susceptibility to the antibiotic TMP-SMX had increased.

A better understanding of the extent of biocidal action of disinfectants is extremely important in order to apply more adequate disinfection strategies and deliver efficient microbial control within DWDS [211]. The inefficiency of these treatments, is of utmost concern, since it hampers the biological control of DW, constitutes a biological hazard and contributes to the spread of antibiotic resistance, and selection and proliferation of ARBs [212, 213]. This work reveals that CIP and IBP exposure may cause a slight increase on *A. calcoaceticus* tolerance to NaOCl, while CBZ exposure may be responsible for a slight increase in its susceptibility. Similarly, Gomes et al. [22] determined that *B. cepacia* became more susceptible to NaOCl after biofilm exposure to CBZ (258 ng/L). However, although an increase in susceptibility could be beneficial for microbial control in DWDS, chlorine disinfection can select antibiotic resistant bacteria and increase relative abundance of ARGs [141]. In DWDS, the minimum recommended residual free chlorine concentration is 0.2 mg/L as per WHO standards [214], and it is expected not to exceed 2 mg/L, due to taste concerns [215]. In Portugal, free chlorine levels are expected to be between 0.2 and 0.6 mg/L, according to law decree no. 152/2017. In general, resulting *A. calcoaceticus* NaOCl tolerance range exceeds these set values, but, seeing that CIP and IBP exposure further expanded *A. calcoaceticus* susceptibility range, EC presence in DWDS could pose as a concerning subject regarding the efficacy of free chlorine on bacterial control in bulk water. Furthermore, the Environmental Protection Agency (EPA) indicates that chlorine levels up to 4 mg/L are considered safe in drinking water, as harmful health effects are unlikely to occur [216]. Therefore, in order to efficiently inhibit *A. calcoaceticus* growth (provided from biofilm exposure to IBP), elevating residual free chlorine levels in DW to 4 mg/L would not compromise DW safety, but, however, could compromise other DW organoleptic properties, such as taste and possibly the formation of harmful disinfection by-products [217]. In addition, chlorine residual concentration declines along its journey in DWDS due to multiple factors (such as: reactions with biofilms, pipe walls, dissolved organic matter, corrosion by-products or bulk-water microorganisms) [36, 218], that could further hamper the disinfection process.

Behind the tolerance to disinfectants, the spread of antibiotic-resistant microorganisms is also a problem faced in DW. Antibiotic resistance can be intrinsic (of natural and predictable occurrence) or extrinsic/acquired (evolution from genetic mutations/changes (unpredictable)) [93].

Also, resistance of bacteria in biofilms is attributed to a combination of the biofilm, as a physical barrier, and physiological (due to genetic modification) resistance [36]. These mutations can be acquired through horizontal transfer of genes from different species [93, 127, 137, 142]. Once resistance is acquired, cells divide and multiply, spreading, exponentially, resulting in their rapid proliferation in aquatic systems [93, 137]. Thus, constituting a concern regarding the quality and safety of DWDS in terms of public health. Several mechanisms are involved in antibiotic resistance, such as: (1) production of enzymes that inactivate the antibiotic molecule, (2) mutation in the site where the antibiotic acts, (3) reduction of absorption, (4) efflux mechanisms that remove the antibiotic from the interior of the cells, (5) excessive production of the antibiotic's target compound, or (6) creation of an alternative metabolic pathway (that performs a similar function to that inactivated by the antibiotic or by limiting cellular requirements for the metabolites produced in this inhibited pathway) [137, 142, 219]. The development of antimicrobial resistance due to trace concentrations of ECs have long and recently been reported in various environments [12, 220, 221]. Furthermore, *A. calcoaceticus* has a tendency to acquire and disseminate antibiotic resistance factors [222], making this a concern for treatments and spread control in DWDS. Increased antibiotic resistance may result in enhanced virulence, pathogenicity, disease outbreaks and transmission, as the consumer is directly exposed to antibiotic-resistant pathogens through consumption of contaminated water [223].

No specific resistance mechanisms of *A. calcoaceticus* isolates were found, and the studies encountered mostly refer to the resistance mechanism of *A. calcoaceticus* coupled with *Acinetobacter baumannii*, forming the *Acinetobacter baumannii* - *A. calcoaceticus* complex (Acb) [224-227]. However, the present work revealed that exposure to CAF, CBZ, CIP and IBP for 7 days can change the susceptibility of *A. calcoaceticus* to CIP, LEV, TET and TMP-SMX. These results confirm that individual exposure to PhACs, non-antibiotic and antibiotic, had an influence on *A. calcoaceticus* behaviour. Additionally, it was mentioned earlier that sub-inhibitory concentrations are the major contributors to promoting resistance. In accordance with these expectations, exposure to CIP decreased *A. calcoaceticus* susceptibility to CIP and LEV. Furthermore, IBP exposure decreased *A. calcoaceticus* susceptibility to LEV and TET, and CAF exposure decreased *A. calcoaceticus* susceptibility to TMP-SMX. Alternatively, CBZ and CIP exposure have increased *A. calcoaceticus* susceptibility to TMP-SMX. Other works refer that CBZ exposure (50,000 ng/L) has enhanced the conjugative transfer of multi-resistance genes within and across

bacterial genera, increased cell membrane permeability, and pilus generation, and expression of genes related to these processes were significantly upregulated [228]. However, in the present work, the increase on the susceptibility to TMP-SMX after biofilm exposure to CBZ could not be correlated with such observations. In summary, these results suggest that selected ECs may have induced alterations, that could be related to increased or decreased activities of resistance mechanisms to CIP, LEV, TET and TMP-SMX. However, tolerance is a concept that differs from resistance. Resistance is the genetic ability of bacteria to encode resistance genes that counteract the inhibitory effect of antimicrobial agents [229]. In turn, tolerance refers to the ability (of genetic origin or not) of a microorganism to survive when exposed to high concentrations of an antimicrobial agent [230]. Therefore, in order to confirm EC induced changes in the genomic structure, that lead to the development of *A. calcoaceticus* resistance to tested antibiotics, further genetic or molecular analysis should be conducted. Furthermore, selected ECs concentrations in DWDSs are in the nanograms per litre (ng/L) range, which is six orders of magnitude below the MICs, minimal biofilm inhibitory concentrations (MBIC), and minimal biofilm eradication concentration (MBEC) that typically reside in the micrograms per mililitre ($\mu\text{g/mL}$) range [61]. These results reiterate how non-antibiotic PhACs, at sub-inhibitory concentration, have a role in the manifestation of antibiotic tolerance/susceptibility.

LEV and CIP are antibiotics belonging to the class of fluoroquinolones [231]. Fluoroquinolones are a group of antibacterial chemotherapeutic agents, that block bacterial DNA replication, through DNA gyrase and topoisomerase IV enzymes inhibition [232, 233]. In general, the mechanisms of quinolone resistance in members of the *Acb* complex reside in mutations in the quinolone resistance-determining region [234, 235]. The mechanisms of resistance to this class of antibiotics lies in: target molecule modification (*gyrA*, *gyrB* and *parC* code for modifications in antibiotic target enzymes) and decreased membrane permeability (in Gram-negative bacteria, resistance to fluoroquinolones is related to the reduction of porins and over-expression of efflux pumps) [232, 236-238]. Furthermore, the *qnrA*, *qnrB*, and *qnrS* genes confer resistance to CIP [127]. On the other hand, TET inhibits protein synthesis by binding to the 30S ribosome subunit, preventing the association of t-RNA during translation [137], and their resistance is usually governed by *tet* genes [239]. The resistance mechanisms to TET comprise of: ribosomal protection, *tet* efflux pumps and enzymatic drug modification [137, 240, 241]. Lastly, regarding TMP-SMX resistance, this antibiotic is a combination of two antibiotics. When combined, they are meant to exert bactericidal

action, creating a synergistic anti-folate effect (acting upon the synthesis of folate, required for DNA and protein production)^[242]. Bacterial resistance mechanism is associated with efflux pumps, changes in the target enzymes, mutational or recombinational changes in the target enzymes, and acquired resistance by drug-resistant target enzymes^[243].

Aside from resistance acquirement, the possibility of induced changes in biofilm structure, composition and formation due to EC exposure have been brought up and observed by many^[126, 128, 244]. Given the presence of a multitude of contaminants in DW and all the issues related to biofilm formation, an increase of *A. calcoaceticus* ability to form biofilms due to EC exposure may be detrimental to DW quality. On the contrary, a decrease in biofilm formation is favourable to DWDS, and lessens the risk for distributed water. In this work, it was possible to observe that bacteria from biofilms exposed to ECs for 7 days had alterations in their biofilm formation ability. Specifically, an increase on the *A. calcoaceticus* ability to form biofilms after MIX exposure was observed, and a decrease after IBP exposure. Alternatively, Gomes et al.^[24] determined that *S. maltophilia* exposure to ECs, including CBZ (258 ng/L) and IBP (3 ng/L), had no remarkable effect on its ability to form biofilms at environmentally relevant concentrations. However, remarkable effects were found for concentrations at 100 times higher than that of DWDS, namely, diclofenac (600 ng/L), IBP (300 ng/L) and tylosin (170 ng/L) individually, and CA (170,000 ng/L) + CBZ (258,000 ng/L) + IBP (300 ng/L) mixtures exposure^[24]. This also proves that ECs and DWDS microbiome interactions have unique, diverse and unpredictable outcomes. Furthermore, DMSO (ranging from 0.0002 to 0.0005 % (v/v)) exposure increased *A. calcoaceticus* biofilm formation ability, and for this reason, IBP and MIX results were compared with solvent control. Similarly Gomes et al.^[23] and Lim et al.^[245] observed that 1% DMSO and 4% DMSO, respectively, increased biofilm formation ability of *S. maltophilia* and *E. coli*, respectively. Since DMSO exposure manifested in bacterial behaviour changes, this proves the importance of providing a negative control in analysis, so that solid conclusions can be drawn from the true effect of the tested EC alone, without solvent interference.

Microbial adhesion to DWDS surfaces is indispensable for biofilm formation. This phenomenon depends on physical, chemical and biological interactions between bacteria and DWDS pipe surfaces, where motility takes part in early stages of biofilm formation^[22], including the adhesion process^[165]. Furthermore, autoaggregation and EPS production are also contributors to primordial biofilm development^[246]. Specifically, EPS plays an important role on bacterial

adherence to surfaces [247] and mediates biofilm-forming bacteria's coaggregation, through expression of proteins and lipids, under stress-conditions [248].

The types of bacterial motility have been reported to be: gliding, swimming, swarming, sliding, twitching and darting [165, 249]. In this study, swimming, swarming and twitching motility were assessed. Bacterial motility corresponds to its ability to move in a liquid media or solid surfaces, whose mechanisms vary between species, being optimized for their respective habitats [250]. Cells can change their modes of motility when stimulated or faced with unfavourable conditions (e.g., nutrients or pollutants), to move to optimal environments for their growth and proliferation [251-253]. Motility is characterized by the presence of cellular structures called flagella, pili or fimbriae [9, 165]. Swimming motility, related to movement in a liquid medium, is flagella-dependent [23, 250] and is important in the primary steps of biofilm (microcolony) formation [254], allowing bacteria to overcome repulsive forces between the cell and surfaces, promoting contact and translocation [49, 255, 256]. Swarming motility mechanism, that provides cells to locomote on solid surfaces, is also related to the flagellar complex [250, 257, 258]. However, *Acinetobacter* spp. do not codify this appendix and the mechanisms associated with this motility need further investigation [192]. As for twitching motility, according to Henrichsen (1984) [190], the phenomena of propagation on solid surfaces and movements of individual cells of *A. calcoaceticus*, is due to twitching motility. Possession of type IV pili, through the action of extension and retraction of pili [259] can mediate this type of motility [258, 260, 261]. Furthermore, this structure, in addition to motility, is related to horizontal gene transfer, adhesion to host cells and biofilm formation [262].

Previously, diclofenac (600 ng/L) and TMP-SMX (170 and 820 ng/L, respectively) were proven to have increased *B. cepacia* swarming motility by 13 and 12%, respectively, and CAF (11,900 ng/L) and galaxolide (220 ng/L) decreased *B. cepacia* swarming motility by 10 and 14%, respectively [22]. This proved that some ECs can impact DW bacteria motile abilities. However, in this study, EC exposure had no remarkable effect ($P < 0.05$) on *A. calcoaceticus* expression of swimming, swarming nor twitching motility in general. Consequently, the increase in biofilm formation does not correlate with results obtained for bacterial motility expression. Additionally, flagella in pathogens also functions as adhesins [256]. Thus, this property confers advantages to pathogenic organisms in proliferating in DWDS environments and overcoming the barriers of the host (i.e., DW consumer). Motility is not only related to biofilm formation, but may also be a crucial attribute to the virulence of pathogenic bacteria [250], since motility also facilitates the

interaction of bacteria with host cells. Thus, the exposure to selected ECs does not seem to alter *A. calcoaceticus* virulence, specifically those factors related to motility

The information regarding the roles of intra and inter species aggregation and biofilm development on DW is still limited [263], much like the simultaneous effect of EC exposure on this aspect. Furthermore, intergeneric coaggregation interactions are related to the formation of heterologous/multi-species biofilms in DWDS [264]. Simões et al. [265] determined that *A. calcoaceticus* plays a very important role in DWDS bacterial communities, co-aggregating with other existing species within DW. This increases biofilm formation and facilitates the association of other species that do not aggregate with each other [265]. Thus, a pertinent question is what effect does ECs presence have on this behaviour of *A. calcoaceticus* and how it could affect the biodiversity and stability of the microbial community in DWDS. This work's findings have proven that no significant changes were observed in *A. calcoaceticus* autoaggregation and coaggregation capacity, and exposure to selected ECs does not further add to DWDS issues regarding this behaviour/characteristic of *A. calcoaceticus*. To that end, and once again, the increase in biofilm formation, in this study, is not related to *A. calcoaceticus* autoaggregation abilities.

Biofilms, aside from microorganisms, contain EPS excreted by cells, constituting up to 50% to 90% of the total organic carbon contained therein [49]. EPS secretion is a consequence of microbial adaptation to environmental conditions [266] and there are many bacterial activities related to EPS that impact microorganisms and overall DWDS biological stability and disinfection processes, aside from biofilm formation. EPS delays the diffusion of antimicrobial agents [267] and is actively involved in the tolerance and resistance of bacterial communities in biofilms [268], hindering the disinfection processes. Furthermore, EPS attributes to the cohesive forces within the biofilm, providing mechanical stability [269, 270] (when shear stresses of a system exceed the forces of EPS, biofilm shedding is promoted [163], causing aesthetic degradation of DW, and is a source of potential pathogenic organisms to the final consumer, leading to disease outbreaks [3]). EC presence has been proven to influence EPS production of biofilms [271, 272]. Therefore, it becomes important to study EC effects regarding EPS production, and how it affects or correlates with other changes in bacterial behaviour observed on a DWDS scenario.

Wang et al. [134], determined, by quantifying proteins present in the polymer matrix, that DWDS bacteria exposed to CIP (1 µg/L – 2 µg/L) had greater enzymatic activity in contaminated DWDS, resulting in a greater production of EPS and promotion of bacterial aggregation and

absorption on pipe surfaces (increased hydrophobicity of bacterial surfaces, due to a higher protein and β -sheet content). In spite of these observations, in this study, no remarkable changes were detected in protein content of extracted EPS, after biofilm exposure to the mixture of selected ECs.

The polysaccharide content in biofilms provides many benefits, such as: attachment (cell to cell and cell to surfaces, promoting colonization of biotic and abiotic surfaces), protection, structure (allowing stratification of the bacterial community and establishing gradients of nutrients and waste products), nutrient sequestration, alternative carbon source, stabilization of sediments, organic carbon transformations, metal cycling and overall support for biochemical interactions between the bacteria and their surroundings ^[273-275]. And so, polysaccharide content supports a heterogeneous population, resistant to adverse environmental conditions that bacteria may encounter ^[273]. But, seeing that MIX resembles realistic environmental conditions of DWDS, and no significant changes were observed, it is possible to conclude that the presence and combination of ECs did not dictate *A. calcoaceticus* behaviour (through production of polysaccharides nor proteins).

Changes in bacterial viability, due to the presence of EC in the aquatic environment, has been reported by Osorio et al. ^[276]. They determined that the translocation of biofilms to more polluted sites of the Llobregat river decreased bacterial density and significantly increased bacterial mortality ^[276]. Determining the content of viable cells in biofilms is a useful tool to assess whether an inhibitory effect has occurred, due to ECs exposure, and the content of viable cells allows estimating the risk of a pathogenic organism ^[277]. In this study, exposure to MIX induced changes in live and total cell content of biofilms. Higher cell density may represent a threat to DW safety and public health, since higher cell number in biofilms may also contribute to ARG dissemination ^[44, 141]. Cell density also has a role in the resistance phenotype of cells embedded on biofilms ^[193], and changes in bacterial abundance, viability and/or community composition due to EC exposure could be indicators of biological instability in DWDS.

Chapter 6. Concluding remarks and perspective for future research

6.1. General conclusions

Biofilm formation in DWDSs causes many problems in the control of water quality and system integrity. Although the concentrations of ECs found in DW are small, their presence could impact the microbiota of DWDSs, and consequently, affect the safety and microbiological quality of the DW distributed. The present work provided insight into possible changes in *A. calcoaceticus* behaviour after biofilm exposure to selected ECs (CAF, CBZ, CIP, IBP and mixture of ECs) and proved that the bacterial behaviour observed has potential for estimating the impact of selected ECs on the DWDS microbiome. In summary, significant results suggest that:

- IBP exposure decreased *A. calcoaceticus* biofilm formation ability by 11%, while MIX exposure increased by 16%;
- Biofilm exposure to EC mixtures induced an increase in biofilm cellular density;
- CIP exposure increased *A. calcoaceticus* growth rates;
- Biofilm exposure to residual, and sub-inhibitory, concentrations of the tested PhACs, antibiotics and non-antibiotics was associated with an increased and decreased *A. calcoaceticus* susceptibility to antibiotics (CIP, LEV, TET and TMP-SMX) and NaOCl. Particularly, CAF exposure decreased *A. calcoaceticus* susceptibility to TPM-SMX, while CBZ and CIP exposure caused an increase. CIP exposure also decreased *A. calcoaceticus* susceptibility to CIP and LEV, and IBP exposure decreased LEV and TET susceptibility. The ranges of minimum bactericidal concentration (MIC) of NaOCl increased for CIP and IBP exposure and decreased for CBZ exposure.

In sum, IBP and CIP are the ECs that caused the most alterations in *A. calcoaceticus* behaviour, specifically their impact is of concern regarding bacteria susceptibility to antibiotics and NaOCl.

On that account, the studied ECs are potential threats to DW quality, consumer health and DWDS infrastructures functional and operational integrity. On a macroscopic scale of events, even though in few particular cases EC exposure could prove beneficial for biofilm management in DWDS (*i.e.*, observed decrease in biofilm formation and tolerance to antimicrobial agents), EC exposure could be responsible for DW quality control failures (by promoting regrowth in biofilm within DWDS walls and water phase, as well as favour antimicrobial resistance). Facing these

results, studying the biofilm structure and behavioural characteristics of *A. calcoaceticus* is essential to better understand ecological repercussions of EC occurrence in DWDS and overcome knowledge limitations regarding this aquatic environment. Furthermore, the methodologies used to achieve these results proved to be effective for early warning and pro-active water quality management purposes.

Additionally, this work highlights that advanced monitoring of EC occurrence in aquatic environments, assessment of their fate and ecotoxicological risks, and implementation of pollution control policies (including: emission control (point-source) and advanced water treatment (in DWTPs and WWTPs)) are necessary to prevent undesired consequences.

6.2. Future work

This work aimed to study ECs effect on biofilm composition and on bacteria phenotypic characteristics. However, obtained information is still not enough to characterize biofilm structure and composition. Subsequently, to further characterize exposed and non-exposed biofilms to ECs, quantifying the dry and wet mass, as well as the thickness^[270] of the biofilms could be the next step to refining the assessment of biofilm structure and morphology. Moreover, the use of microscopy techniques such as confocal laser scanning microscopy (CLSM) and/or optical coherence tomography (OCT) may be helpful on the analysis of biofilm structure as well as the spacial distribution of bacteria^[163, 278, 279]. Fluorescence *in situ* hybridization (FISH) could also be used to identify and visualize cell distribution in single or mixed biofilms of different microbial groups^[280].

Changes in antimicrobial susceptibility were also observed in this study. However, the tests done do not provide concrete information regarding the resistance mechanisms at the genomic level. These can only be detected by genetic, or molecular, testing^[219], by targeting specific genetic sequences from the ARG coding region, based on the fact that the presence of resistance genes equates to the resistance to a particular antibiotic. For the detection of resistance genes there are hybridization and amplification systems^[219, 281]. Furthermore, DNA sequencing allows the identification of point mutations associated with resistance^[219]. These results could compliment the present work, as the presence/absence of a certain gene determinant of resistance to an antibiotic^[281] does not necessarily dictate the susceptibility of an organism to that particular antibiotic, as observed in the disk-diffusion method used, and vice-versa.

Additionally, BAPs (biofilm-associated proteins) are proteins that play an important role in the formation of biofilms [282]. And, according to literature, type-1 and type-2 BAPs are present in *A. calcoaceticus* [283]. Therefore, if EC exposure could induce changes on *A. calcoaceticus* ability to express BAPs, would impact its ability to form microbial communities in DWDS?

Microbial adhesion involves a complex interaction of physical and chemical forces (receptor-ligand, hydrophobic and other nonspecific) [284, 285]. Whereas external factors (such as flow rate, temperature, and nutrient concentration) can also influence adhesion [49]. Besides the production of EPS and presence of fimbriae or flagella, cell surface hydrophobicity influences the ability of bacteria to attach to surfaces [49]. Although changes in the ability to adhere to biotic surfaces indicate pathogenicity, changes in the ability of bacteria to adhere to abiotic surfaces may add to the difficulties that exist in controlling biofilm formation within DWDS. So, it would be interesting to test the extent and capacity of *A. calcoaceticus*, exposed to ECs, to adhere to abiotic and biotic surfaces. This would allow us to predict whether, through prolonged exposure to residual concentrations in DW, these DW bacteria would express higher pathogenicity and would pose increased risk to the safety of DW (as water is a vehicle for the transport of pathogenic organisms to the human body).

In a study carried out by Mortensen et al. [286], it was reported that CIP at sub-inhibitory concentrations, induced an inhibitory effect on the ability of the enteroaggregative *E. coli* strain (EAEC) to adhere to glass surfaces and in HEp-2 mammary cells. Likewise, Gomes et al. [23], determined that exposure to CA reduced the ability of *S. maltophilia* to invade HT-29 cells (human colon adenocarcinoma cells). HT-29 cells served as a model to estimate the potential effects of consuming DW contaminated with EC-exposed bacteria, and how they might interact with mammalian intestinal cells. Furthermore, the type of material that makes up the pipes can influence adhesion and determine the density of bacteria attached to its walls, as well as the composition of microbial communities [39, 44, 69]. To evaluate the mechanisms that control the adhesion of bacteria to surface substrates, there are methods such as electron microscopy, atomic force microscopy, surface charge analysis (measurement of zeta potential) and measurement of surface hydrophobicity (measurement of contact angles) [39, 165, 285]. Therefore, this is another proposition for future experiments: study surface influence in *A. calcoaceticus* ability to form biofilms and structure.

The study of virulence factors, such as: proteases, iron chelators (siderophores) and gelatinases, was also primarily aimed to be studied in the present work, with the intent to evaluate the impact of EC exposure on virulence development/ expression. However, *A. calcoaceticus* was not able to grow on culture media. **Appendix III** provides the records of preliminary laboratorial work done. Therefore, changes in protocol are needed in order to provide proper growth media that promotes *A. calcoaceticus* growth in the future, or even resort to different methodologies.

Virulence describes the ability of an organism to infect another host organism and cause disease. Virulence factors are molecules that assist in infectious organisms colonization [287]. A cycle of infection consists of discovery and penetration into the host organism, spread, development of symptoms and transmission to a new organism., with its success depending on high coordination of virulence factors produced by the pathogen [288]. Virulence factors of pathogenic organisms are essential components of a secretory (membrane) or cytosolic nature [287], having the ability to facilitate adhesion to host surfaces, and evade the host by breaking down physical barriers, mediating movements of the infectious agent, modulation of the host's immune system response, among others [288].

Siderophores are low molecular weight iron (Fe^{3+}) chelating molecules [289-291]. In aerobic environments, iron is insoluble at biological pH [292], and many Gram-positive and Gram-negative bacteria produce and secrete siderophores to collect iron from the extracellular medium [22]. Siderophores, like other iron acquisition systems, are considered virulence factors [289, 293]. These pathogens secrete siderophores to solubilize iron, whose affinity is greater than that of molecules and proteins excreted by the host, competing and decreasing the availability of this essential ion to the hosts [23]. As for, proteases, these enzymes contribute to the pathogenicity of bacterial cells by manipulating the immune response of the host organism [294], attributing pathogenic organisms the ability to degrade structural barriers by attacking host tissues (physical protection barrier) [295], facilitating bacterial invasion and proliferation [288]. Regarding gelatinases, these are enzymes considered to be virulence factors [296, 297], capable of hydrolysing gelatin, collagen, haemoglobin and, other host compounds, facilitating invasion [298]. If EC exposure were to influence *A. calcoaceticus* virulence, coupled with potentiated regrowth in DWDS and antimicrobial resistance, public health and safety would be compromised.

Lastly, new and more robust platforms for biofilm formation should be used. As previously mentioned, microtiter plates are very limited in terms of recreation of multi-stress environments

of DWDS. However, this work's results could function as selection criteria (according to relevancy, and conditions that appeared to have a bigger impact on *A. calcoaceticus* behaviour) to later collectively study the effect of EC exposure with other distinguished environmental factors of DWDS, such as hydrodynamics, temperature, surface/pipe material, residual disinfectants, and biodiversity (mixed biofilms or from real DWDS). To achieve that, a rotating cylinder reactor (RCR) could be used ^[186]. This platform is composed of 3 PVC cylinders (although other surface materials could also be tested), which are submerged in the bacterial suspension, joined by a chain that guarantees the synchronized rotation of the cylinders (speed and direction). Thus, mimicking real DWDS conditions.

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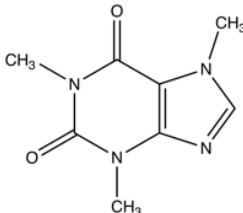
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References

Appendix

Appendix I. Supplementary selected EC information:**Table A I. CAF information.**

EC		Description
Caffeine 	Pharmaceutical class	Stimulant
	Use	Psychoactive stimulator, that affects the central nervous system, acting as a cardiac, cerebral, and respiratory stimulant ^I . CAF intake results in increased alertness, stamina, reduced drowsiness, and increased anxiety levels ^{II} .
	Action mechanism	Intracellular mobilization of calcium, inhibition of phosphodiesterase, antagonism at the level of adenosine receptors and interaction with benzodiazepine binding sites ^{II} .
	Natural sources	Tea leaves, cacao and coffee beans , guarana, and certain nuts ^{III} .
	Anthropogenic sources	Approximately 5% of the CAF consumed is not metabolized, being eliminated and excreted by the body ^{IV} . High consumption of products with caffeine (both food (coffees, soft drinks, teas, and other food products (<i>e.g.</i> , chocolate)) and pharmaceutical ^{V-VII}) leads to an increase in the emission of this compound to domestic wastewater ^{VI} . Direct dumping of expired drugs to sewage systems ^{VIII} , unloading of coffee and food waste in landfills, and industrial decaffeination processes are also sources of CAF emissions to the environment ^{VI} .
	Prevalence and fate in aquatic environments	When in the aquatic environment, CAF can undergo photolysis or biodegradation ^{VI} . Thus, the concentration oscillates temporally and spatially, and according to environmental factors such as: physicochemical characteristics (<i>e.g.</i> , light, depth, temperature) and biological properties (microbiota) ^{VI} . CAF has a half-life of 1.5 days ^{IX} . Studies have suggested the accumulation of CAF in organisms ^X .

Note: EC molecules were designed with ChemDraw software, version 20.1

^I Moore, M.T., et al., Assessing Caffeine as an Emerging Environmental Concern Using Conventional Approaches. Archives of Environmental Contamination and Toxicology, 2008. 54(1): p. 31-35.

^{II} Nehlig, A., J.-L. Daval, and G. Debry, Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. Brain Research Reviews, 1992. 17(2): p. 139-170.

^{III} Frary, C.D., R.K. Johnson, and M.Q. Wang, Food sources and intakes of caffeine in the diets of persons in the United States. Journal of the American Dietetic Association, 2005. 105(1): p. 110-113.

^{IV} James, J.E., Critical review of dietary caffeine and blood pressure: a relationship that should be taken more seriously. Psychosomatic medicine, 2004. 66(1): p. 63-71.

^V Kot-Wasik, A., A. Jakimska, and M. Śliwka-Kaszyńska, Occurrence and seasonal variations of 25 pharmaceutical residues in wastewater and drinking water treatment plants. Environmental monitoring and assessment, 2016. 188(12): p. 661.

^{VI} Bruton, T., et al., Fate of caffeine in the environment and ecotoxicological considerations, in Contaminants of emerging concern in the environment: Ecological and human health considerations. 2010, ACS Publications. p. 257-273.

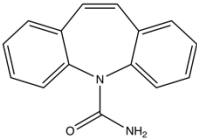
^{VII} Nawrot, P., et al., Effects of caffeine on human health. Food Additives & Contaminants, 2003. 20(1): p. 1-30.

^{VIII} Júnior, C.A.M., N. da Costa Luchiari, and P.C.F.L. Gomes, Occurrence of caffeine in wastewater and sewage and applied techniques for analysis: a review. Eclética Química Journal, 2019. 44(4): p. 11-26.

^{IX} Lam, M.W., et al., Aquatic persistence of eight pharmaceuticals in a microcosm study. Environ Toxicol Chem, 2004. 23(6): p. 1431-40.

^X Li, S., et al., Risks of caffeine residues in the environment: Necessity for a targeted ecopharmacovigilance program. Chemosphere, 2020. 243: p. 125343

Table A II. CBZ information.

EC	Description	
Carbamazepine 	Pharmaceutical class Use Action mechanism Anthropogenic sources Prevalence and fate in aquatic environments	Anticonvulsant This compound has antiepileptic and psychotropic properties ^{XI} . CBZ is used in psychotherapeutic treatments, treatment of psychomotricity and epilepsy (anticonvulsant for seizure disorders), glossopharyngeal and trigeminal neuralgia, neuropathic pain, schizophrenia, personality disorder, manic seizures, bipolar disorder and attention deficit hyperactivity disorder ^{XI-XIV} . Blocks voltage-gated sodium channels, inhibiting synaptic glutamate and other neurotransmitters release ^{XI} . CBZ metabolized in the liver, but this compound is eliminated in its metabolized form (10,11-epoxy-carbamazepine and other metabolites) and only 1-3% is excreted unchanged and pharmacologically active form ^{XI, XII} . CBZ emissions are due to hospital and household waste water discharge ^{XV} . Furthermore, in WWTPs, CBZ removal rates on waste water and sludge are very low ^{XVI} . As a result, CBZ can be found in treated wastewater ^{XVII} . Landfill and Soil leaching also leads to CBZ contamination of water resources ^{XVIII} . CBZ and its metabolites are very stable ^V , since it is resistant to microbial ^{XIX} and photodegradation ^{XX} . CBZ has a 88 days photolysis and 234 days biodegradation half-life ^{XXI} . CBZ is biotransformed and has a potential to bioaccumulate in fish ^{XXII} .

Note: EC molecules were designed with ChemDraw software, version 20.1

^{XI} Houeto, P., et al., Assessment of the health risks related to the presence of drug residues in water for human consumption: Application to carbamazepine. *Regulatory Toxicology and Pharmacology*, 2012. 62(1): p. 41-48.

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^{XVI} Almeida, Â., et al., Occurrence of the antiepileptic carbamazepine in water and bivalves from marine environments: A review. *Environmental Toxicology and Pharmacology*, 2021. 86: p. 103661.

^{XVII} Hai, F.I., et al., Carbamazepine as a Possible Anthropogenic Marker in Water: Occurrences, Toxicological Effects, Regulations and Removal by Wastewater Treatment Technologies. *Water*, 2018. 10(2): p. 107.

^{XVIII} Durán-Álvarez, J.C., et al., Environmental fate of naproxen, carbamazepine and triclosan in wastewater, surface water and wastewater irrigated soil — Results of laboratory scale experiments. *Science of The Total Environment*, 2015. 538: p. 350-362.

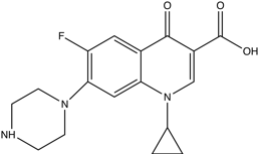
^{XIX} Tixier, C., et al., Occurrence and fate of carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, and naproxen in surface waters. *Environmental science & technology*, 2003. 37(6): p. 1061-1068.

^{XX} Dordio, A., V. Silva, and A. Carvalho, Carbamazepine in the environment: Sources, fate and adverse effects. 2017. p. 37-90.

^{XXI} Yamamoto, H., et al., Persistence and partitioning of eight selected pharmaceuticals in the aquatic environment: laboratory photolysis, biodegradation, and sorption experiments. *Water Res*, 2009. 43(2): p. 351-62.

^{XXII} Valdés, M.E., et al., Bioaccumulation and bioconcentration of carbamazepine and other pharmaceuticals in fish under field and controlled laboratory experiments. *Evidences of carbamazepine metabolization by fish. Science of The Total Environment*, 2016. 557-558: p. 58-67.

Table A III. CIP information.

Ciprofloxacin 	Pharmaceutical class	Antibiotic (fluoroquinolone)
	Use	CIP has a bactericidal activity used to treat bacterial infections ^{XXIII} , including the treatment of respiratory tract infections, urinary tract infections, gastrointestinal disorders, and typhoid fever ^{XXIV} .
	Action mechanism	CIP acts upon chromosomal DNA, targeting DNA gyrase and DNA topoisomerase IV ^{XXV} , causing double strand breakage, ultimately leading to bacterial cell death ^{XXIV} .
	Anthropogenic sources	Of the dose administered to the human body, 45-62% is not metabolized and excreted with urine, and 15-25% by faeces ^{XXVI} . The discharge of untreated or treated wastewater from pharmaceutical industries, WWTP effluents, hospital effluents, application of manure on agricultural land, livestock activities, incomplete metabolism of antibiotics, leaching from landfills, and direct disposal are possible sources of CIP contamination in aquatic environments ^{XXVI, XXVII} .
	Prevalence and fate in aquatic environments	CIP is not readily biodegradable, nor is it degraded by abiotic factors in aquatic ecosystems ^{XXVIII} . However, CIP is light sensitive, being characterized for having a short photodegradation half-life ^{XXIX} .

Note: EC molecules were designed with ChemDraw software, version 20.1

^{XXIII} Wang, Y., et al., Removal of ciprofloxacin as an emerging pollutant: A novel application for bauxite residue reuse. Journal of Cleaner Production, 2020. 253: p. 120049.

^{XXIV} Mortensen, N.P., et al., Effects of sub-minimum inhibitory concentrations of ciprofloxacin on enteroaggregative Escherichia coli and the role of the surface protein dispersin. International journal of antimicrobial agents, 2011. 38(1): p. 27-34.

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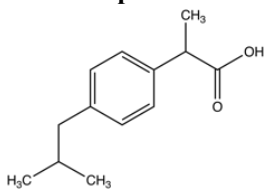
^{XXVI} Girardi, C., et al., Biodegradation of ciprofloxacin in water and soil and its effects on the microbial communities. Journal of hazardous materials, 2011. 198: p. 22-30.

^{XXVII} Tran, N.H., et al., Occurrence and risk assessment of multiple classes of antibiotics in urban canals and lakes in Hanoi, Vietnam. Science of the Total Environment, 2019. 692: p. 157-174.

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^{XXIX} Rodríguez-López, L., et al., Photodegradation of Ciprofloxacin, Clarithromycin and Trimethoprim: Influence of pH and Humic Acids. Molecules (Basel, Switzerland), 2021. 26(11): p. 3080.

Table A IV. IBP information.

EC	Description	
Ibuprofen 	Pharmaceutical class	Non-steroidal anti-inflammatory drug (NSAID)
	Use	Used in the treatment of rheumatoid arthritis, osteoarthritis, pain relief, reduce inflammation and mild to moderate fever ^{xxx, xxxi} .
	Action mechanism	Non-selective, reversible inhibition of the cyclooxygenase (COX) enzymes COX-1 and COX-2, blocking the synthesis of proteinoids ^{xxxii} .
	Anthropogenic sources	IBP is amongst the most consumed, prescribed and non-prescribed, pharmaceuticals in the world ^{xxxiii} , however, it is not entirely metabolized and is excreted in their unchanged form (10-80%) ^{xxx, xxxi} , together with its metabolites (more toxic than the original molecule) ^{xxxiv} . Furthermore, pharmaceutical industry waste ^{xxx} , hospital effluents, landfill leachates ^{xxxv} , WWTP effluents ^{xxxvi} , livestock treatment and inadequate disposal ^{xxxiv} are also possible sources of emission of this contaminant.
	Prevalence and fate in aquatic environments	In controlled laboratorial experiments, IBP could reach a 413 days photolysis and 20 days biodegradation half-life ^{xxi} . IBP was reported to accumulate on fish ^{xxxvii} .

Note: EC molecules were designed with ChemDraw software, version 20.1

^{xxx} Davarnejad, R., et al., Ibuprofen removal from a medicinal effluent: A review on the various techniques for medicinal effluents treatment. Environmental Technology & Innovation, 2018. 11: p. 308-320.

^{xxxI} Davids, M., et al., The effects of ibuprofen on activated sludge: Shift in bacterial community structure and resistance to ciprofloxacin. Journal of hazardous materials, 2017. 340: p. 291-299.

^{xxxII} Mazaleuskaya, L.L., et al., PharmGKB summary: ibuprofen pathways. Pharmacogenetics and genomics, 2015. 25(2): p. 96-106.

^{xxxIII} Bu, Q., et al., Assessing the persistence of pharmaceuticals in the aquatic environment: Challenges and needs. Emerging Contaminants, 2016. 2(3): p. 145-147.

^{xxxIV} Chopra, S. and D. Kumar, Ibuprofen as an emerging organic contaminant in environment, distribution and remediation. Heliyon, 2020. 6(6): p. e04087.

^{xxxv} Paíga, P., et al., Pilot monitoring study of ibuprofen in surface waters of north of Portugal. Environmental Science and Pollution Research, 2013. 20(4): p. 2410-2420.

^{xxxvi} Jiménez-Silva, V.A., et al., Study of the ibuprofen impact on wastewater treatment mini-plants with bioaugmented sludge. Process Safety and Environmental Protection, 2019. 123: p. 140-149.

^{xxxvII} Lahti, M., et al., Uptake from water, biotransformation, and biliary excretion of pharmaceuticals by rainbow trout. Environ Toxicol Chem, 2011. 30(6): p. 1403-11.

Appendix

Appendix II. Image processing and automated cell counting for high density images

In some cases, manual cell counting can be quite impractical, particularly in large datasets. FIJI/ImageJ provides an automated cell function, that can be used in Live/Dead cell quantification. This section provides a step-by-step guide on this works custom cell counting routine, following the processing of the file presented on **Figure A I**.

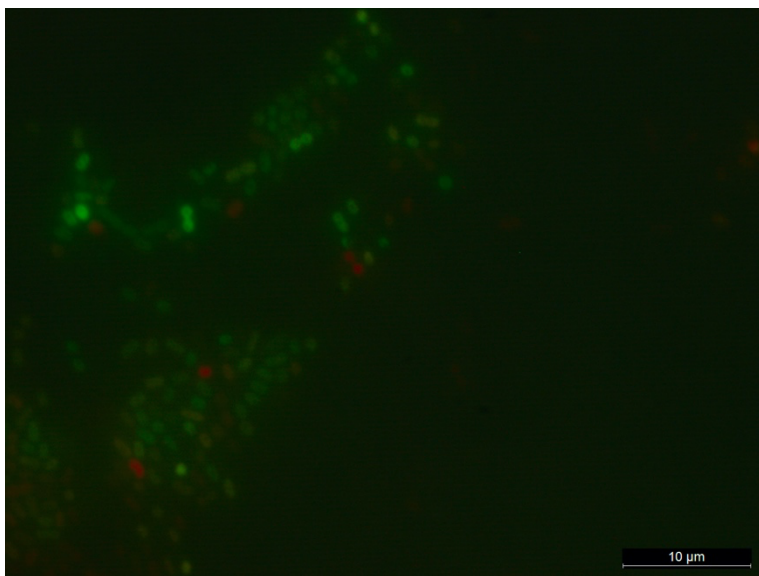


Figure A I. *Original image.*

The first step is image clean-up, aiming to obtain a higher quality image (better signal, focus and low background noise) and make image processing of separate colour channels easier and quicker. To subtract image background, as it is common for obtained images to possess background noise, the mean fluorescence of the background was subtracted to original image. To determine the background value, a snippet of the background was selected, from which fluorescence values were measured (Analyse > Measure). According to the example, a value of 12.188 was subtracted (Process > Math > Subtract) from the original image. Resulting image is presented on **Figure A II** (“MIX sample BG subtraction result”). The final step of the image clean-up process was brightness and contrast adjustments (Image > Adjust > Brightness/Contrast), presented on **Figure A III**.

Appendix

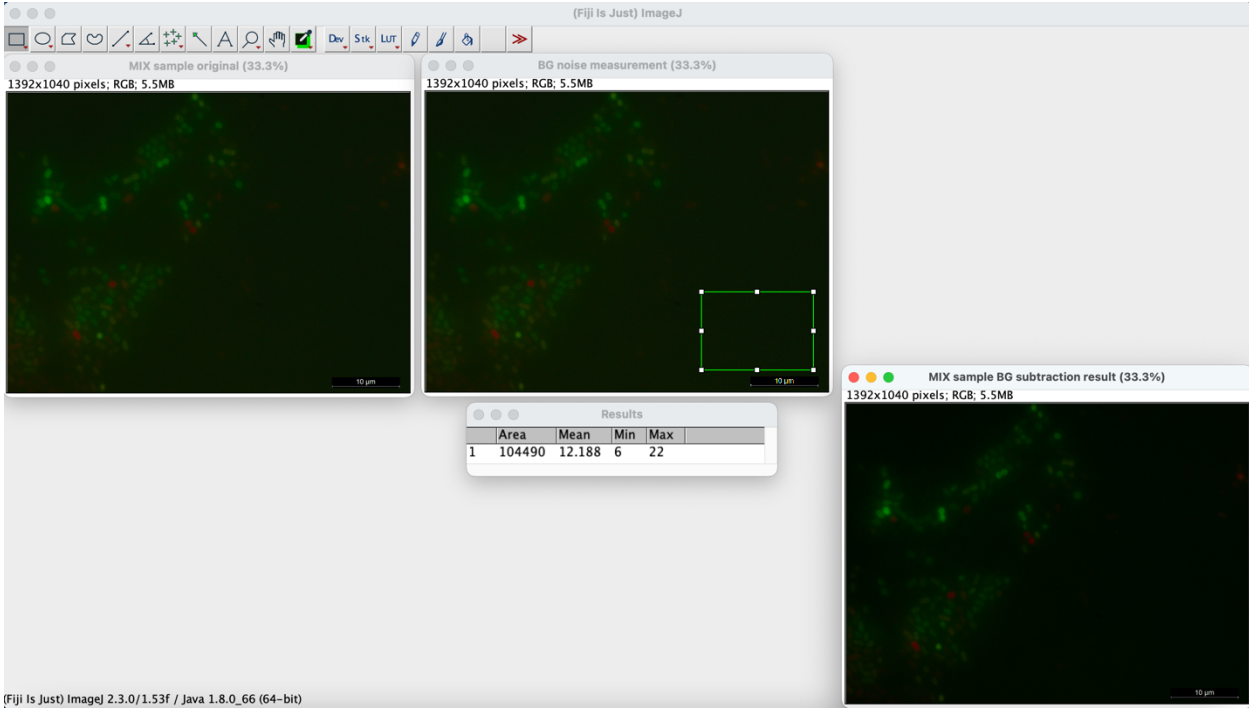


Figure A II. Background subtraction results in a less noisy image

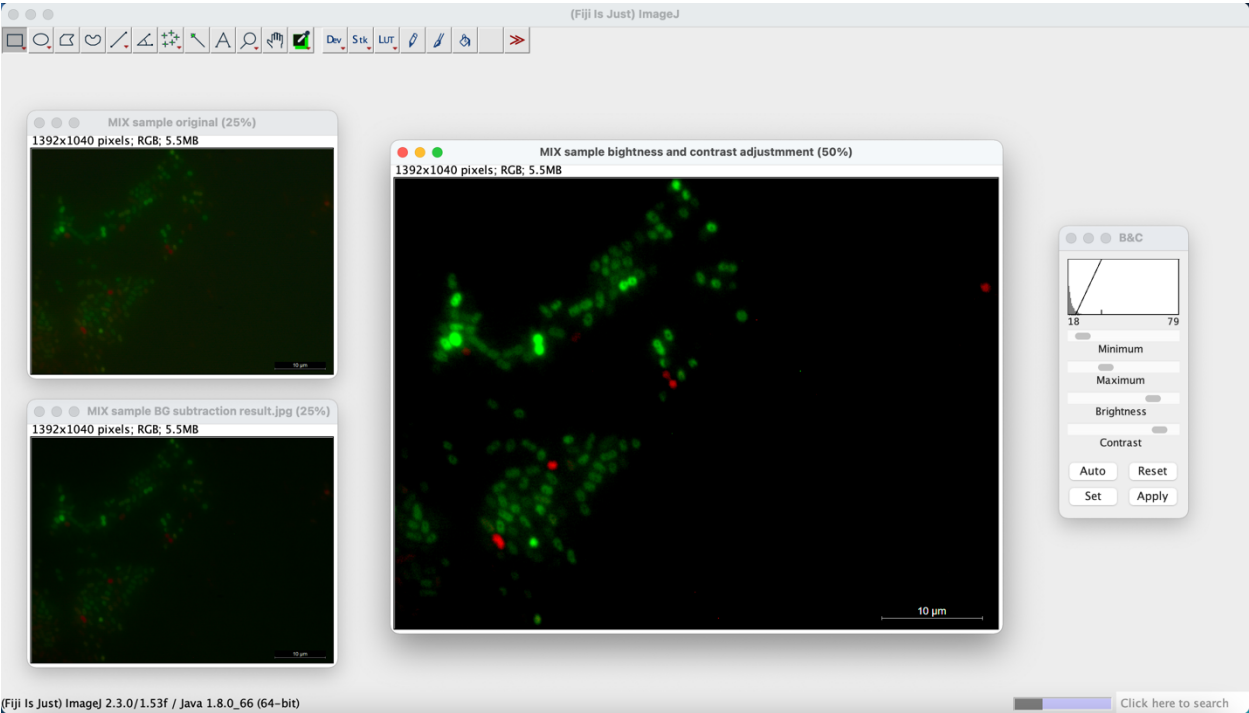


Figure A III. Brightness and Contrast adjustment results in better fluorescence.

The final processed image was later split into separate colour channels (Image > Color > Split channels), obtaining a 8-bit greyscale image for red, green and blue signals, as presented on **Figure A IV**. Only red and green channels were used.

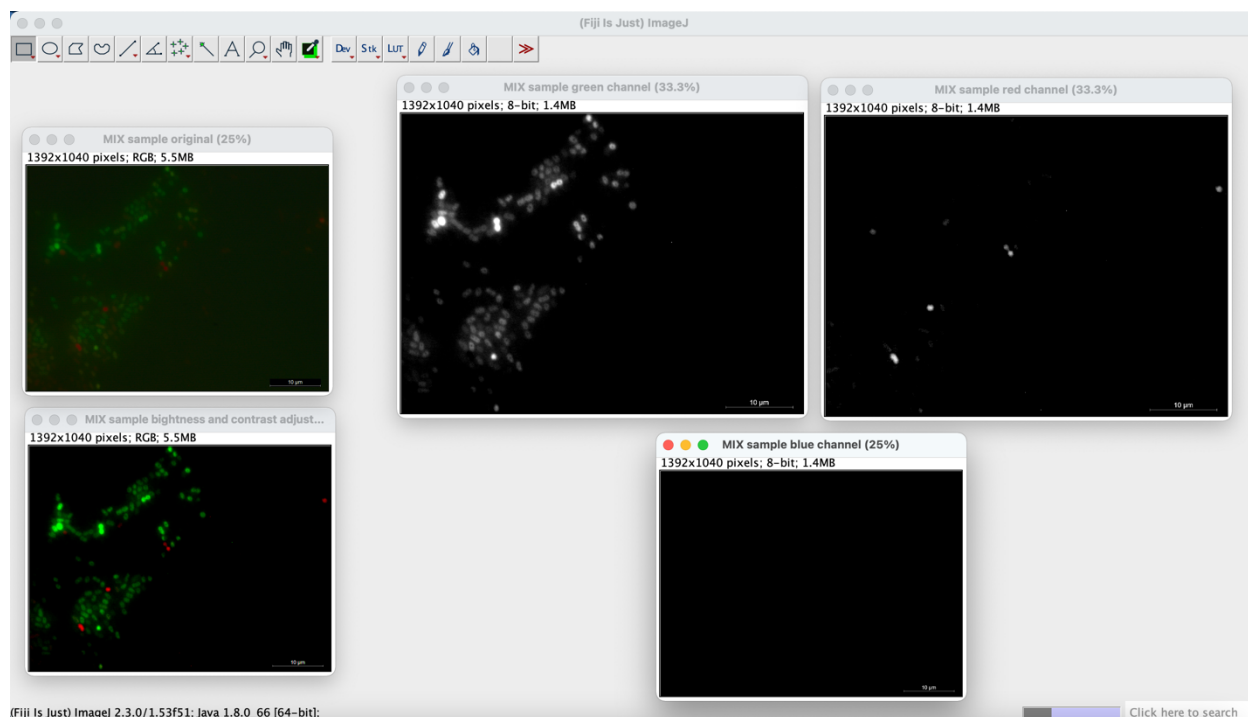


Figure A IV. Image colour channel splitting.

The next step consists of image segmentation, starting by adjusting the threshold (Image > Adjust > Threshold) to highlight cellular structures (**Figure A V**, following the green channel adjustments as an example). However, this is not the best example of optimal threshold adjustment, as cell agglomerates are fusing together in a section of the image. This example was chosen to epitomize this effect. Even after manually adjusting threshold values to better fit exported image, this happens due to different signal densities distributed and high cell density in that particular area. In those cases, cell counting was proceeded manually for that section. Nonetheless, afterwards, in an attempt to further separate cells, a line was created by using watershed function (Process > Binary > Watershed).

Finally, automated cell counting was achieved with the Analyse Particles function (Analyse – Analyse Particles). Counted parameters/criteria were left according to default settings, without restriction on particle size or circularity, to count all particles present in the image (**Figure A VI**).

On **Figure A VII** is the program output for cell counting, with a list of measurement results and counted particles with numbered outlines drawn in (useful for troubleshooting).

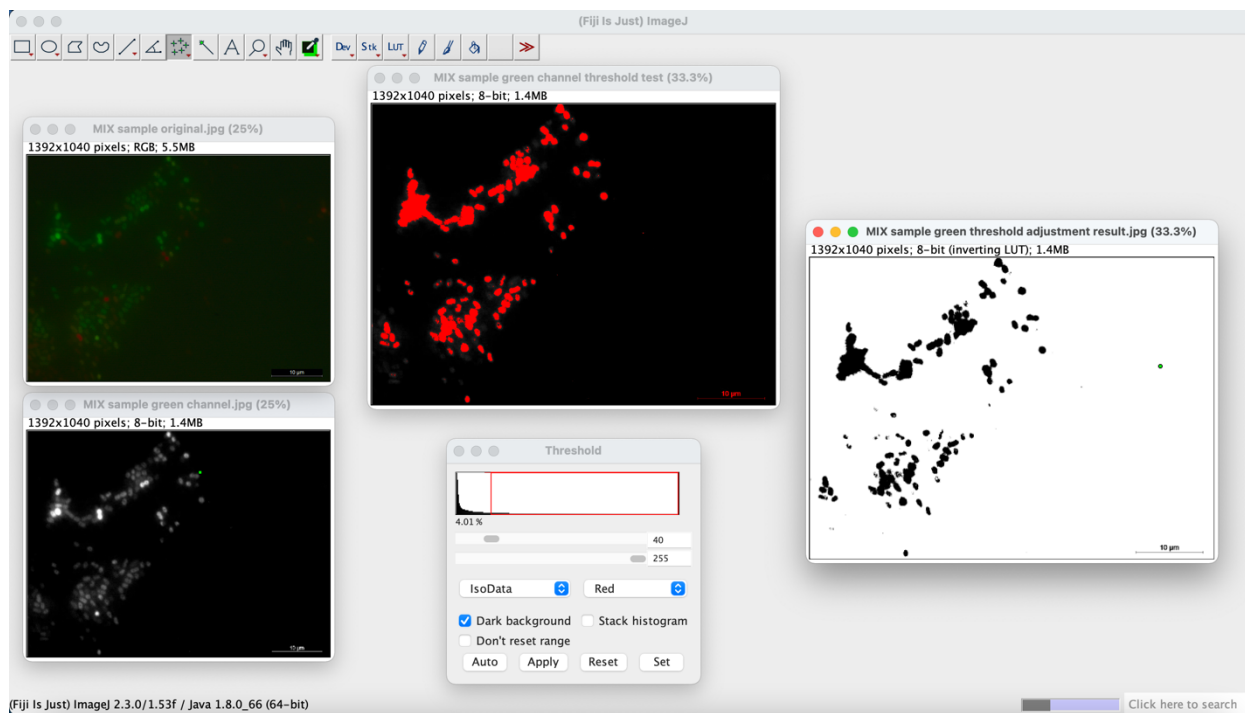


Figure A V. Threshold adjustments.



Figure A VI. Automated cell counting function.

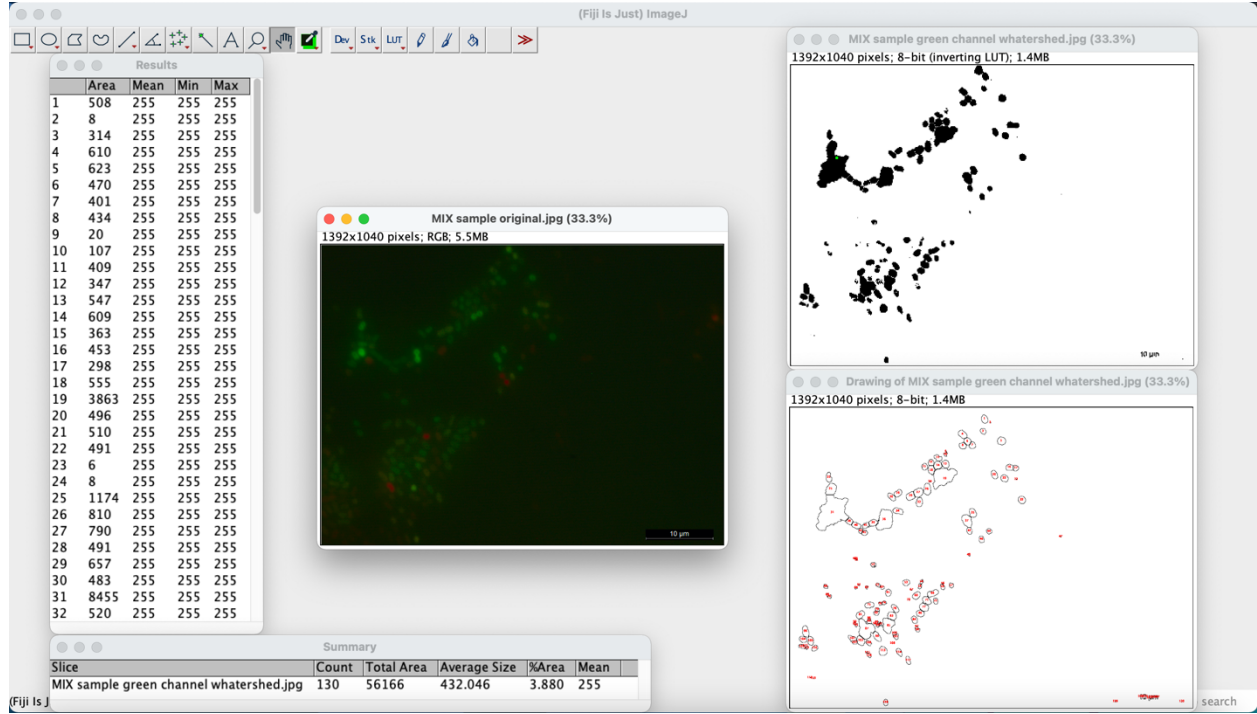


Figure A VII. Automated cell counting results.

To calculate the number of live or total cells per unit area, **Equation A I** was used:

$$N_{cells} (cells.cm^{-2}) = N_{counted\ cells} \times F \times \frac{A_{nucleopore\ filter}}{A_{image} \times V_{sample}} \times \frac{V_{inocule}}{A_{coupon}} \quad (I)$$

Where N_{cells} is the number of cells per unit area, $N_{counted\ cells}$ corresponds to the number of cells counted, F is the dilution factor (in this case, 1), $A_{nucleopore\ filter}$ is the area of the Nucleopore filter ($2.27\ cm^2$), A_{image} is the equivalent area covered by the image captured by the microscope ($6.03 \times 10^{-5}\ cm^2$), V_{sample} is the sample volume (1 mL), $V_{inocule}$ is the volume of biofilm sample (5 mL) and A_{coupon} is the surface area of a single coupon ($1.8\ cm^2$).

Appendix III. Complimentary laboratorial work - Virulence factor assessment:

I. Methods

I.I. Gelatinase assay:

The gelatinase production identification protocol follows the method described by Meireles et al.^{XXXVIII}. An aliquot of 10 µL of 10⁶ cells/mL bacterial suspension resulting from **section 3.4** were placed in the centre of a Gelatin agar plate (prepared with 5 g/L peptone, 3 g/L yeast extract, 30 g/L gelatin, 15 g/L of agar). The plate was incubated at 25°C for 48h and translucent halos were then measured (mm). Tests were done in duplicates.

I.II. Protease assay:

Protease production identification protocol follows the method described by Meireles et al.^{XXXVIII}. An aliquot of 10 µL of 10⁶ cells/mL bacterial suspension resulting from **section 3.4** were placed in the centre of PCA plates (with 10 g/L of skim milk powder). Plates were incubated at 30°C for 72h and halos were then measured (mm). Tests were done in duplicates.

I.III. Siderophore assay:

Siderophore production was assessed according to Gomes et al.^{XXXIX}. Three aliquots of 15 µL of 10⁶ cells/mL bacterial suspension resulting from **section 3.4** were positioned in CAS agar plates, prepared according to Schwyn and Neilands (1987)^{XL}. Plates were then incubated at 25°C for 48h. In the presence of siderophores, orange halos are formed.

^{XXXVIII} Meireles, A., et al., Characterization of the heterotrophic bacteria from a minimally processed vegetables plant. LWT-Food Science and Technology, 2017. 85: p. 293-300.

^{XXXIX} Gomes, I., et al., Prolonged exposure of *Stenotrophomonas maltophilia* biofilms to trace levels of clofibric acid alters antimicrobial tolerance and virulence. Chemosphere, 2019. 235: p. 327-335.

^{XL} Schwyn, B. and J. Neilands, Universal chemical assay for the detection and determination of siderophores. Analytical biochemistry, 1987. 160(1): p. 47-56.

II. Results and comments

A. calcoaceticus virulence factors were assessed, with positive controls (*S. maltophilia* and *B. cepacia*). The results are shown on **Table A V** and **Figure A VIII**.

Table A V. Virulence factor production assay results (final halo – initial halo diameter).

	CONT	Solv	<i>S. maltophilia</i>	<i>B. cepacia</i>
Gelatinase	NGO ^a	NGO ^a	+	+
Protease	–	–	+	+
Siderophore	NGO ^a	NGO ^a	+	+

^a NGO- no growth observed after incubation period.

– = not detected; + = detected

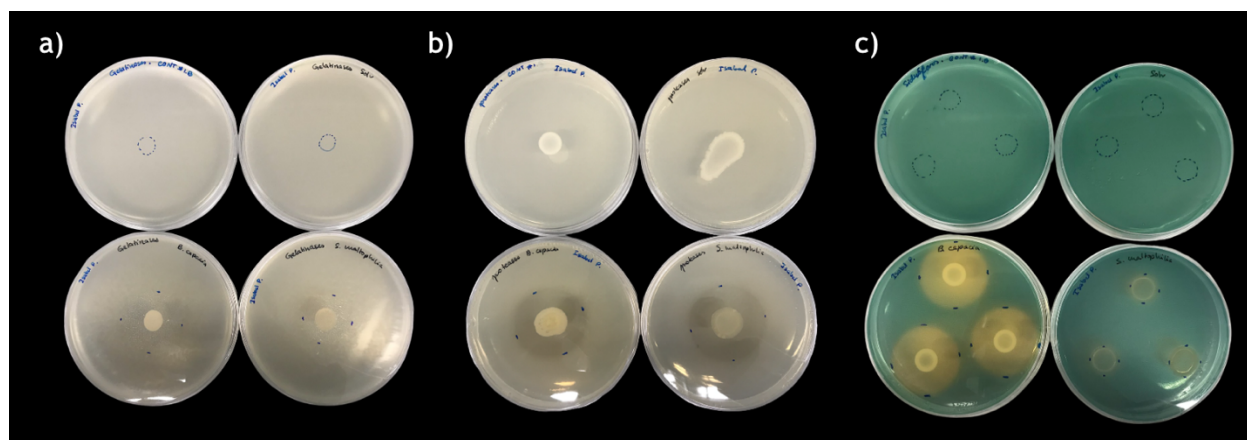


Figure A VIII. Photographic record of virulence factor assay (Legend: a) gelatinase results; b) protease results; c) siderophore results).

A. calcoaceticus are able to produce these proteins^{XL1, XLII}. However, as shown on **Figure A VIII**, *A. calcoaceticus* (Control (non-exposed) and solvent control, top plates) did not grow on Gelatin agar plate and CAS agar plates media. On the other hand, *S. maltophilia* and *B. cepacia* are capable of producing gelatinase, protease and siderophores, as the observed halos indicate the presence of these virulence factors. Positive control results are in accordance with found literature, as these bacterial strains produce and exhibit gelatinase, protease and siderophores activity^{XLIII-XLV}.

^{XL1} Poh, C.L. and G.K. Loh, Enzymatic profile of clinical isolates of *Acinetobacter calcoaceticus*. *Med Microbiol Immunol*, 1985. 174(1): p. 29-33.

^{XLII} Smith, A.W., et al., Characterisation of a siderophore from *Acinetobacter calcoaceticus*. *FEMS Microbiology Letters*, 1990. 70(1): p. 29-32.

^{XLIII} Thomas, R., R.A. Hamat, and V. Neela, Extracellular enzyme profiling of *Stenotrophomonas maltophilia* clinical isolates. *Virulence*, 2014. 5(2): p. 326-330.

^{XLIV} García, C.A., et al., Siderophores of *Stenotrophomonas maltophilia*: detection and determination of their chemical nature. *Rev Argent Microbiol*, 2012. 44(3): p. 150-4.

^{XLV} Malešević, M., et al., Virulence traits associated with *Burkholderia cenocepacia* ST856 epidemic strain isolated from cystic fibrosis patients. *Antimicrobial resistance and infection control*, 2017. 6: p. 57-57