The monitoring of pharmaceutical pollutants in wastewater treatment plants in the Algarve, Portugal

By

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July 2024

UNIVERSIDADE DO ALGARVE
FACULDADE DE CIÊNCIAS E TECNOLOGIA

The monitoring of pharmaceutical pollutants in wastewater treatment plants in Algarve, Portugal

Thesis for partial fulfilment of the Erasmus Mundus Master in Quality in Analytical Laboratories

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July 2024

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Declaration of authorship of work

I declare I am the author of this work, which is original and unpublished. The sources consulted have been duly cited in the text and included in the list of references.

Hopolang J. Mathaba, July 2024

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the Centre of Marine Sciences (CCMAR) and the University of Algarve for giving me the opportunity and resources to conduct my research. The invaluable support and guidance from my supervisors has been instrumental in executing this duty.

I have the utmost gratitude to the Erasmus Mundus program for providing this exceptional academic experience.

To my family and friends, I am indebted to your unwavering support and encouragement. Your companionship, love, patience, and belief in my abilities have come a long way in making this journey a success.

ABSTRACT

The occurrence of pharmaceutical compounds in wastewaters of Faro-Olhão and Faro-Noroeste wastewater treatment plants was monitored over summer and winter seasons from 2021 to 2024, to contribute to monitor marine pollution. A total of 26 pharmaceutical pollutants were analysed in the influent wastewater and in treated effluents by liquid-chromatography coupled with tandem mass-spectrometry (LC-MS/MS). The results showed quantifiable occurrences of 17 of the 26 pharmaceutical pollutants with outstanding levels of acetaminophen and caffeine (average 50-55 and 63.8-65 µg/L, respectively), and no quantifiable presence of sex hormones or contraceptives. These influent and effluent occurrence concentrations were used to compare the treatment performance between the classical activated sludge and the aerobic granular sludge wastewater treatment systems deployed in Faro-Noroeste and in Faro-Olhão wastewater treatment plants, respectively. The conventional activated sludge treatment exhibited relatively higher treatment efficiencies for most of the monitored pollutants than the aerated granular sludge. The majority of the quantifiable pharmaceutical pollutants were removed effectively by the wastewater treatment plants, with over 60% removal for ten pharmaceutical pollutants in Faro-Noroeste and five in Faro-Olhão. The high occurring caffeine and recalcitrant diclofenac were the only pollutants found at quantifiable levels in the surrounding Ria Formosa environment. Resistance to removal from the wastewater was observed greatly for the anticonvulsants carbamazepine and diclofenac leading to the investigation of carbamazepine, phytoestrogen coumestrol, and wastewater effects on the marine invertebrate *Artemia sp.* using an immobilisation/lethality assay. The assay showed high toxicity potential for coumestrol and lower for carbamazepine with LC_{50} values of 39.87 and 94.53 mg/L, respectively, in artificial seawater, and 5.09 and 26.47 mg/L in natural seawater, respectively. The toxicity levels were, however, multiple times (approximately $1.9x10^4$) higher than their existence in the wastewater.

Key words: Environmental monitoring, contaminants of emerging concern, wastewater treatment, marine pollution, ecotoxicology

SUMÁRIO

A ocorrência de compostos farmacêuticos nas águas residuais das estações de tratamento de águas residuais de Faro-Olhão e Faro-Noroeste foi monitorizada durante os períodos de verão e inverno de 2021 a 2024, para contribuir para a monitorização da poluição marinha. Um total de 26 poluentes farmacêuticos foram analisados nas águas residuais afluentes e nos efluentes tratados por cromatografia líquida acoplada à espectrometria de massa em tandem (LC-MS/MS). Os resultados mostram ocorrências quantificáveis de 17 dos 26 poluentes farmacêuticos com níveis excecionais de paracetamol e cafeína (média de 50-55 e 63,8-65 µg/L, respectivamente), e nenhuma presença quantificável de hormonas sexuais e contracetivos. Estas concentrações de ocorrência de afluentes e efluentes foram utilizadas para comparar o desempenho do tratamento entre os sistemas de tratamento de lamas ativadas clássicas e de lamas granulares aeróbias implantados nas estações de tratamento de águas residuais de Faro-Noroeste e de Faro-Olhão, respetivamente. O tratamento convencional com lodo ativado exibiu eficiências de tratamento relativamente elevada para a maioria dos poluentes monitorizados do que o lodo granular aerado. A maioria dos poluentes farmacêuticos analisados foi removida de forma eficaz pelas estações de tratamento de águas residuais. A cafeína de elevada ocorrência e o diclofenac recalcitrante foram os únicos poluentes encontrados em níveis quantificáveis no ambiente envolvente da Ria Formosa. A resistência à remoção das águas residuais foi observada grandemente para os anticonvulsivantes carbamazepina e diclofenaco, levando à investigação da carbamazepina, do fitoestrógeno coumestrol e dos efeitos das águas residuais no invertebrado marinho *Artemia sp.* usando um *Artemia sp.* ensaio de imobilização/letalidade. O ensaio mostra alto potencial de toxicidade para o coumestrol e menor para a carbamazepina. Estes obtiveram valores de LC_{50} de 39.87 e 94,53 mg/L, respectivamente, em água do mar artificial, e 5.09 e 26,47 mg/L em água do mar natural, respectivamente. Os níveis de toxicidade foram, no entanto, várias vezes (aproximadamente $1{,}9 \times 10^4$) superiores aos que existem nas águas residuais.

Palavras-chave: Monitoramento ambiental, contaminantes de preocupação emergente, tratamento de águas residuais, poluição marinha, ecotoxicologia

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LIST OF ABBREVIATIONS AND ACCRONYMS

℃	Degrees Celsius
ADR	Adverse Drug Reaction
AGS	Aerobic Granular Sludge
AMU	Antimicrobial Use
AMR	Antimicrobial Resistance
APAP	Acetaminophen
API	Active Pharmaceutical Ingredient
AS	Activated Sludge
ATN	Atenolol
AZM	Azithromycin
BZF	Bezafibrate
CA	Concentration Addition
CAF	Caffeine
CBZ	Carbamazepine
CCL	Contaminant Candidate List
CECs	Contaminants of Emerging Concern
CLR	Clarithromycin
COVID-19	Coronavirus Disease 2019
COX	Cyclooxygenase
DIC	Diclofenac
ECDC	European Centre for Disease Control
EDCs	Endocrine Disrupting Compounds
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EPA	Environmental Protection Agency
EQSD	Environmental Quality Standard Directive
ESI	Electron Spray Ionisation
EU	European Union
EUMS	European Union Member States
eV	Collision energy (in electron Volts)
FO	Faro Olhão

FNW	Faro Noroeste/Faro North-West
HOC	Hydrophobic organic compounds
IBU	Ibuprofen
LC	Liquid Chromatography
MERS	Middle East Respiratory Syndrome
MET	Metoprolol
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
NOEC	No effect concentration
NPX	Naproxen
NSAIDs	Non-steroidal Anti-inflammatory Drugs
PhCs	Pharmaceutical Compounds
PPL	Propranolol
RF	Radio Frequency
RPF	Relative Potency Factors
SARS	Severe Acute Respiratory Syndrome
SDWA	Surface Drinking Water Act
SMX	Sulfamethoxazole
SPD	Sulfapyridine
UCMR	Unregulated Pollutant Monitoring Rule
USA	United States of America
UV	Ultraviolet
VLDL	Very low-density lipoproteins
WFD	Water Framework Directive
WHO	World Health Organisation
WTP	Water Treatment Plant
WWTP	Wastewater Treatment Plant

1. Introduction

1.1. Background of the problem and aim of the thesis

The oceans cover over 70% of the world's surface and are a habitat to a vast biodiversity. With an ever increasing urbanisation, industrialisation and chemical production and use that leads to an increased release of contaminants (often through wastewaters) and terrestrial and especially marine environments are continually under threat from the resulting pollution (Landrigan et al., 2018; Ribeiro et al., 2015; Silva et al., 2021; Sousa et al., 2019). The threats to the environment resulting from pharmaceutical compounds (PhCs) micropollution has been an issue of concern lately and has triggered intense research towards their analysis, removal, and monitoring (Martínez-Piernas et al., 2021; Ribeiro et al., 2015; Sousa et al., 2019).

Despite attempts to prevent these pollutants reaching the oceans, micro- and nano-pollutants occur due to their resistance to degradation and ineffective treatment, which is not currently optimised for their removal (Gaffney et al., 2017; Sousa et al., 2019). These compounds exist in small quantities but their presence is significant due to their possible bioactivity at low concentrations; this is the case of many toxicants that can disrupt the endocrine systems of animals. The endocrine-disrupting compounds (EDCs) together with other micropollutants present a global threat to environmental health, including plants and animals and eventually to human health (Aubakirova et al., 2017; Gaffney et al., 2017; La Merrill et al., 2020; Marasco et al., 2019).

Thus, the monitoring of the levels of pollutants in aquatic environments and pre- and post-treatment in wastewater treatment plants (WWTPs), in countries with established sanitation systems, will provide information on the effectiveness of current treatments, and on the diversity and concentrations of pollutants released into these environments. Therefore, environmental monitoring can aid in finding remediation approaches and safeguard aquatic ecosystems (Gonçalves et al., 2014). These kind of monitoring studies can contribute to establish a national registry of micropollutants in WWTPs, efficiencies of removal, and contribute to reduce the re-entry of micropollutants into the water cycle. One of the main challenges lies in the cumulative and interacting impacts of these micropollutants on ecosystems. Recalcitrant compounds ineffectively treated in WWTPs need urgent investigation into their impacts on the environment and living organisms, and for the development of chemical analysis techniques and regular monitoring implementation. Monitoring studies are made possible by current advances in highly sensitive analytical techniques. In the present study, wastewater samples were analysed using an optimized and validated solid-phase

extraction (SPE) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) approach (Gaffney et al., 2017; C. Silva et al., 2021).

1.1.1. Problem statement

The exponential accumulation of pharmaceutical pollutants in the environment (persisting even after treatment in WWTPs) is increasingly under study, but the real impacts of their degradation into micro and nanoparticles and on health and environmental equilibria remain unknown. There are currently limited data on the systematic seasonal monitoring of pharmaceutical waste released into the environment from wastewater treatment plants. Thus, this necessitates comprehensive studies on the types and quantities of pharmaceutical micropollutants introduced into the marine environments from urban wastewater. This should include assessment of the impact of seasonal changes on their concentrations, removal efficiencies by wastewater treatment plants, and their toxicity (lethality) to organisms. An effective marine monitoring strategy still does not exist for the Algarve, Portugal.

1.1.2. Aim

This project aims to monitor pharmaceutical compounds from urban wastewater, the ability of WWTPs to remove them, and their release or potential accumulation in marine environments, in the Ria Formosa natural park, Algarve, Portugal. The present thesis data is gathered to assess the existence and quantities of micropollutants released into the environment (in the frame of the "MicroWaste" project, www.microwaste.pt), to assess the efficacy of WWTPs, and to raise awareness of substances of concern (namely PhCs) present at significant levels in the environment. The monitoring results may be helpful in the optimisation of wastewater treatment and WWTP removal efficiencies and in the long-term contribute to influence legislation on PhCs release, which may contribute to reduce waste and environmental micropolution. This aim was achieved through the set of objectives defined below.

1.1.3. Objectives

1.1.3.1. To carry out seasonal sampling campaigns of wastewater from the two selected regional WWTPs, Faro-Olhão (FO) and Faro Noroeste/Faro North-West (FNW), together with FO-WWTP lagoon and environmental receiving waters in the Ria Formosa;

1.1.3.2. To analyse the wastewater samples for 26 PhCs using optimized SPE-LC-MS/MS techniques;

1.1.3.3. To analyse and compare occurrence profiles of the analysed PhCs between FO or FNW WWTPs, the FO WWTP lagoon and the environmental waters in the receiving environment in the Ria Formosa;

1.1.3.4. To assess and compare the removal efficiencies of the classical activated sludge (AS) and the aerobic granular sludge (AGS) used in FNW-WWTP and FO-WWTP, respectively;

1.1.3.5. To establish and compare the lethal concentrations of recalcitrant anticonvulsant carbamazepine and phytoestrogen coumestrol using an *Artemia sp.* immobilisation/lethality assay.

1.2. Literature Review

1.2.1. Micropollutants: Contaminants of Emerging Concern (CECs)

Micropollutants are a group of potentially hazardous compounds and particles originating from multiple sources, including the breakdown of products such as cosmetic and self-care products, hormones, industrial waste, pesticides, and pharmaceuticals. They derive their collective name from their occurrence in small concentrations, around or less than a microgram per litre (European Investment Bank, 2023).

A group of compounds termed contaminants of emerging concern (CECs) is currently at the centre of research by the scientific community. The CECs refer to chemical substances, their by-products and degradation products, including naturally occurring compounds whose adverse effects were previously not recognised (Barbosa et al., 2016; Čelic et al., 2023). This is a constantly evolving category of chemicals of unknown impact on human and animal health both on land and in water. They include PhCs and personal care products, which have potential adverse biological outcomes in marine organisms, and include promotion of antimicrobial resistance, endocrine disruption or other associated threats, and have high potential of affecting ecosystem health (Barbosa et al., 2016; Couto et al., 2019; Marasco et al., 2019; C. Silva et al., 2021).

With high rates of production and consumption, PhCs and their breakdown products are discharged into wastewater mainly through domestic and industrial wastes (Albendín et al., 2021). These compounds are designed to be effective and biologically active at low concentrations in humans (and some target animals), thus, despite their possible occurrence at low concentrations in aquatic environments they can still pose a biological threat to aquatic life (Alrashood, 2016). The 26 PhCs (Table 1.1) monitored in this study belong to different pharmaceutical classes and were selected based on prior monitoring studies (Gaffney et al., 2017; C. Silva et al., 2021; Sousa et al., 2019) and a list of compounds proposed for monitoring in the EU Water Framework Directive (WFD) 2000/60/EC (European Commission, 2022b).

The European Commission through WFD 2000/60/EC and the Environmental Quality Standards Directive (EQSD) 2008/105/EC on water policy made a provision in Article 8 of the EQSD to establish a “watch list” of substances regarded as contaminants of emerging concern (CECs). This directive that resulted from the amendment and repealing of Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and 2000/60/EC of the European Parliament and of the Council was to incorporate monitoring matrices and methods

of potential use in the analysis of substances on the watch list. The first watch list was composed of ten groups of substances and was set out in a Commission Implementing Decision (EU) 2015/495 (European Commission, 2022b).

Table 1.1: A list of the 26 monitored PhCs, their pharmaceutical classes and abbreviations/acronyms used in this study.

Pharmaceutical Class	**PhC**	**Abbreviation/Acronym**
Analgesic	Acetaminophen	APAP
Anticonvulsant	Carbamazepine	CBZ
Antibiotic	Azithromycin	AZM
	Clarithromycin	CLR
	Erythromycin	ERY
	Sulfadiazine	SFD
	Sulfamethoxazole	SMX
	Sulfapyridine	SPD
Antidepressant	Fluoxetine	FLU
Anti-dyslipidemic	Clofibric acid	CFA
	Bezafibrate	BZF
Beta-blocker	Atenolol	ATN
	Metoprolol	MET
	Propranolol	PPL
Non-Steroidal Anti-Inflammatory Drugs (NSAID)	Diclofenac	DIC
	Ibuprofen	IBU
	Naproxen	NPX
Psychostimulant	Caffeine	CAF
Corticosteroid	Cortisone	E
Sexual hormone	Testosterone	TEST
	17β-Estradiol	E2
	Estriol	E3
	Estrone	E1
Contraceptive	17α-Ethinylestradiol	EE2
	Diethylstilbestrol	DES
	Gestodene	GSD

The WFD encompasses water pollutants of greatest concern in surface water, identified as "priority substances". These directives together with the Groundwater Directive are aimed at

managing and mitigating pollution caused by chemicals in European water bodies by ensuring an integrated approach to the management of water (Backhaus, 2023). This is expected to be achieved through the preservation of ecosystem integrity by proper testing of individual pollutants and setting their regulatory standard (European Commission, 2022b).

The substances on the watch list should be monitored by the member states at least once per year in the four years' monitoring phase. This monitoring inspired by the EQSD is meant to act as a counteractive approach against the negative effects of CEC micropollutants in the environment and to address growing public concern. Monitoring is meant to provide data about CEC micropollutants to assess if they should be included in the EU priority list of wide concern substances (Backhaus, 2023).

In a similar manner, the United States of America (USA) through its Environmental Protection Agency (EPA) has established under the Safe Drinking Water Act (SDWA) two regulations to monitor CECs in water. First, there is the Contaminant Candidate List (CCL) comprising compounds not subject to regulations, but setting priorities to survey their occurrence and toxicity. The CCL could be likened to the EU priority list of wide concern substances. The second regulation is the Unregulated Pollutant Monitoring Rule (UCMR). Through the UCMR, the EPA gathers data about contaminants potentially present in drinking water but without established limits by SDWA. Similar to the contaminants on the EQSD watch list, the risk assessment and eco-toxicological results of the contaminants on the UCMR are assessed to determine if the contaminant should be added to the EQSD priority list or SDWA CCL, respectively (EPA, 2024; European Commission, 2022b).

The occurrence of the micropollutants in WWTPs raw sewage, effluents, and receiving environments occur in varying concentrations that can be influenced by the frequency and intensity of usage by the population in the vicinity of the WWTP, the weather conditions, the efficiency of the WWTPs, and the resistance of the substances to degradation and treatment (Rodrigues et al., 2019; Silva et al., 2021). The resistance to treatment of some substances is thought to be mainly due to their polarity (Lajeunesse et al., 2008; Ternes, 1998). Their concentrations usually vary from micrograms per litre to nanograms per litre, and some have toxicity at low levels in animals (e.g Alrashood, 2016)). These substances can also be assessed as mixtures according to the commission's proposal with an aim to improve the monitoring of chemical mixtures to better assess combination effects and take account of seasonal variations in pollutant concentrations (Backhaus, 2023).

In this case, the combined effect of the chemicals is taken into consideration when evaluating their toxicity or adverse biological outcome. In most cases the toxicity levels of the mixtures are assessed by concentration addition (CA) of the individual components making up the mixture (Backhaus, 2023). The CA concept is applied by adding relative potency factors (RPFs) used to give proportional concentrations of the chemical components contributing to a mixture, usually based on their relative environmental concentrations. In a study by Melvin et al., (2014), the combined toxicity of carbamazepine, naproxen, and sulfamethoxazole to Australian striped marsh frog tadpoles was higher compared to individual compound exposure proving the synergistic effects chemical mixtures have compared to their individual effect. A similar outcome was observed by Cleuvers (2003) on the combined effects of clofibric acid and carbamazepine on the freshwater flea *Daphnia magna*; where the individual effects were significantly lower compared to the much stronger than expected combined effect.

In depth research on the substances on the international watch lists can help to establish risks of these substances to humans and the environment, and thereby aid in establishing quality recommendations for policy makers responsible for chemical regulations. The data and information collected from monitoring studies such as the IMPETUS project, dedicated to the monitoring and treatment optimization for PhCs in some Portuguese WWTPs, can contribute to the development and implementation of legislation on CECs in the European member states (LIFE IMPETUS, 2019).

1.2.1.1. Pharmaceutical Compounds (PhCs)

PhCs are compounds of natural or synthetic origin found in over-the-counter therapeutic drugs, prescription medicine and veterinary drugs containing active ingredients meant to treat particular illnesses in human and animals (Adeleye et al., 2022; Kock et al., 2023; Ortúzar et al., 2022). The production of PhCs is a highly regulated process to ensure patient safety. This is assured through good manufacturing practices, clinical trials, and further studies of biological activities, contributing to the understanding of the impacts of these substances on their target patients. However, their distribution to the patients and environmental fate is not as controlled since some of them are sold without pre-registration or prescription (Gil et al., 2017; Ortúzar et al., 2022). The ever increasing development and use of PhCs and their release (directly or by excretion) and incomplete removal in WWTPs raises concerns about their impacts to the environment (Ortúzar et al., 2022). This problem is exacerbated by the limited knowledge about their impact on animals and their ecosystems (most of them with poorly characterized physiology), and PhCs form a large portion of the currently defined CECs. Most of the WWTP

systems were established prior to the production of these CECs, and are not designed to remove them.

PhC pollutants includes drugs meant to treat human and animal pathologies (Jaffrézic et al., 2017; Ramírez-Morales et al., 2021). In relation to animals a study on veterinary PhC contamination of mixed land use watersheds, Jaffrézic et al. (2017) reported that several drugs meant for veterinary use occur in the environment at concentrations exceeding that of drugs intended for human use.

Major sources of pharmaceutical waste in surface waters are illustrated in Figure 1.1 and include urban wastewater enriched with human and livestock excreta, the landfill leachates of sludge or disposed expired and unused pharmaceuticals as well as from farm lands where livestock excreta is used as manure (Barrios-Estrada et al., 2018). The release of pharmaceutical waste from manufacturing industries is subject to strict rules about waste treatment prior to its release into urban wastewater. However, this is still reported as one of the major sources of active pharmaceutical ingredients in water (Cardoso et al., 2014). Although metabolites rather than the parent active pharmaceutical ingredient (API) are excreted and reach the WWTPs (Khetan & Collins, 2007).

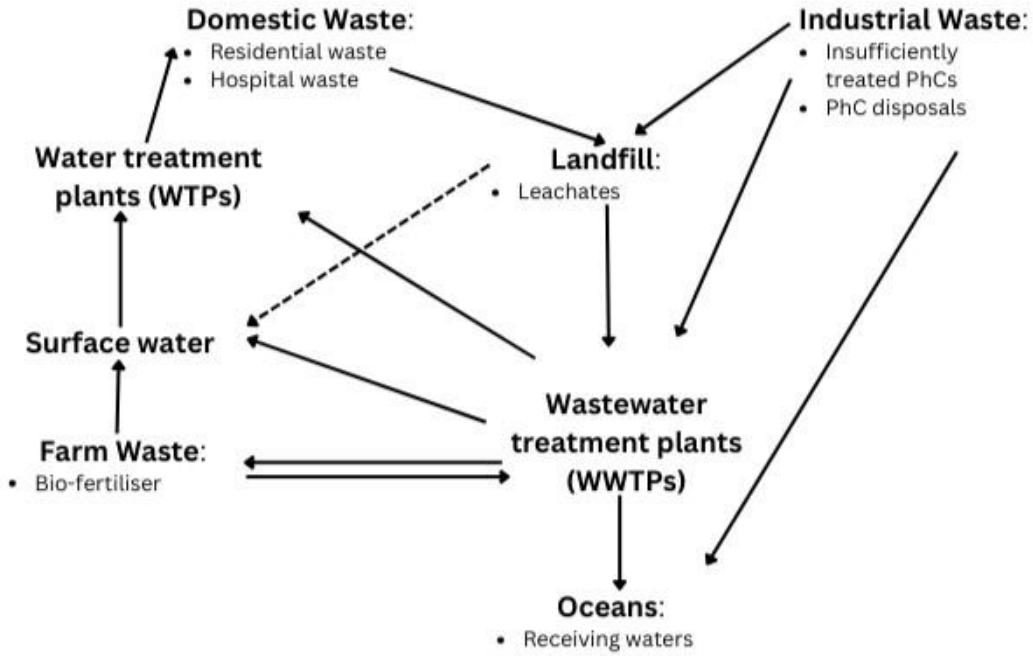

Figure 1.1: Flow and fates of PhCs from source to marine and surface waters (modified from (Yang et al., 2017)).

1.2.1.1.1. Antibiotics/Antimicrobials and Antimicrobial Resistance

Gaining popularity amongst the CECs are antimicrobial/antibiotics. Throughout the ages since the discovery of the first antibiotic, penicillin, in 1928 by Sir Alexander Fleming (Adedeji, 2016; Porrit, 1951), antibiotics are widely used for treatment of bacterial infections, significantly improving health conditions and controlling infectious diseases. Antibiotics are used to treat humans and animal, especially used as additives in animal feed (Ghimpețeanu et al., 2022; Mutuku et al., 2022). The present study is focused on monitoring sulphonamide antibiotics and macrolide antibiotics mentioned in the panel of 24 PhCs justified above.

The earliest effective antibiotics used for human medicine in the 1930's were the **sulfonamide group**, which include sulfadiazine, sulfamethoxazole, and sulfapyridine (Figure 1.2). They are broad-spectrum bacteriostatic antibiotics that inhibit the biosynthesis of folic acid by the bacteria, which is required for their cell growth. Bacterial resistance to sulfonamides is commonly reported in humans, and has led to a decrease in their use, although they are still used for treatment of urinary and parasitic infections. This group of drugs has also been associated with drug-induced liver injury with symptoms such as lymphocytosis, fever, facial edema, etc (Bethesda, 2012).

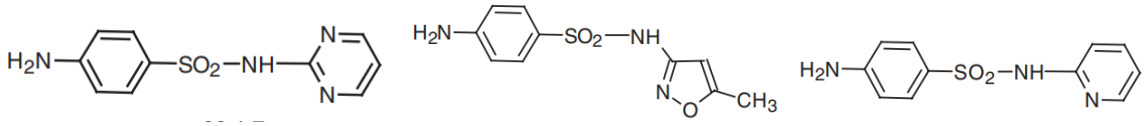

Figure 1.2: Chemical structures of sulfonamide antibiotics, sulfadiazine, sulfamethoxazole, and sulfapyridine from left to right in that order (Vardanyan & Hruby, 2006)

The **macrolide antibiotics** are naturally occurring antibiotics made up of a lactone ring with deoxy sugars attached to it. The macrolides targeted in this study are azithromycin, clarithromycin, and erythromycin shown in Figure 1.3. They are used for treatment of bacterial infections such as pneumonia, sinusitis, and some sexually transmitted diseases such as chlamydial and gonococcal infections, by inhibiting protein synthesis in the causative bacteria.

The introduction of antibiotics into water bodies poses a threat to aquatic organisms inhabiting freshwater and marine environments, as well as to human beings using the water for consumption and recreation. Most of these affected organisms are not target organisms to whom the antibiotics are meant for but get impacted (Ignoto et al., 2022).

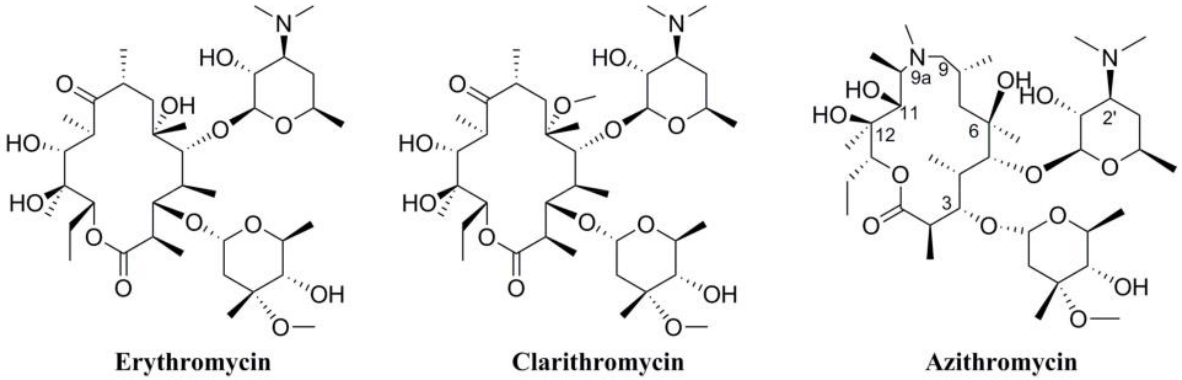

Figure 1.3: Chemical structures of macrolide antibiotics erythromycin, clarithromycin, and azithromycin (C. M. Wang et al., 2017)

The excessive, and inappropriate use of antibiotics has led to drug-resistant and harmful bacteria, a phenomenon termed antimicrobial resistance (AMR) (Paíga et al., 2019; Wu et al., 2023). The inappropriate use of antibiotics can cause adverse drug reactions (ADR) including organ toxicity, allergic reactions, destruction of beneficial microorganisms, and can be lethal to the users. The wide monitoring of the use of antimicrobials, their occurrence in wastewater and WWTPs effluents to the environment and surface waters will strengthen the knowledge and the data that will aid the World Health Organisation's (WHO) global action plan on AMR and the One Health approach. The One Health approach is an approach coined to recognise the close connection between the health of people and animals and their shared environment (CDC, 2024). The surveillance of AMR and antimicrobial use (AMU) together with the environmental pollution monitoring studies such as the present study on WWTPs and their dissemination to the environment form part of the collaborative, multisectoral, and interdisciplinary approach pursued by the One Health approach.

The European Union (EU) council in 2016 adopted the recommendations on the steps to take under the One Health approach. The council set up a surveillance of antimicrobial consumption and antimicrobial resistance in humans and animals for food-production through a collaborative initiative between the European Centre for Disease Control (ECDC), European Food Safety Authority (EFSA), and European Medicines Agency (EMA). The surveillance is enabled by an established list of proposed harmonised outcome indicators for human antimicrobial consumption, indicators for AMR in humans, and indicators for AMR in animals for food-production. It serves as a support for EU member states in their efforts to address AMR (ECDC et al., 2017). Amongst the indicators for AMR in humans is a macrolide resistant bacteria *Streptococcus pneumonia*, which is one of the significant causes of community-acquired infections (ECDC et al., 2017). It is reported to have acquired resistance to macrolide

antimicrobials of which some are part of the current monitoring study, namely azithromycin, clarithromycin, and erythromycin. The EU council in 2019 further stressed the need for strengthening and widening the scope of the surveillance of AMR and healthcare-associated infections, together with the surveillance of the consumption of antimicrobials in both animals and humans (Council of the European Union, 2019). The monitoring of antimicrobial consumption is regarded an important factor towards the prevention and control of AMR (ECDC et al., 2017). The threats of AMR do not only stop at the direct consumption of antimicrobials but extend to indirect consumption from contaminated drinking water due to inefficient water treatment. In a similar manner that AMR surveillance should be frequent due to its association to the fluctuating use of antimicrobials, frequent antimicrobial waste monitoring plans may be essential in staying up to date with the exposure of organisms to antimicrobials and associated microbial resistance development.

An extract of the European Investment Bank (2023) report describes the WWTPs as vectors of antibiotic resistant bacteria from polluted drainages to humans. The entry route of the disposed pharmaceuticals into humans can be directly through drinking polluted surface waters and secondary through eating contaminated fish in both surface and marine waters (Moermond, 2014). Main pollution sources for surface waters are WWTPs, in European Union member states. Other routes through which the antimicrobial pollutants enter surface water bodies are direct drainages from households, industries, and farms (European Investment Bank, 2023).

Recent reports by the ECDC indicated a fluctuating trend in the use of antimicrobials in Portugal and Europe as a whole for the past five years (2017-2022) in the total care (community and hospital) sector. This reveals a decline in the usage of antimicrobials for systemic use from 2018 to 2020, a slight rise in 2021 followed by a sharp increase in 2022 (ECDC et al., 2017). A study by Ferreira et al. (2022) indicates an increase in the reports of ADR cases associated with the most widely used antibiotics including azithromycin and clarithromycin and the reported cases were more prevalent in females (52.2%) than in men (41.3%).

1.2.1.1.2. Analgesics

Pain is one of the main indicators of an illness's severity and a major health concern. According to international estimates, 20% of adults worldwide suffer from pain and 10% new cases of chronic pain are diagnosed each year (Ahmed et al., 2021; Goldberg & McGee, 2011). The aetiology of pain is often complex due to its multiple causes. Excess of chronic pain can further

lead to other issues such as depression, physical incapacity, social withdrawal, and suicidal thoughts (Goldberg & McGee, 2011). Analgesics are typically used to relieve pain.

Among analgesics drugs, since 1955 acetaminophen (N-acetyl-p-aminophenol) is one of the most extensively used globally. Acetaminophen (Figure 1.4), known as paracetamol, is considered an effective and safe analgesic and antipyretic available as a subscription or over-the-counter drug. The daily dose recommended by the manufacturers is 1 to 4 g and it is an active ingredient in most of the inti-influenza drugs (Bunchorntavakul & Reddy, 2013; Quesada et al., 2019).

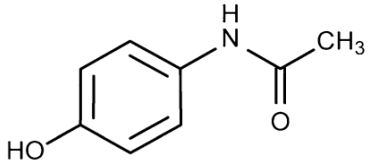

Figure 1.4: Chemical structure of acetaminophen (Seal et al., 2017)

However, overdose associated risks include acute liver failure and fatal liver damage and can occur even when the recommended safe dose limits were not exceeded greatly (Bunchorntavakul & Reddy, 2013; Larson et al., 2005; Lee, 2004). Following their consumption, the analgesics are secreted from the body as the main compound or metabolite in urine, and ultimately being released into wastewater for treatment.

1.2.1.1.3. Antidepressants

The antidepressant monitored in this study was fluoxetine (Figure 1.5), a drug classified as medium risk to the environment (Gaffney et al., 2017). Antidepressants combat depression by targeting specific neurotransmitters that are categorised by their biochemical activities and activities in mood regulation. Categories include dopamine reuptake inhibitors (DRI), monoamine oxidase inhibitors (MAO-I), selective noradrenaline reuptake inhibitors (NARI), noradrenergic and specific serotonergic antidepressants (NaSSA), selective serotonin reuptake enhancers (SSRE) and selective serotonin reuptake inhibitors (SSRI), to which fluoxetine belongs (Melchor-Martínez et al., 2021).

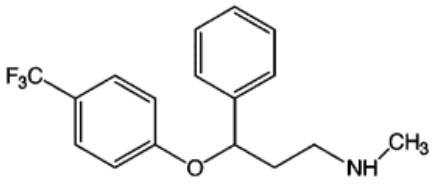

Figure 1.5: Chemical structure of fluoxetine (Khetan & Collins, 2007)

The SSRIs are used in the treatment of anorexia, anxiety, depression, eating disorders, obsessive compulsive disorder (OCD), and panic disorders. They improve the activity of serotonergic neurons by blocking the function of serotonin transporters, so that higher levels of serotonin, a mood enhancer accumulates (Khetan & Collins, 2007).

Recommended doses of fluoxetine for adults is up to 80 mg per day and is characterised by high absorption properties when administered orally. Fluoxetine is metabolised in the liver to the main metabolite norfluoxetine by N-demethylation and to other minor metabolites. It has the elimination half-life of 48-72 hours, and an estimation of 2.5% of it is excreted renally unchanged while 10% is excreted as norfluoxetine (Wexler et al., 2005).

1.2.1.1.4. Anticonvulsants

The anticonvulsant monitored in this study was carbamazepine (Figure 1.6), a dibenzoazepine that is a derivative of 5H-dibenzo[b,f]azepine with a carbamoyl substituent at the azepine nitrogen (National Center for Biotechnology Information, 2023). According to Silva et al. (2021), over 95 % of carbamazepine is metabolised in humans to the major metabolite 10,11-epoxy-carbamazepine/carbamazepine-10,11-epoxide, which is further hydrolysed to diol-derivatives and mainly excreted as glucuronides (Bahlmann et al., 2009, 2014; Khetan & Collins, 2007; C. Silva et al., 2021; Ternes, 1998).

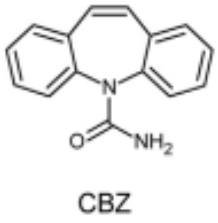

Figure 1.6: Chemical structure of carbamazepine (CBZ) (Bahlmann et al., 2014)

Carbamazepine is administered for the treatment of epileptic seizures and neuropathic pain and can be used to treat bipolar disorders as a second-line treatment drug. It may be used in clinical applications on bipolar and unipolar disorders as an antidepressant, antimanic, and a

prophylactic against mental disorders. Alrashood (2016) indicates carbamazepine is a promising treatment for schizoaffective and personality disorders.

Several studies (Albendín et al., 2021; Oetken et al., 2005) have demonstrated the toxicity of carbamazepine on aquatic organisms. In addition to being toxic as a pure compound, carbamazepine shows toxicity in combination with other pharmaceutical compounds and generates more toxic degradation products when exposed to ultraviolet radiation (Donner et al., 2013). This means carbamazepine in wastewater exposed to sunlight may results in more ecologically toxic compounds although toxicity effects are not fully known yet.

1.2.1.1.5. Anti-dyslipidemics drugs/Lipid regulators

The lipid regulators, also called anti-dyslipidemics, are PhCs used for the reduction of lipids in the blood to alleviate risks of fat deposition in the blood vessels; this fat deposition is termed atherosclerosis, and is encouraged by high levels of cholesterol and triglycerides in the blood. The lipid regulator PhCs work by inhibiting the production of very low-density lipoproteins (VLDL), thus reducing the levels of triglycerides in plasma (Khetan & Collins, 2007). They can be either natural or synthetic, and the most widely used groups are fibrates and statins. In the current monitoring study, two lipid regulators of the fibrate group were surveyed, bezafibrate and clofibric acid. Since most of the high blood lipid problems or hyperlipidemia are a result of a poor lifestyle and unhealthy diet, except congenital hyperlipidemia caused by genetic predisposition, a change in lifestyle and diet are recommended practises. However, in cases where these control and preventive measures are impractical, lipid regulators may be used despite their serious risks and side effects. These risks to human health include development of gallstones, muscle pain, and stomach abnormalities, and they have also been reported as environmental pollutants although impacts in marine life are largely unknown (Khetan & Collins, 2007; Rezka & Balcerzak, 2015).

1.2.1.1.6. Beta-Blockers

Beta-Adrenergic receptor blockers (β-blockers) constitute a class of pharmaceutically active compounds used in the treatment of some of cardiovascular disorders known to be chronic to human. These include high blood pressure (hypertension), chest pains (angina), irregular heartbeats (arrhythmias) and recurring heart attacks (Khetan & Collins, 2007; Yi et al., 2020). By 2020, at least 20 β-blockers had been detected in wastewater and drinking water at concentrations within the nanograms and micrograms per litre (Yi et al., 2020).

Commonly detected beta-blockers in wastewater are atenolol, metoprolol, and propranolol (Figure 1.7). Our current study focuses on the profiling of these three. The majority of the β-blockers are highly polar weak bases and are known to exist as racemic mixtures, a mixture of enantiomers, even though usually the beta-blocking activity is exerted by only one enantiomer. The chirality nature influenced by the enantiomers determines the phase distribution of the compounds according to (Sanganyado et al., 2016), which then determines the hydrophobicity of a compound and its retention in water due to the adsorption properties therein.

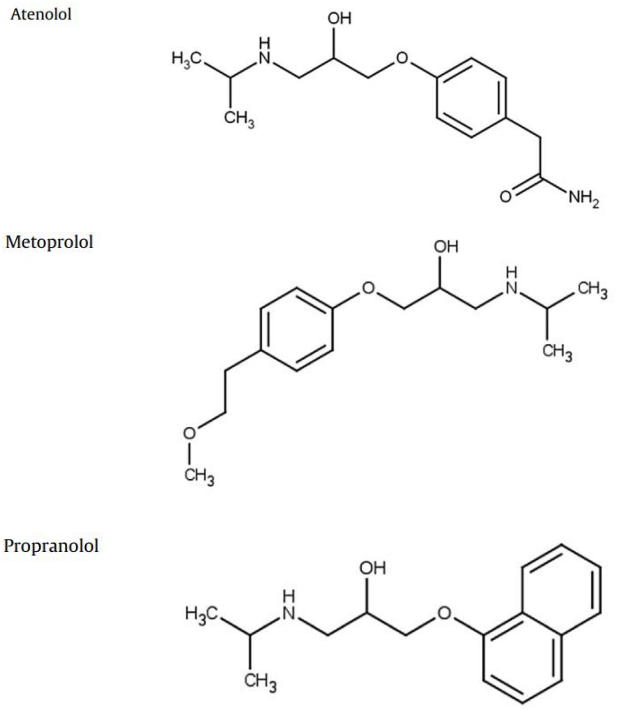

Figure 1.7: Beta-blockers studied in the monitoring study (Sanganyado et al., 2016)

1.2.1.1.7. Non-Steroidal Anti-inflammatory Drugs (NSAIDs)

Amongst the commonly consumed drugs globally are the NSAIDs (Brennan et al., 2021; Ortúzar et al., 2022). These are compounds responsible for the inhibition of the prostaglandins producing an enzyme called cyclooxygenase (COX) which are largely found in the endoplasmic reticulum of prostanoid-forming cells, an action that forms the basis of their therapeutic action. The COX-1 enzyme produces prostaglandins responsible for protecting the kidneys and the stomach from damage, while the COX-2 enzyme induced by inflammatory stimuli produces prostaglandins responsible for pain and inflammation swelling. Thus, the anti-inflammatory property of the NSAIDs acts on pain and inflammation by inhibiting the COX-2 enzyme. They are therefore prescribed for acute pain as analgesics for similar reasons described in the "Analgesics" section 1.2.1.1.2.

The NSAIDs are also reported to delay the progress of Alzheimer's disease (Vane & Botting, 1998). The common NSAIDs included in this monitoring study are diclofenac, ibuprofen, and naproxen illustrated in Figure 1.8. Some of these compounds such as diclofenac are reported to be removed with low efficiencies in WWTPs depending on the type of treatment system in place (Aydın et al., 2023).

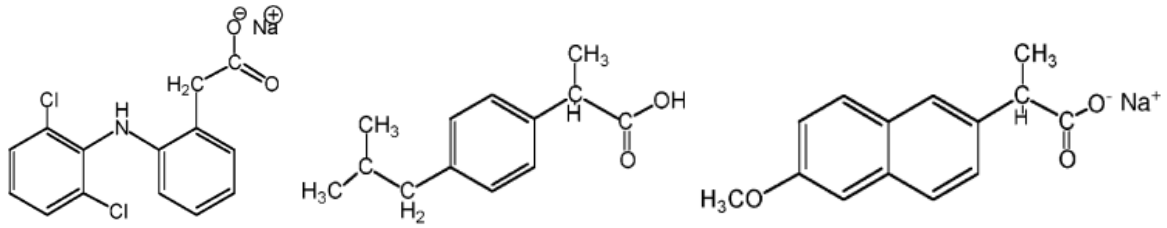

Figure 1.8: Common NSAIDs diclofenac, ibuprofen, and naproxen (from left to right) (Khetan & Collins, 2007).

1.2.1.1.8. Psychostimulants

Caffeine, scientifically termed 1,3,7-trimethylxanthine, is one of the most commonly used psychostimulants. It is an alkaloid of the xanthine group with a molar mass of 194.19 g/mol, a molecular formula $C_8H_{10}N_4O_2$ (Marasco et al., 2019), and a chemical structure shown in Figure 1.9. Caffeine has a negligible volatility, low octanol-water partition coefficient, and high solubility of 13.5 g/L, thus enabling its detection in environmental waters (Marasco et al., 2019). Due to its relative stability in the environment with a half-life of 30 days, it can be detected for extended periods in the environment post the initial release (Gonçalves et al., 2014). For pharmaceutical purposes, it serves as an active ingredient for stimulant drugs. Additionally, caffeine is classified as food and can be naturally found in products such as coffee, tea, energy drinks, chocolates, soda drinks, etc. Some sources of caffeine include coffee beans, cocoa beans, tea leaves, and fruits such as guarana berries (Korekar et al., 2020).

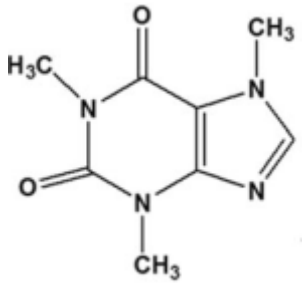

Figure 1.9: Chemical structure of caffeine (Korekar et al., 2020)

The consumption rate of caffeine in Europe was estimated at 271.7-288.58 mg/person/day in 2013 and 193-380 mg/person/day in the United States of America in 2005, and it was the most consumed drug and stimulant, with more than 80% of the world's population consuming on a

daily basis (James, 1997; Korekar et al., 2020; Zucconi et al., 2013). The high caffeine usage in the European Union Member States (EUMS) is characterised by the high increase in the imports of caffeine products, up to 14% over ten years prior 2020. According to Eurostat (2020) the EUMS imported approximately 3 million tons of coffee in 2020 at an estimated cost of 7.5 billion Euros. In a study by Batista et al. (2022), a sample of Portuguese people in 2020 were reported to consume 2656.71 mg/week of caffeine, almost 380 mg/person/day.

While caffeine is metabolised and absorbed into the human gastrointestinal system upon consumption, approximately 0.4-5% of it is said to be excreted unmetabolised (Arnaud & Advisor, 1987), thereby ending up in WWTP influents together with higher quantities from other sources other than human excreta. Due to its considerably high occurrence in wastewater, it is argued to be a marker of anthropogenic activity and domestic contamination (Marasco et al., 2019).

1.2.1.1.9. Corticosteroids

Corticosteroids have widely been in use for treatment of inflammatory and autoimmune disorders and various other illnesses since the mid-90s (D. Liu et al., 2013). They are the synthetic analogues of the naturally occurring body hormones produced in the adrenal cortex of mammals. Corticosteroids exhibit the characteristics of natural hormones such as possessing glucocorticoid responsible for the metabolism of fat and protein, anti-inflammation, immunosuppression and vasoconstriction. They may also show mineralocorticoid properties, which is responsible for the electrolyte regulation and water balance by influencing the ion transport in epithelial cells of the renal tubules (D. Liu et al., 2013).

The synthetic corticosteroids are PhCs used in human and veterinary medicine for the treatment of illnesses such as allergies, rheumatic arthritis, inflammatory diseases, and asthma (Kugathas et al., 2012). They are non-volatile substances relatively soluble in water, and this allows for their excretion from the body and presence in wastewater. Despite their pharmaceutical importance, corticosteroids pose several side effects in humans that include avascular bone necrosis, cardiovascular diseases, immunosuppression, osteoporosis, amongst others (Dias et al., 2023; Liu et al., 2013).

The corticosteroid cortisone, monitored in this study, is considered to be one of the least potent corticosteroids and is preferably used on patients with adrenal deficiency owing to its possession of both mineralocorticoid and glucocorticoid activities (Liu et al., 2013).

1.2.1.1.10. Endocrine Disrupting Compounds (EDCs): Contraceptives and Phytoestrogens

The EDC are a diverse group of compounds with the potential of disrupting the normal physiological functions of natural hormones in animals. The initial definition by the International Programme on Chemical Safety states an EDC as,

> "…an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations." (International Programme on Chemical Safety - World Health Organization et al., 2002)

The classification of the EDCs stems from their disruptive actions which include: 1) mimicking the natural hormones, 2) acting as receptor antagonists of hormone receptors leading to failure of hormonal action, and 3) interfering with hormones to reach their receptors thereby leading to metabolic alterations (Barrios-Estrada et al., 2018; Kabir et al., 2015; La Merrill et al., 2020). They can be of a natural or synthetic origin and are known to cause the disruption by mimicking the functions of hormones (Kim et al., 2007). The natural EDCs include phytoestrogens and hormones produced and secreted by animals and humans, while those of synthetic origin include some industrial pesticides and pharmaceuticals (Barrios-Estrada et al., 2018).

In this study, the focus (included in the LC-MS/MS panel) was to monitor sexual hormones (testosterone, β-estradiol, 17 β-estradiol, estrone) and contraceptives (17α-ethinylestradiol, diethylstilbestrol, gestodene), some of which are shown in Figure 1.10. However, some of the compounds classified under separate classes of PhCs are also reported to have endocrine disruption potential e.g. sulfamethoxazole, bezafibrate, etc. (Barrios-Estrada et al., 2018; Isidori et al., 2009)).

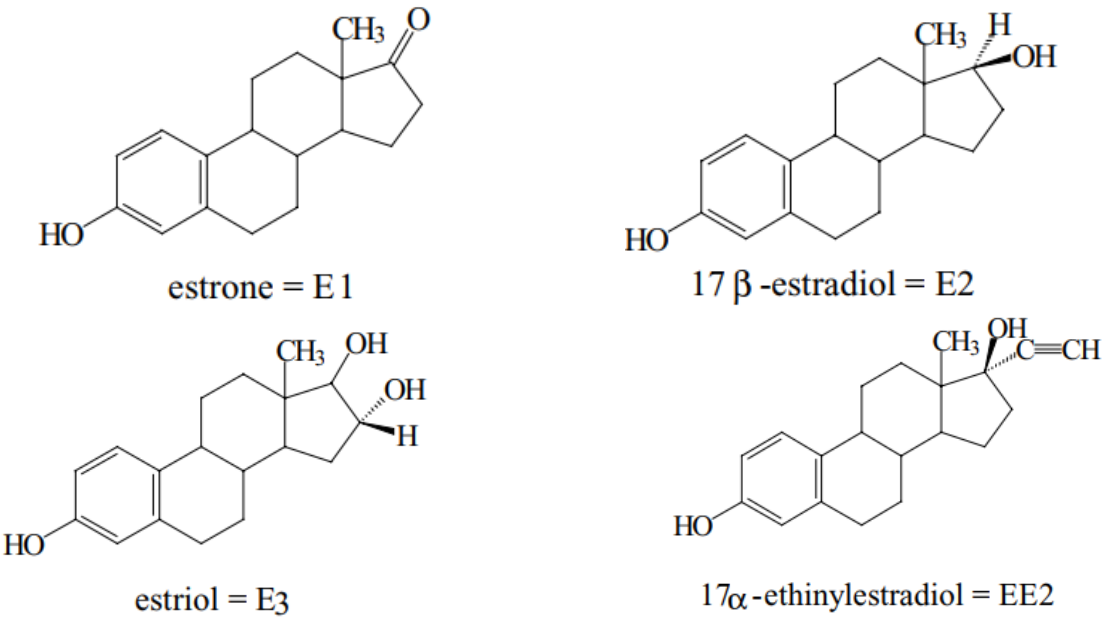

Figure 1.10: Chemical structures of some of the human sexual hormones estrone, 17 β-estradiol, estriol, and 17α-ethinylestradiol (Laganà et al., 2004)

The definition of priority hazardous substances of the WFD does not specifically include endocrine activity or endocrine disruptors, however according to the review by Backhaus (2023), the specific inclusion of endocrine disruption in the definition of priority hazardous substances Article 1 (2c) of the European Union's Commission's proposal may facilitate them being prioritised in future political discussions. According to Article 13 of the EU Directive 2020/2184 of the European parliament and the council on the quality of water intended for human consumption, the commission shall adopt an implementation of the acts meant to establish and update a watch list addressing substances or compounds of concern to either the public or the scientific community on health grounds. These substances and compounds include PhCs, EDCs and microplastics (European Union Council, 2020). The article further states that the addition of the substances and compounds to the watch list will be on the basis that they possess a potential of occurrence in water intended for human consumption and could potentially pose a threat to human health (European Union Council, 2020). With an inclusion of EDCs in the watch list, a consideration of those of plant origin (e.g. phytoestrogens) is worth pursuing due to their readily available nature and for purposes of investigating more of their effect to both water organisms and human hormone system.

1.2.1.1.11. Phytoestrogen – Coumestrol

Though not classified under pharmaceutical compounds, coumestrol is a natural estrogen, from the coumestan class of phytoestrogens that promise potential therapeutic applications as an antioxidant, potential cancer chemoprevention agent and in hormone replacement therapy. It is

a polyphenol with two hydroxyl groups on its structure (Figure 1.11) orientated in a similar fashion to the estrogenic compound estradiol, a property that gives it the capacity for estrogenic and antioxidant activity (Bacaloni et al., 2005; Fekri et al., 2023; Montero et al., 2019). Phytoestrogens are estrogenic compounds of plant origin being the most common sources of coumestrol the leguminous plants such as soy, red clover, and alfalfa. They can act as mimics of estrogens and have endogenous estrogen receptor binding affinity (Bacaloni et al., 2005; Wexler et al., 2005).

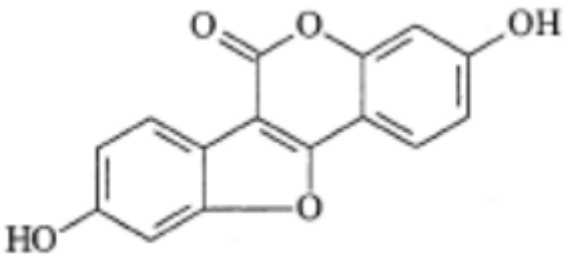

Figure 1.11: Chemical structure of coumestrol (Cos et al., 2003)

Phytoestrogens such as coumestrol, genistein, and daidzein have been reported to affect animal metabolic, reproductive, immunologic, mineral and growth functions (Pinto et al., 2016, 2019). The inclusion of phytoestrogens in the toxicity studies though not part of the monitoring study is for possible inclusion in the watch-list of contaminants of concern due to their reported adverse effects.

1.2.2. Wastewater Treatment

According to environmental regulations, wastewater must undergo treatment before being released into the environment. This ensures proper sanitation and safety to the environmental ecosystem.

The municipal WWTPs were initially not designed and purposed specifically to treat the growing amount of micropollutants, but rather to retain particulate matter, nutrients such as carbon, nitrogen, and phosphorus and pathogenic organisms from municipal waste (Adeleye et al., 2022; Aydın et al., 2023; Melchor-Martínez et al., 2021; Rodrigues et al., 2021). However, several of the micropollutants end up being significantly removed by wastewater treatment (Arun et al., 2022; Barbosa et al., 2016; J. Wang & Wang, 2016). The advent of the micropollutants pollution and detection has recently led to higher attention and to projects aiming at improving WWTPs efficiencies in treating these emerging compounds e.g. the production of new eco-friendly adsorbents tested for the adsorption of recalcitrant pharmaceuticals carbamazepine, diclofenac, and sulfamethoxazole (LIFE IMPETUS, 2019).

The removal efficiency of these compounds vary vastly depending on their chemical properties, solubilities in water, ability to be degraded by biological means, and on their affinities to the treatment adsorbents used in the WWTPs, and thus they are more or less efficiently treated according to their chemical and physical properties (LIFE IMPETUS, 2019; Loganathan et al., 2023). This makes it challenging to develop a one-size-fits-all treatment technique; hence, the measures taken to improve treatment efficiencies would have to be specific for each WWTP depending on the main type of pollution it receives (together with micropollutants content monitoring) or a combination of treatments should be designed that target a wide range of pollutants. The failure to effectively remove these pollutants may lead to their accumulation and persistence in the environment, thereby posing much greater threats to the ecosystem. Despite the fact that most known micropollutants appear to be significantly removed (in the cases this was evaluated), WWTPs are reported to be the main sources of micropollutants entry in water bodies, in part also because of the large volumes of effluents produced (Loganathan et al., 2023; J. A. Rodrigues et al., 2021; C. Silva et al., 2021).

The different WWTP processes usually include a biological treatment such as activated sludge (AS), physical systems such as initial mechanical separations, membrane separations and activated carbon, and chemical systems such as advanced oxidation processes.

Conventional biological treatments are widely employed for the degradation and removal of organic matter. These include the use of activated sludge, as it is the case in the Faro Noroeste WWTP (FNW-WWTP) studied in this thesis, and they are widely applied globally as the main biological treatment of wastewaters (Seth Portugal, 2024). The AS treatment makes use of microbial cultures suspended in an aerated bioreactor for the biodegradation of organic matter from the wastewater. Experimental work on the use of AS was first mentioned over a century ago by Ardern and Lockett (1914) for the remediation of wastewater. The experiments described the application of a biological treatment in the form of heterotrophic biomass for assimilating or oxidising dissolved organic matter in an aerated reactor (Sheik et al., 2014). The microbial cultures used in WWTP biological treatments are now known to release enzymes that play a role in the detoxification of contaminants, rendering them less toxic to the environment. As a typical treatment method in WWTPs, the effectiveness of AS varies between different pollutants and is reliant on several treatment parameters such as the reactor pH, size, and retention times (Lama et al., 2022).

The biological treatment stage is termed "secondary wastewater treatment", following the primary treatment which involves the physical removal of debris and particulate matter. After this aerated biological treatment, in the next treatment reactor, the biomass/AS is retained by gravity filtration and recycled while the treated wastewater is released for further tertiary treatment. The tertiary treatment stage is responsible for the removal of biological contaminants, and it is usually carried out by means of chemical sterilants, such as hypochlorite, or physically by ultraviolet radiation. Though conventional AS treatments with their various modifications has for ages served as a successful means of wastewater treatment, it is currently making way to the advent of a new technology or AS improvement, called the "aerobic granular sludge" system (AGS).

The AGS technology, such as one used in the Faro-Olhão WWTP (FO-WWTP) monitored in this work, overcomes some of the challenges of activated sludge that may render it an unsustainable wastewater treatment process. The AGS consists in the assemblage and self-immobilisation of self-growing phylogenetically distinct groups of treatment microbiota, including the nitrifying bacteria, anammox bacteria, polyphosphate accumulating organisms and glycogen accumulating organisms. The hydrolysis of biodegradable organic matter, the fermentation of organic matter to fatty acids, and the accumulation of volatile fatty acids occurs in the aerobic filling phase in the reactor. The removal of the chemical oxygen demand then occurs during the slow feeding of wastewater influent through the biomass by up-flow transport under anaerobic conditions (Nancharaiah & Sarvajith, 2019). In comparison to the AS process where vast amounts of energy are used for the removal and recycling of biomass and which has large land area requirements, complex process design and generates significant amounts of potent greenhouse gases, the AGS technology offers a more sustainable treatment. This is supported by its minimal energy consumption and process design complexities, operational costs, water-biomass separation challenges and land area requirements (Giesen et al., 2016; Nancharaiah & Sarvajith, 2019; Pronk et al., 2015; Sarvajith & Nancharaiah, 2021; Sheik et al., 2014).

As illustrated in Figure 1.12b, the simplicities of the AGS technology are proven by its capacity to combine biological treatment and water-biomass separation in a single reactor. The AGS granules are more compact and granulated compared to AS flocs, as shown in Figure 1.13. AGS technology does not require secondary clarifiers as in AS treatment due to the improved settling velocities of the granules. The reduced multiple process units make the AGS process less demanding in space in a magnitude of 25-75% as reported by (Giesen et al., 2016).

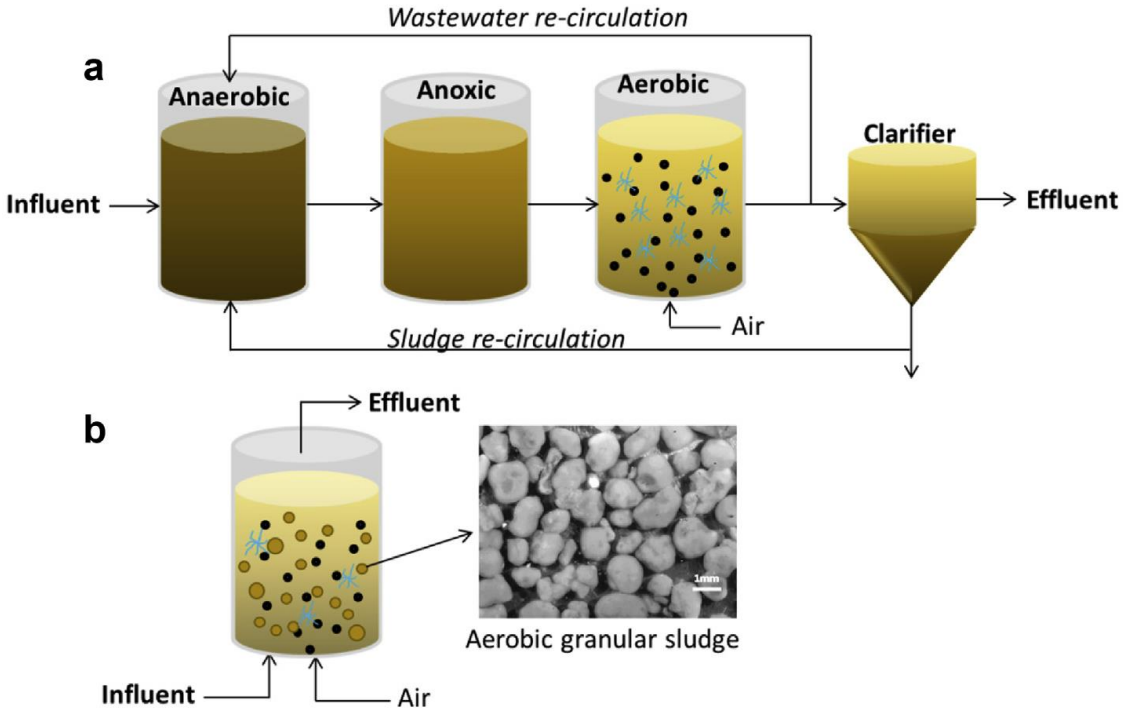

Figure 1.12: Principal units of (a) activated sludge and (b) aerobic granular sludge (From Nancharaiah & Sarvajith, 2019).

Figure 1.13: Aerobic granular sludge (AGS) – left, and activated sludge (AS) – right

The AGS provides microenvironments of aerobic, anoxic, and anaerobic digestion within the granules, as illustrated in Figure 1.14. This eliminates the need to have different anoxic and aerobic reactor compartments for the removal of biological nitrogen, thereby ensuring a much lower use of space (Nancharaiah & Sarvajith, 2019).

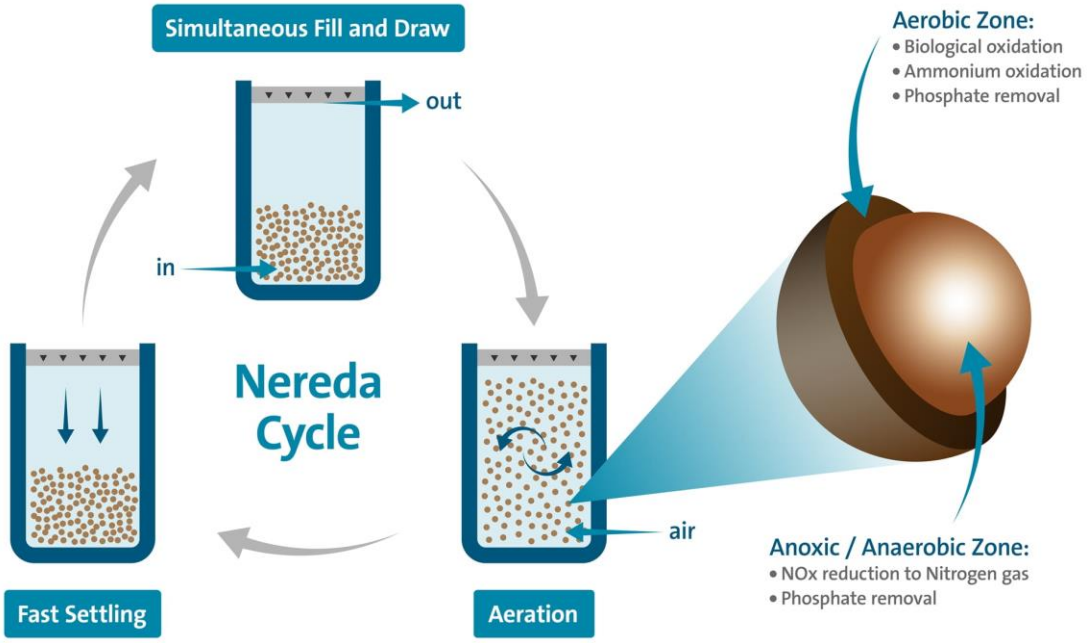

Figure 1.14: A summarised treatment cycle of the aerobic granular sludge using the patented Nereda technology (Nereda, 2024).

In addition to the AS and AGS processes, other biological treatment approaches involve the use of enzymes and biological filters. In as much as biological treatment is referred to as a cost-effective approach, biodegradation is argued to be a slower treatment method compared to physical and chemical methods. Moreover, some of the pollutants such as antimicrobials are toxic to the typical treatment microorganisms, thus affecting the treatment efficiency, which would require physical and chemical interventions if removal of more recalcitrant pollutants or heavy metals is pursued (Du & Zhou, 2021; Loganathan et al., 2023).

The physical approaches developed or under development to improve micropollutants removal from wastewater mainly involve the use of a solid material on which the pollutants are attracted and attached to, hence the use of "adsorption" for this category of wastewater treatment. Adsorption is applied for the recovery of organic pollutants occurring in trace amounts in water. It is preferred over other approaches such as coagulation-flocculation and photo catalysis for its cost-effectiveness, simplicity, efficiency even at low pollutant concentrations and production of minimal waste on reverse osmosis concentrate (Jamil et al., 2019). For adsorption-based removal of micropullutants from wastewater, carbon-based adsorptive materials such as activated carbon have widely been used. The physical and chemical systems take advantage of the large surface area created by the porous nature and powdered form of activated carbon; small and large organic molecules with higher affinity for the activated carbon than for water have the tendency to pass through its pores and adsorb leading to a separation of micropollutants

from the wastewater. Compounds with higher charges, bigger sizes, and more hydrophobic are more retained by the activated carbon. The optimization and improvement of treatment efficiency can be carried out by manipulating operating conditions, such as contact time, or employing different carbon-based adsorptive materials including graphene and carbon nanotubes (Jamil et al., 2019; LIFE IMPETUS, 2019).

Various studies (Gaffney et al., 2017; J. Rodrigues et al., 2019; C. Silva et al., 2021) focusing on the monitoring of the CECs, particularly pharmaceutical compounds, have reported a portion of these compounds to withstand conventional treatment approaches used by the WWTPs, thereby leading to their release into the environment posing a threat to the ecosystem.

A comparison between the different WWTPs ought to be carefully carried out with a consideration of both the initial (influent) and final (effluent) concentrations.

Thus, a removal efficiency Eq.1. below is employed;

$$Removal\ Efficiency\ (\%) = \frac{C_{inf.} - C_{eff.}}{C_{inf.}} \times 100$$

Eq. 1: (Gaffney et al., 2017)

Where $C_{inf.}$ is the concentration of the measured influent pharmaceutical compound and $C_{eff.}$ is the concentration of the measured effluent pharmaceutical compound for a specific phase of wastewater treatment (Gaffney et al., 2017).

In a similar manner to the monitoring carried out by (Gaffney et al., 2017), the monitoring study carried out by the MicroWaste project and described in this thesis serves both to investigate the occurrence (and seasonal variation) of pharmaceutical microwaste entering the two WWTPs, as well as making an evaluation of their removal efficiency. This will aid in identifying and optimizing the better treatment approaches for different PhCs based on their waste removal efficiencies comparing one AS with one AGS treatment plants.

1.2.3. Presence and effects of pharmaceutical pollutants in marine organisms

PhCs or active pharmaceutical ingredients are of significant therapeutical importance to their target organisms but can be detrimental to those unintended for. This section explores the various studies where PhCs were shown to act as toxicants to non-target marine organisms, which may have high exposure incidences due to long-term daily and lifelong exposure to contaminated waters (and potential bioaccumulation).

As example, a study by Vera-Chang et al. (2018) outlines the adverse effects of an antidepressant fluoxetine on zebra fish, *Danio rerio*. Their findings revealed a reduction in basal and stress-induced cortisol levels in adult *D. rerio* resulting from a six days exposure to fluoxetine during a critical brain developmental stage, at human physiological and environmental doses. The effect was transferred down to three generations of the zebra fish unexposed to fluoxetine. Male *D. rerio* was also reported to have reduced courtship displays when exposed to common anticonvulsant carbamazepine and gemfibrozil at 10 µg/L for six days. The effects were also observed on sperm shape and size (Galus et al., 2014).

Fish species are widely reported to be affected by disruption changes in the functions of their reproductive systems due to the presence of estrogenic/endocrine compounds released into their marine habitats through wastewater release. A well-known synthetic estrogenic compound, 17α-ethynylestradiol (EE2), used as a birth control in humans, was revealed to feminise male fish *Pimephales promelas* (fathead minnow). Male *P. promelas* produced a protein produced by female fish during oocyte maturation, vitellogenin, while females had altered oogenesis when exposed to low concentrations of EE2 at 5-6 ng/L (Kidd et al., 2007). Disruption of liver synthesis of vitellogenin in immature and male fish is one of the most known biomarkers of endocrine disruption and "feminization", demonstrated for many environmental pollutants recognized as estrogenic EDCs at research and drug testing levels and also one of the most used biomarkers in monitoring programs to survey micropollutants biological impacts (Pinto et al., 2014).

As an example of impact by PhC pollutants, Ericson et al. (2010) investigated and revealed the bioaccumulation of diclofenac and propranolol in tissues of Baltic sea blue mussel, *Mytilus edulis trossulus*, over time and the decline in the combined available energy for growth and reproduction. The bioaccumulation of PhC pollutants was also reported by Ali et al. (2018) in a study on detecting PhC and personal care products in marine organisms in coastal waters of the Saudi Red Sea. The study reported detectable levels of caffeine and carbamazepine, 41.3 and 1.7 ng/g, respectively in microalgae and barnacles.

1.2.4. Economic threats of PhC pollutants and CECs

Amongst the CECs, antibiotics are some of the extensively studied pharmaceuticals posing a threat to human health through the development of antimicrobial resistance by pathogenic bacteria targeted by them. A study by Cosgrove (2006) established an association between AMR in *Staphylococcus aureus*, *enterococci*, and gram-negative *bacilli* with an increase in

patient mortality, the length of hospitalisation, and the associated costs of health care. This shows the effect of AMR on both health and the economy. The human mortality rate due to AMR in the European Union/European Economic area in 2019 was estimated to be around 33,000 people per year, and 1,27 million per year globally, thus posing a threat of further mortalities in the future if unresolved (Cassini et al., 2019). This high impact of AMR is estimated at 1.5 billion Euros costs annually related to losses in production and in health care (European Commission, 2022a).

The presence of pharmaceutical pollutants in the environment also pose threats to food production. The adsorption and accumulation of the PhCs onto the treatment sludge poses a risk to food safety as in many instances there is a reuse of treated sewage and sludge for agricultural purposes as biofertilisers and upcycling of treated wastewater for drinking or domestic use (Vaz-Moreira et al., 2022). Despite their occurrence at low concentrations in the environment or in WWTPs' effluents, the large volumes involved and the uptake of these micropollutants by plants and animals (in particular the aquatic ones) leads to them accumulating up the trophic level, by the "bioaccumulation" phenomenon (Dolliver et al., 2007; Rajapaksha et al., 2014). This may lead to adverse effects if these micropollutants are not effectively removed by treatment systems and prevented from reaching the environment. Despite most of these pollutants degrading easily in water, the constant addition to the aquatic environment at rates beyond that of their degradation may lead to their accumulation and thus be assimilated by aquatic species.

1.2.5. Ecotoxicology of PhCs and Toxicological assays

Most scientific studies on the environmental impacts of chemical substances on aquatic organisms focus mainly on the use of invertebrates and fish (mainly laboratory fresh water models) to establish the toxicities of the said substances (Kock et al., 2023; Pecoraro et al., 2021; Road & Lewis, 1994; J. Rodrigues et al., 2019).

In most studies, the reported toxic concentrations of the pharmaceutical compounds are above the existing environmental concentrations. Albeit seemingly insignificant now, the risks associated with the accumulation of these pollutants at any trophic level may soon be realised, hence the importance of these studies. A study on the impact of ibuprofen on diatom *Phaeodactylum tricornutum* showed that higher concentration at 100-300 µg/L lead to negative effects on the growth of *P. tricornutum* and inhibited the chloroplastic energy transduction in the electron transport chain resulting in lower energy (M. Silva et al., 2020).

Since the residues of the PhCs are usually found in mixtures rather than as individually occurring compounds in the environment and WWTPs, the ecotoxicological assays ought also to include assessments of mixtures on marine life. The concentration addition and independent action concepts have been in use for decades to predict the toxicities of mixtures. Concentration addition is based on the concept that substances applied below their individual 'no effect concentration' (NOEC) would nonetheless contribute to the total effect of their mixture (Cleuvers, 2003). The concept further assumes that the components of the mixture have the same behaviour or mode of action for the combined effect they produce. On the other hand, the dissimilar action concept assumes that the mode of action of each component is different and remains unchanged in the presence of the others (Cleuvers, 2003; Pöch, 1993). The complexity of mixtures which lead to either more or less intense effects on organisms in comparison to their individual effects highlights the importance of exploring the effects caused by pharmaceutical pollutants in mixture assays in addition to studying them on their individual level.

The OECD has established standardised methods or assays for acute or chronic toxicity of chemicals, mostly on freshwater organisms. The more common established endpoints for these assays include mortality, heart rate, and fecundity (Morgan et al., 2019; OECD, 2004). Arthropod mortality is a commonly used endpoint for acute toxicity, for instance by using *Artemia sp.* in the second to third instar stage, which are recently hatched nauplii (Nunes et al., 2006). According to the OECD Test Guideline 202 on "*Daphnia sp.*, Acute Immobilisation Test" adopted in 2004 (OECD, 2004), the validity of the test is based on a set of test performance criteria. These demand that in the negative control and the control containing the solvent/solubility agent, the immobilisation of the nauplii should not exceed 10% of the total nauplii in a particular assay. This includes not showing signs of disease or stress by the nauplii such as unusual behaviour and or discolouration. Furthermore, the dissolved oxygen concentration at the end of the test should be more or equal to 3 mg/L in the control and in the test vessel. These guidelines apply to *Daphnia sp.* (freshwater) and may also apply to other crustaceans such as *Artemia sp.* (marine). In addition to being applied as a validity criteria, the negative control is used for ensuring that the mortality observed at the different pollutant concentrations are not due to starvation since the tests are performed without feeding of the test animals (Carballo et al., 2002). During the assay length (48 h), the nauplii are believed to be feeding from the yolk-sacs from which they were hatched (Pelka et al., 2000), and they have been reported to survive beyond 48 hours without supply of food (Otang et al., 2013).

Artemia is a genus of primitive crustaceans found in salty aquatic environments and is also known as brine shrimp. These organisms have a high capacity for osmoregulation thereby enabling them to tolerate and live over a wide range of salinity (Croghan, 1958). They are easy to culture in the laboratory and maintain and can be hatched in artificial saltwater to mimic natural saltwater (Pecoraro et al., 2021). The artificial saltwater differs from the natural sea or lake water in that it has less diversity of microorganisms, less chemical contaminants besides the standard ones used as typical saltwater constituents, as well as a regulated pH, maintaining the salt composition of the natural sea water. Is is also easier to reproduce the conditions of an artificial saltwater in the laboratory and optimise the experimental conditions in controlled assays, such as those testing the exposure to a given pollutant (Ignoto et al., 2022).

The predetermined lethal toxicity of these chemicals on marine or freshwater organisms can aid in the use of these organisms as bioindicators of micropollutants impacts. Their short life cycle, tolerance for a wide range of salinity and temperatures and their abundance in marine water makes *Artemia sp.* a good model for use as bioindicators of marine micropollution, in ecotoxicity assays (Alishahi & Tulaby Dezfuly, 2019; Morgan et al., 2019). The commonality of *Artemia sp.* and being a well-studied organisms allows for better understanding of their reaction to the environmental stresses and provide measurable response as Holt and Miller, (2011) describe qualities of a good bioindicator, with the added advantage of being representative of the marine environment, contrary to *Daphnia magna*.

The OECD guidelines state that the test be carried out with test animals not more than 24 hours old for reduction of variability amongst the brood. The stock they are selected from should be healthy with no signs of stress and high mortality of the brood, or the discolouration of the animals, and in particular, they should be of the same culture origin. The culture conditions of these test animals should maintained and kept similar to the test conditions (OECD, 2004).

In a case where the toxicity of the test substance is unknown or unavailable, a range-finding test is performed for the determination of the concentration range required for the definitive test. The test animals are exposed to widely spaced test substance concentrations for a 48 hours period or less depending on the quality of the data obtained (Alishahi & Tulaby Dezfuly, 2019; OECD, 2004). Information from the range-finding test should aid in establishing the highest concentration, which preferably should result in 100% immobilisation, and the lowest concentration showing no observable effect on the test animals.

The lethal concentration of the substance that kills half of the population, $LC_{50,}$ is inversely proportional to the toxicity of the substance. The system for the classification of substances as 'hazardous to the aquatic environment' adopted by the European Commission (2008) for crustacea considers the substances hazardous according to the following criteria:

Table 1. 2: Classification categories for substances hazardous to aquatic crustaceans

Acute (short-term) aquatic hazard: Acute Category 1	**Chronic (long-term) aquatic hazard: Chronic Category 1**	**Chronic Category 2**	**Chronic Category 3**	**Chronic Category 4:**
≤ 1 mg/L	≤ 1 mg/L	> 1 to ≤ 10 mg/L	> 10 to ≤ 100 mg/L	Safety net classification*

*Cases when data do not allow classification under former criteria, whereas there are nonetheless common grounds for concern such as poor solubility of the substance and potential to bioaccumulate. This includes, for example, poorly soluble substances for which no acute toxicity is recorded at levels up to the water solubility.

Together with the use of bioindicators such as *Artemia sp.*, targeted and quantitative methods of testing such as chromatography hyphenated with mass spectrometry can be utilised to monitor the levels of pollutants. These would work as complementary tests to confirm the level of pollutants in the environments.

2. Materials and Methods

2.1. Sample Collection and Preservation

The monitoring study was carried out at two WWTPs and proximate environmental waters in the Ria Formosa Natural Park, in the Algarve region, Portugal.

The FO-WWTP is located between the cities of Faro and Olhão, and receives wastewater from a public hospital (Centro Hospitalar Universitário do Algarve, CHUA, Faro) and a highly populated residential area of 113 200 people (in population equivalents) from parts of Faro, Olhão, São Brás de Alportel and surrounding areas (Águas do Algarve, 2023). The FO-WWTP is designed to receive up to 28 149 L per day of raw sewage, and uses the patented Nereda AGS system for biological treatment, in two bioreactors. Since this new WWTP substituted an older, smaller WWTP using lagoon treatment, the inactivated lagoon was maintained and serves as a buffer, receiving the treated effluents before they are discharged into the Ria Formosa.

Three samples were taken in this WWTP, the influent raw sewage fed into the WWTP (designated as "Inf."), the treated effluent from the WWTP ("Eff.1") and the treated effluent from the buffering lagoon (Eff.2). Both Inf. and Eff.1 were representative samples of hourly samples collected over 24 hours, using an automatic sampler, while Eff.2 was a single point sample collected on the sampling day. Additional exploratory samples from the sediment of the lagoon was taken in summer 2022, to investigate the presence and/or accumulation of PhCs in sediments, using a beaker placed at the end of an extensible stick. Finally, "Ria-Formosa" water samples were collected from one of the lagoon system channels, located close to the discharge point from the buffering lagoon of the FO-WWTP. One grab sample was taken about 30 metres from the release point of treated water from the lagoon into the environment, using a beaker at the end of an extensible stick (a commonly used collection tool at the WWTP).

The FNW-WWTP is located near Faro airport, treating urban waste water from the airport (that receives approximately 8 million visitors annually mainly from Europe, US and Canada), a small private hospital and 44 530 people from urban/countryside populations around Faro and Loulé (Águas do Algarve, 2023), treating up to 13 221 L per day of raw wastewater. Samples were taken from the influent (Inf.), raw sewage fed into the WWTP, and from the treated effluent from the WWTP (Eff.1) into the channel and ocean, both samples were 24h-composite samples of hour fractions, using an automatic sampler (Águas de Portugal).

In collaboration with the team of regular samplings from Águas do Algarve (AdA, group "Águas do Algarve"), the CCMAR researchers collected the raw sewage, effluents or

environmental samples above specified into clean 1 L amber glass bottles, which were stored in ice-cooled sample transport boxes. These samples were then sent by AdA for delivery in Lisboa at EPAL (Empresa Portuguesa das Águas Livres SA), using the internal transport of the Águas de Portugal group to which both companies belong. Additional samples of 250 ml were transferred to autoclaved flasks and transferred to CCMAR and stored for later metagenomics and microplastics investigations (not part of this thesis). At EPAL, the 1 L samples were immediately filtered and stored refrigerated (5 ± 3 ℃) for LC-MS/MS analysis within 7 days of receipt (Refer to 2.3.1 for sample preparation details). The sampling was carried out in summer (June-July) and winter (Jan-Mar) over four years of monitoring, 2021-2024.

2.2. Reagents and Materials

2.2.1. LC-MS/MS

All the used solvents (methanol) and standards of the PhCs were of analytical grade (highest purity available, ≥ 95%) meeting the British Pharmacopoeia and/or the United States Pharmacopoeia specifications, thus suitable for chromatography analysis. The methanol (J.T. Baker, Denver, Netherlands) was of LC/MS grade, acetone and acetonitrile (Fisher Scientific, Leicestershire, UK) of HPLC grade, formic acid of LC grade and 97%, p.a. ammonium acetate (Merck, Darmstadt, Germany). The following pharmaceutical standards were used for method development: acetaminophen, atenolol, bezafibrate, carbamazepine, clofibric acid, cortisone, diclofenac, erythromycin, fluoxetine, ibuprofen, naproxen, propranolol, sulfadiazine and sulfapyridine (Sigma-Aldrich, Spain); caffeine, sulfamethoxazole and testosterone (Fluka, Spain); azithromycin and clarithromycin (European Pharmacopoeia Reference Standard, France); gestodene and metoprolol (LGC, Spain); 17-α-ethinylestradiol, diethylstilbestrol, estradiol, estriol and estrone (Dr. Ehrenstorfer, GmbH, Germany).

Individual stock solutions were prepared in methanol for each compound and a joint standard solution was prepared by diluting each individual standard solution in methanol to have a concentration of 2 mg/L for ibuprofen, 6 mg/L for the hormones and 1 mg/L for the rest of the compounds. All working solutions for the calibration and standard addition curves were prepared in adjusted matrix (wastewater) to compensate for matrix effects that may influence the mass spectrometry results.

2.2.2. Toxicity Assays

Analytical grade solvent Methanol (>95%) and ultra-purified water produced using Milli-Q EQ 7000 (Merck, Germany) were used for solvent preparations. Analytical grade PhC standards

carbamazepine (Sigma-Aldrich, Spain), caffeine (Merck, Germany), and diclofenac (Cayman chemical Company, USA) were used in toxicity assays. The Motic SMZ-171 (Motic, China) stereo microscope was used for viewing and counting test organisms. *Artemia sp.* (Inve Technologies, Belgium) was used as test organism.

2.3. Analysis of Pharmaceuticals: Solid Phase Extraction – Liquid Chromatography – Tandem Mass Spectroscopy (SPE-LC-MS/MS)

The analysis of the wastewater samples was carried out by an external laboratory, Empresa Portuguesa das Águas Livres, S.A. – EPAL, using the optimised and previously validated SPE-LC-MS/MS methods adapted from Gaffney et al. (2017, 2014) & Silva et al. (2021).

2.3.1. Sample Pre-treatment

The collected samples (500 mL) were filtered in a three-step filtration process. The first filtration step was carried out using gravity filtration on a 5-13 µm pore filter paper (VWR, Germany). The second and the third filtrations were performed using vacuum filtration on a 1.2 µm pore nylon filter paper (VWR, Germany) followed by the 0.45 µm pore glass fibre filter (Cytiva, USA). Next, the solid-phase extraction (SPE) technique using Oasis HLB (200 mg, 6 mL) cartridges on an automated AutoTrace[a] SPE workstation (Thermo Scientific) was employed on each sample for the purification and pre-concentration of the target analytes. The cartridges were conditioned with 8 mL of methanol and 8 mL of ultra-pure water, and then a 50 mL sample of the wastewater sample was passed through at a 5 mL/min flow rate. Cartridges were rinsed at the same flow rate with 5 mL ultra-pure water and dried with a nitrogen stream for 15 min. The target analytes were eluted with a two-step elution with 4 mL methanol per elution at 2 mL/min flowrate. The extract was concentrated to dryness by evaporating under a stream of nitrogen (5 psi, 35℃) using an evaporator (Biotage TurboVap®, Sweden).

2.3.2. Liquid Chromatography – tandem Mass Spectrometry (LC-MS/MS)

The analysis of the pre-concentrated PhCs was carried out using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) pre-validated in terms of linearity, sensitivity, precision, and accuracy. The standard addition quantification method was used on the Dionex Ultimate 3000 system with an automatic injector, binary pump, and a thermostated chromatographic column compartment coupled to a triple quadrupole TSQ Endura mass spectrometer (Thermo Scientific, USA). A Kinetex EVO C18 column (2.1 x 50 mm, 2.6 µm) from Phenomenex (USA) was employed for chromatographic separation.

The optimised and validated acidic and basic chromatographic methods were employed for the selected PhCs. A gradient elution was carried out using 95% solvent A (water + 0.01 mM ammonium acetate + 0.5% (v/v) formic acid), followed by a linear decrease to 50% of solvent A until 3.0 min, 30% until 5.5 min, and 10% until 8.0 min (held 2.0 min) with solvent B (methanol) for the acidic method. An increase of solvent A to 95% was performed in 1.0 min (held 3.0 min) to re-equilibrate the system. For the basic method, the gradient elution was performed with 70% of solvent C (water + 0.05% ammonia at 25% (v/v)), followed by a decrease to 30% of solvent C until 3.0 min, and 10% until 5.0 min (held 2.0 min) along with solvent B. An increase of solvent C to 70% was performed in 1.0 min (held 3.0 min) to re-equilibrate the system.

The mass spectrometer was operated in positive and negative modes, specific for each compound using standardized methods, with electron spray ionisation (ESI). Triple quadrupole operating conditions were optimised to work in multiple reaction monitoring (MRM) mode with the optimisation based on the selection of ionisation mode, optimum collision energy (eV), ion transfer tube and vaporizer temperatures (°C) and accurate radio frequency (RF) lens for each compound. Validation studies for the SPE-LC-MS/MS are reported in Silva et al. (2021).

The chromatography retention times for each drug, their precursor ion masses, the product ions corresponding to the quantification transition (MRM1) and qualification transition (MRM2) and their respective collision energies are presented in Table 2. 1, as well as the limit of quantification, to demonstrate the selectivity and sensitivity of the LC-MS/MS method for each compound.

Table 2. 1: Retention times of PhC chromatographic peaks, and precursor ion masses and collision energies applied to the formation of product ions for quantification and qualification.

PhC	Retention Time (Min)	Ionization Mode	Transition MRM Precursor ion - Product ion	Limit of quantification (µg/L)	Collision Energy (eV)
Acetaminophen	0.2 - 3.5 (0.9)	ES+	MRM1, 152.3 to 110.1	0.04	18
			MRM2, 152.3 to 93.2		25
Carbamazepine	6.0 - 10 (7.3)	ES+	MRM1, 273.1 to 194.0	0.04	20
			MRM2, 273.1 to 191.9		23
Azithromycin	4.0 - 9.0 (?)	E+	MRM1, 749.4 to 573.3	0.07	32.2
			MRM2, 749.4 to 591.4		27
Clarithromycin	4.0 - 9.0 (?)	ES+	MRM1, 748.4 to 157.9	0.07	26.3
			MRM2, 748.4 to 590.1		17.6
Erythromycin	4.0 - 8.0 (5.3)	ES+	MRM1, 716.4 to 558.3	0.07	16.3
			MRM2, 716.4 to 158.1		27.7
Sulfadiazine		ES+	MRM1, 251.1 to 156.0	0.04	15

	0.2 - 3.5 (1.0)		MRM2, 251.1 to 92.1		25
Sulfamethoxazole	3.0 - 7.0 (4.6)	ES+	MRM1, 254.0 to 156.0	0.04	15
			MRM2, 254.0 to 92.1		25
Sulfapyridine	0.4 - 4.0 (1.3)	ES+	MRM1, 250.1 to 156.0	0.04	15
			MRM2, 250.1 to 92.1		15
Fluoxetine	4.5 - 9.0 (5.6)	ES-	MRM1, 310.1 to 148.1	0.07	10
			MRM2, 310.1 to 44.5		10
Clofibric acid	7.0 - 11 (8.0)	ES-	MRM1, 213.1 to 127.0	0.2	15
			MRM2, 213.1 to 85.1		10.2
Bezafibrate	0.3 - 3.5 (0.5)	ES-	MRM1, 360.2 to 274.0	0.03	16
			MRM2, 360.2 to 154.0		28
Atenolol	0.2 - 3.5 (0.6)	ES+	MRM1, 267.1 to 190.1	0.04	18
			MRM2, 267.1 to 145.1		25
Metoprolol	4.0 - 8.0 (4.8)	ES+	MRM1, 268.2 to 116.2	0.04	18
			MRM2, 268.2 to 98.1		20
Propanolol	4.0 - 8.5 (5.9)	ES+	MRM1, 260.2 to 183.1	0.04	18
			MRM2, 260.2 to 116.2		18
Diclofenac	0.3 - 4.0	ES+	MRM1, 296.0 to 215.0	0.035	20
			MRM2, 296.0 to 250.0		16
Ibuprofen	0.2 - 4.0 (0.5)	ES-	MRM1, 205.2 to 161.1	0.07	10.2
			MRM2, -		-
Naproxen	7.0 - 11 (8.1)	ES+	MRM1, 231.1 to 185.1	0.035	13
			MRM2, 231.1 to 170.1		25
Caffeine	3.0 - 7.0 (4.6)	ES+	MRM1, 195.1 to 138.1	0.035	20
			MRM2, 195.1 to 110.2		23
Cortisone	6.0 - 10 (7.3)	ES+	MRM1, 361.2 to 163.1	0.04	23
			MRM2, 361.2 to 121.1		30
Testosterone	7.0 - 12 (8.5)	ES+	MRM1, 289.2 to 97.1	0.03	20
			MRM2, 289.2 to 109.1		25
17β-Estradiol	2.5 - 6.5 (4.1)	ES-	MRM1, 27.1 to 183.0	0.4	40.4
			MRM2, 27.1 to 145.0		40.8
Estriol	1.5 - 5.0 (2.2)	ES-	MRM1, 287.2 to 171.0	0.35	36.7
			MRM2, 287.2 TO 145.1		41.9
Estrone	25 -6.5 (4.0)	ES-	MRM1, 269.2 to 145.0	0.5	384
			MRM2, 269.2 to 143.0		53.7
17α-Ethinylestradiol	3.0 - 7.0 (4.2)	ES-	MRM1, 295.2 to 267	0.4	26.2
			MRM2, 295.2 to 159.1		34.3
Diethylstilbestrol	3.0 - 7.0 (4.3)	ES-	MRM1, 267.1 to 251.0	0.4	26
			MRM2, 267.1 to 237.0		28.6
Gestodene	7.0 - 11.5 (8.4)	ES+	MRM1, 311.2 to 109.1	0.04	25
			MRM2, 311.2 to 83.1		28

2.3.2.1. Removal Efficiency calculations

The efficiency of the WWTP and the lagoon to remove the PhCs from the water samples was estimated using Eq. 2 , assuming the flow rates of the influent and the effluent were constant. This was estimated by subtracting the effluent concentration (C_{inf}) from the influent concentration (C_{inf}), dividing by C_{inf} and expressed in percentage.

$$\text{Removal Efficiency (\%)} = \frac{C_{inf} - C_{eff}}{C_{inf}} \times 100$$

Eq. 2: (Gaffney et al., 2017)

2.4. Toxicity Assays

2.4.1. Acute toxicity of Carbamazepine and Coumestrol on *Artemia sp.*

The *Artemia sp.* based lethality assay (brine shrimp lethality assay, BSLA) is a rapid, convenient, and low cost toxicity assay. The test was carried out following specifications by the OECD Test Guideline 202 on "*Daphnia sp.*, Acute Immobility Test" (OECD, 2004).

The culture medium and test substance diluent used were natural seawater and artificial seawater, that were used in separate assays. The tests were carried out in triplicate for repeatability purposes and two controls were used to ensure the validity of the tests: seawater (artificial/natural depending on the assay) as a negative control and 1% (v/v) methanol in seawater as a solvent control.

Seawater collected from Faro offshore (maintained in the CCMAR "Leoa" marine facility) was adjusted with tap water to obtain 30 parts per thousand salinity, following commercial *Artemia* manufacturer's instructions. The artificial seawater was prepared according to method by Rojo-nieto et al. (2012). The preparation was carried out by dissolving NaF (3 mg/L), $SrCl_2$ x 6H2O (20 mg/L), H_3BO_3 (30 mg/L), KBr (100 mg/L), KCl (700 mg/L), $CaCl_2 \times 2H_2O$ (1470 mg/L), Na_2SO_4 (4000 mg/L), $MgCl_2 \times 6H_2O$ (10780 mg/L), NaCl (23500 mg/L), Na_2SiO_3 x $9H_2O$ (20 mg/L) and $NaHCO_3$ (200 mg/L) in MilliQ water.

The test compounds evaluated in the assay were an anticonvulsant carbamazepine (CBZ) and a phytoestrogen coumestrol (CMT) at a range of concentrations 5, 25, 50, 75, 100, and 1000 mg/L, with the exception of 1000 mg/L for coumestrol due to limited compound availability.

First tests at concentration ranging 25, 50, 75, and 100 mg/L were carried out to establish the effective doses ranges. The the highest concentration (100 mg/L), was also used as a limit test to establish whether at this concentration a complete immobilisation was achieved. For a test

substance that does not produce complete immobilisation at 100 mg/L, a complete test with the maximum of 1000 mg/L concentration was be used.

With reference to Rojo-nieto et al. (2012) on, "recreating the seawater mixture composition of hydrophobic organic compounds (HOCs) tests with *Artemia franciscana* by passive dosing", the determined toxicity of methanol concentration on *Artemia franciscana*, 2.5% methanol does not exert significant mortality. In this study, methanol concentrations in all test solutions were kept below 1% (v/v).

The *Artemia sp.* cysts (1 g) were hatched in 1 L of seawater (natural/artificial) at an incubation temperature of 30℃ with a constant supply of air and lighting for 24 hours, according to the supplier's recommended procedures (INVE Technologies, Belgium). After 24 hours, the magnetised cyst shells and unhatched cysts were removed using a magnet and the *Artemia sp.* nauplii filtered from the culture medium using a 150 µm sieve. The new brood was acclimatised in fresh 2 L of natural seawater to room temperature, 25±2℃, in darkness for a further 12 hours to aid in their transition to developmental stages instar II and III from instar I. The toxicity assays were performed using *Artemia sp.* nauplii 48 hours post hatching, which were at instar II and III, which are the stages more sensitive to toxins/pollutants (Personne et al., 1980; Road & Lewis, 1994). The values of pH and temperature of the natural seawater were measured before each assay to confirm the defined temperature (30℃) and salinity (30‰) were maintained.

To isolate the 48 hours old *Artemia sp.* nauplii, a 15 mL Pasteur/transfer pipette was used to dispense the seawater with *Artemia* from the batch and placed in drops onto a large glass Petri dish, under the DM9 digital microscope (Shenzen Mai Kelong Technology Co.Ltd, China). Before transferring the nauplii, triplicate 5 mL glass Petri dishes had been prepared for each experimental group (compound and concentration, defined above) by diluting the stock solutions in the seawater. The desired number of nauplii (10) were counted and added to the assay vessels, covered with lids to prevent evaporation and kept at room temperature in the dark. At 24 hours and 48 hours, the immobilisation of *Artemia sp.* was assessed at each compound concentration and for the controls using a SMZ-171 (Motic, China) zoom stereo microscope (10X magnification). Mortality was adopted as the end-point for toxicity evaluations and considered to happen if immobilisation of the test organisms was observed for 10 continuous seconds, after gentle shaking.

The percentage immobilisation/mortality was calculated as follows:

$$\%\ Immobilisation = \frac{number\ of\ immobilised\ test\ organisms}{total\ number\ of\ test\ organisms} \times 100$$

Eq. 3: (Pecoraro et al., 2021)

During the counting process, the movement of live/mobile nauplii was observed and recorded. The remaining unused *Artemia sp.* nauplii were recovered from the culture medium on a 150 µm sieve, air-dried, and stored in a storage tube for other experimental purposes. One immobilization experiment was carried out to define optimal test doses for compounds CBZ and CMT in natural or in artificial sea water (25-100 mg/L). The experiments were then repeated with each compound independently tested in natural and artificial seawater between 5-1000 mg/L (precise concentrations of each assay presented in the results section).

2.4.2. The combined effect of the pharmaceutical compounds on *Artemia sp.*

Here, the combined effect of three PhCs, caffeine (CAF), carbamazepine (CBZ), and diclofenac (DIC) selected from the monitoring list results was performed. The PhCs were applied at influent and effluent concentrations that were based on the summer 2023 FO-WWTP samples (CAF 84 and 3.2 µg/L, CBZ 0.5 and 0.5 µg/L, DIC 1.6 and 1.5 µg/L). The selection was based on the established high occurrence for CAF, and the high recalcitrance of CBZ and DIC, in the WWTP influents and effluents. The preparation of the solutions, the *Artemia sp.* incubations, the test experiments and the data analysis were carried out as in the previous section (2.4.1) on the acute toxicity of single compounds. However, in this case a conjoined solution of CAF, CBZ, and DIC was also prepared instead of only single compounds. The tests were carried out at concentrations simulating the actual FO-WWTP influent and effluent concentrations detected in the FO-WWTP and FNW-WWTP monitoring study results for winter 2023. The effect of the combined PhCs' solutions were compared against the effect of each individual test substance, thus, the functional assay was comprised of the individual test substances and their mixture in influent concentrations and the individual test substances and their mixtures in the effluent concentrations.

2.4.3. Effect of wastewater on *Artemia sp.*

A preliminary experiment was carried out to evaluate the effects of the real wastewater samples from the FO-WWTP's influent and effluent (raw and treated wastewater, respectively) on *Artemia sp.*, using the immobilisation assay. This was carried out using the non-diluted samples, their ten-fold dilution and their hundred-fold dilution. The immobilisation assay was carried out as described in the acute toxicity assay above (see section 2.4.1), in triplicates at all dilutions.

The assay served to investigate the effect of the wastewater pollutants on the *Artemia sp.* nauplii in real wastewater samples, the effects of the wastewater treatment on the toxicity of the wastewater and whether the treatment can reduce pollutants (or other components) toxicity. The success of this assay would later be used as a presumptive test for the test of efficiency of the wastewater treatment to expedite long waiting periods for confirmatory analytical tests on the quantity of the PhCs in the wastewater, but also the global effects of the whole wastewater or effluent in case of exposure to marine organisms.

2.4.4. Immobilisation Recovery of *Artemia sp.*

The immobilisation recovery test was meant to investigate whether the immobilisation effect of CBZ on *Artemia sp.* is temporary (reversible) or permanent (irreversible). This was achieved by exposing the test organisms to the test compound CBZ and removing it after 12 hours. Following the procedure of the *Artemia* immobilisation assay in section 2.4.1, the *Artemia sp.* nauplii were introduced into two 50 mL solutions of 50 and 100 µg/L concentrations CBZ in beakers overlaid with a 150 µm sieves. After 24 hours at room temperature in the dark, the nauplii's behaviour was observed. The nauplii were then removed from the test substance, rinsed with and placed in fresh non-polluted artificial seawater. Their immobilization behaviour was later observed (as above) after an hour of rinsing and after 24 hours.

2.5. Data Treatment

2.5.1. LC-MS/MS

GraphPad Prism 8.0.2 was employed for processing PhCs' occurrence profiles, their comparable concentrations, and for plotting the removal efficiency data.

2.4.1. *Artemia sp.* Toxicty Assays

The toxicities of the test substance was estimated and expressed in the form of LC_{50} values using the Quest GraphTM LC_{50} Calculator based on the Hill equation non-linear regressions and correlation graph between the concentrations of the substance and the lethality/immobilisation (%) (AAT Bioquest Inc., 2023). The graphs were constructed with the mean values of the replicate tests and reported with the standard error of the mean.

3. Results and Discussion

The study was carried out to monitor the seasonal occurrences of the pharmaceutical pollutants in the influent and effluent wastewater of two WWTPs in the Algarve region (inside the Ria Formosa natural park) as well as their removal efficiencies. The discussion is based on the frequencies of occurrence and the concentrations of the pharmaceutical compounds. It further focuses on the removal efficiencies of the WWTPs, the remediation effect of the lagoon (Effluent 2 concentration comparing to Effluent 1 concentration) as well as a comparison between the two treatment plants. Additionally, the study explores the impact of these wastewater pollutants on marine invertebrates, *Artemia sp.*, using the *Artemia* immobilisation assays.

3.1. Occurrence and trends of pharmaceutical compounds in wastewater treatment plant influents

The occurrence of the 26 pharmaceutical pollutants in the FO and FNW-WWTPs was investigated over the summer and winter seasons from 2021 to 2024. The monitoring study indicates the presence at detectable levels of 17 pollutants from which the following were present in all the monitoring seasons: the analgesic acetaminophen, the anticonvulsant carbamazepine, the antibiotics azithromycin, clarithromycin, erythromycin, sulfamethoxazole, and sulfapyridine, the anti-dyslipidemic bezafibrate, the beta-blockers atenolol and metoprolol, the NSAIDs diclofenac and naproxen, as well as the psychostimulant caffeine.

Amongst these, in agreement with prior studies in Portuguese and international WWTPs (Gaffney et al., 2017; Paíga et al., 2019; C. Silva et al., 2021; Ternes, 1998), the compounds that had higher concentrations in the influent (raw sewage) along the seasons (between 2021 and 2024) were caffeine and acetaminophen. They were detected in the concentration ranges of 46-84 µg/L and 18-79 µg/L, respectively (Figure 3.1). Winter 2023 showed the highest occurrence concentrations for both compounds in the FO-WWTP. The same was observed for the FNW-WWTP, where the concentration ranges were 47-80 µg/L and 31-74 µg/L for caffeine and acetaminophen, respectively Figure 3.2. Their presence in high concentrations could be due to their consumption, excretion and/or disposal in higher amounts compared to other compounds prescribed and found in low concentrations. The general recommended intake of acetaminophen by an adult per day is 4000 mg, that is 1000 g dose four times per day every 4-6 hours (Bunchorntavakul & Reddy, 2013), and the average coffee intake per day per person in Portugal is estimated to be 380 mg (Batista et al., 2022), thus explaining their occurrence in high concentrations in the wastewater.

In the FO-WWTP influent (with more sampling times, thus allowing temporal analysis; Figure 3.1) the caffeine concentrations showed a slight rise from winter 2021 (46 µg/L) to winter 2023 (84 µg/L), but no apparent difference between summer and winter, with a slight drop to a stable concentration (~64 µg/L) in the last two sampling campaigns in FO-WWTP.

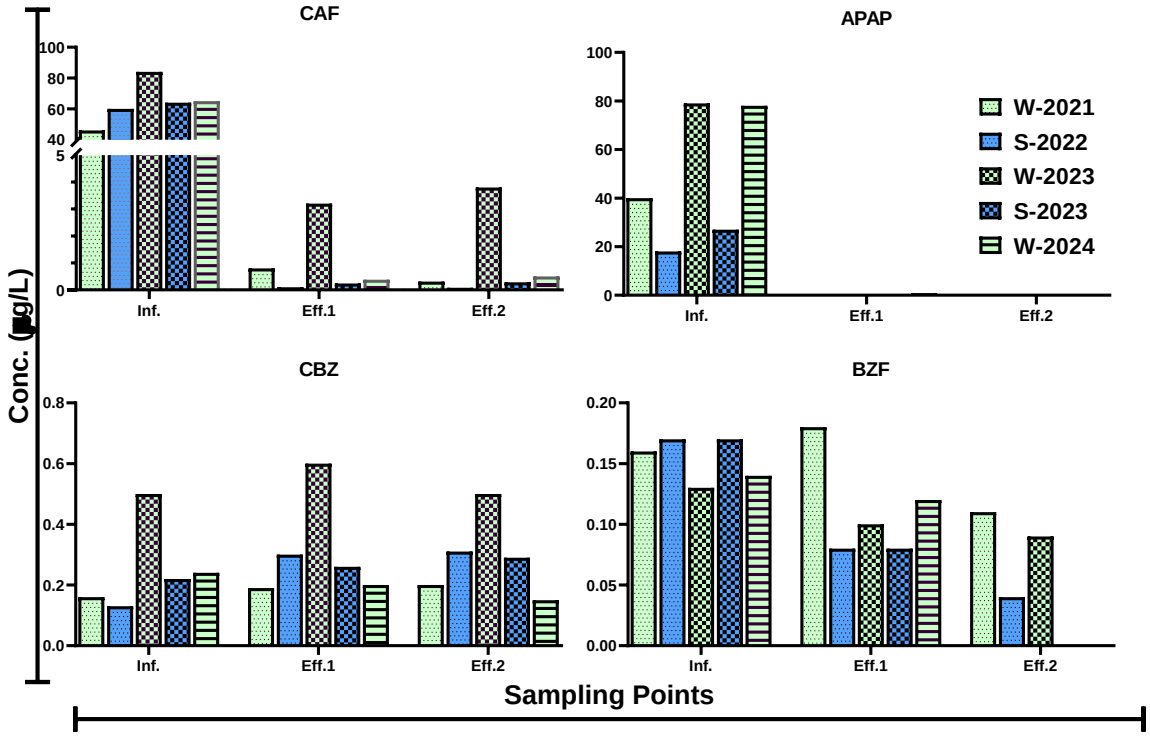

Figure 3.1: Occurrence concentrations of caffeine (CAF), acetaminophen (APAP), carbamezapine (CBZ), and bezafibrate (BZF) in the Faro-Olhão wastewater treatment plant, across seasons from 2021 to 2024 (S= summer; W= winter). Each point represents the detection by LC-MS/MS (µg/L) in extracts from the raw sewage or influent (Inf., composite sample representing 24h), the treated effluent discharged to the lagoon (Eff.1, composite sample of 24h) and the final treated effluent discharged from the lagoon to the Ria Formosa environment (Eff.2, single sample), (n = 1).

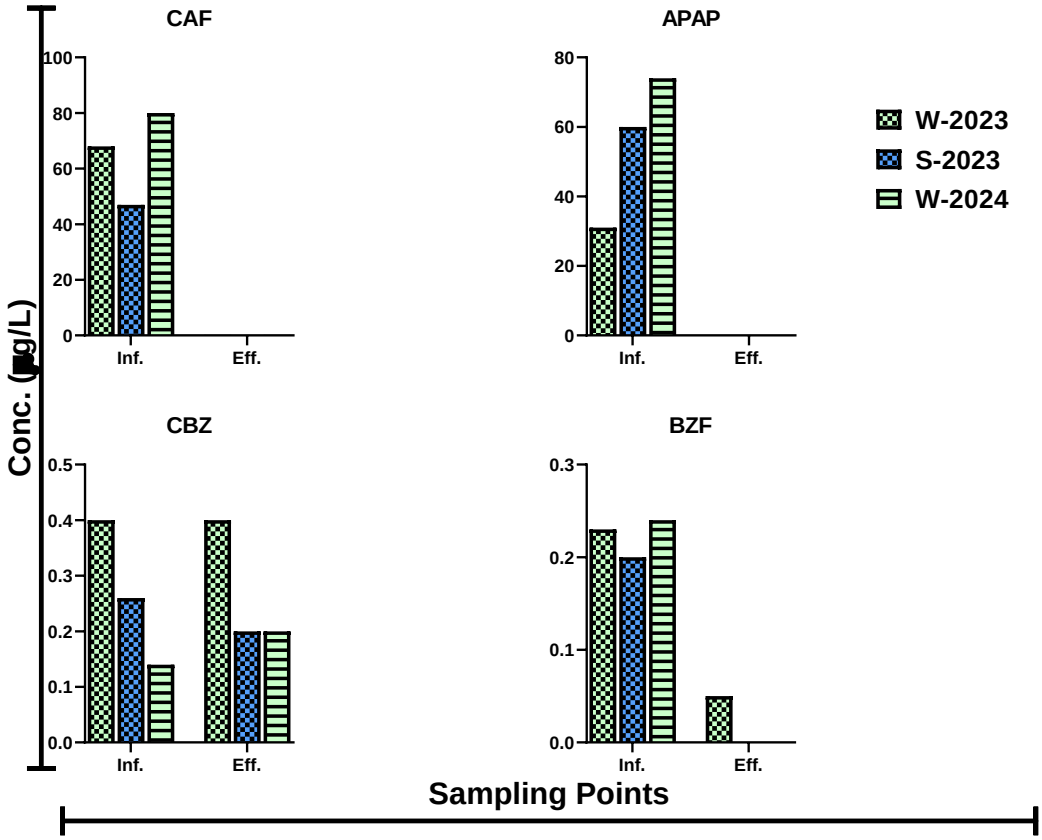

Figure 3.2: Occurrence concentrations of caffeine (CAF), acetaminophen (APAP), carbamazepine (CBZ), and bezafibrate (BZF) in the Faro-Noroeste wastewater treatment plant (FNW-WWTP), in the winter (W) and summer (S) of 2023 and winter of 2024. Each point represents the detection by LC-MS/MS (in µg/L) in extracts from the raw sewage or influent or from the treated effluent discharged to the environment (Eff.1), both of them sampled in composite samples representing 24h, (n = 1).

When comparing in the same graph both WWTP results for the common sampling campaigns (Figure 3.3, winter 2023 to winter 2024) slightly higher levels in the winter compared to summer were detected, with the highest caffeine influent concentrations detected at 80 µg/L, though more sampling campaigns would aid in confirming the trend, while in the treated effluents the levels were very low.

In the Ria-Formosa environmental water samples collected over the same period, caffeine levels up to 0.5 µg/L were detected (Figure 3.3), which together with diclofenac (Figure 3.4) were the only two compounds found at detectable levels in the Ria Formosa waters. This could be due to their high stability leading to persistence in the environment (Gonçalves et al., 2014).

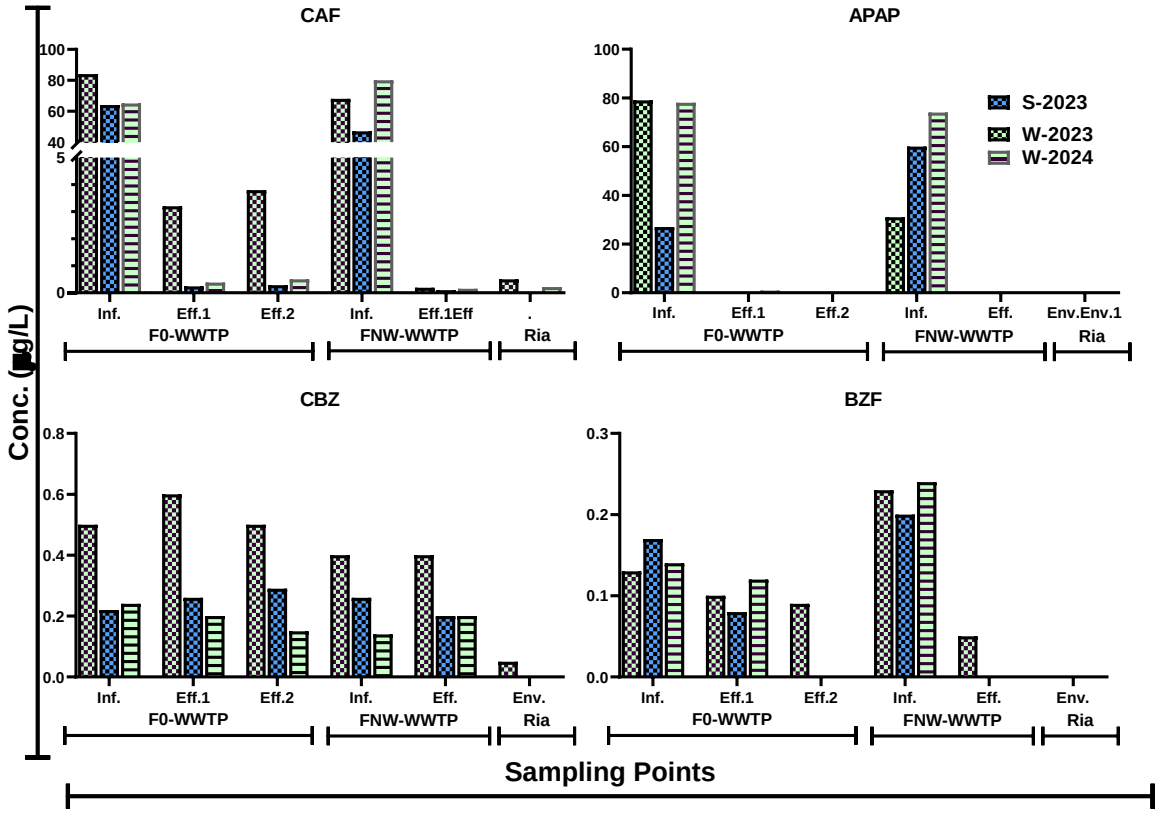

Figure 3.3: Comparison of occurrence concentrations of caffeine (CAF), acetaminophen (APAP), carbamazepine (CBZ), and bezafibrate in Faro-Olhão (FO-WWTP), Faro-Noroeste (FNW-WWTP) and in the environmental waters collected at the Ria Formosa (Ria) for winter (W) or summer (S) samples collected in 2023 and 2024. Each point represents the detection by LC-MS/MS (in µg/L) in extracts from the raw sewage or influents (Inf., composite samples of 24h), the treated effluents (Eff.1, composite sample of 24h) and, in the case of the FO-WWTP, also the final treated effluent discharged from the lagoon to the Ria Formosa (Eff.2, single sample), (n = 1).

Winter being the colder season is usually observed to correlate with the growth in cold and flu infected and affected patients, thereby leading to an increased use of analgesics, antibiotics, and NSAIDs (J. A. Rodrigues et al., 2021). In this study and in the FO-WWTP that had a more extensive sampling period, a sharp increase in the analgesic acetaminophen influent concentrations was observed from winter 2021 (48 µg/L) to winter 2023 (79 µg/L), from which it levelled off until winter 2024 (Figure 3.1). A constant increase was observed in the FNW-WWTP (Figure 3.2) influent from winter 2023 (31 µg/L) to winter 2024 (74 µg/L) with intermediate levels in summer 2023. It appears the pattern of high acetaminophen levels in the winter compared to summer seen in the FO-WWTP were not seen in FNW-WWTP, although fewer sampling times were covered to permit a robust seasonal analysis. The high concentrations of acetaminophen may also be associated with the elevated global use of acetaminophen since the coronavirus disease 2019 (COVID-19) pandemic, with a 198%

increase reported at the beginning of the pandemic from 2019 to 2020 in a study in Greece by (Galani et al., 2020).

Analysing the concentrations of the anticonvulsant carbamazepine in the FO-WWTP influent (Figure 3.1) showed concentrations in the range 0.16-0.5 µg/L, with the winter seasons showing relatively higher concentrations, especially in the winter of 2023, which was twice the next abundant season concentration. In the FNW-WWTP (Figure 3.2), carbamazepine concentrations were found to decline from the first sampling campaign in winter 2023 (0.4 µg/L) to 0.14 µg/L in the last sampling campaign in winter 2024 with no observable impact of seasonality. These findings were found to be in a similar range to the values found in Aveiro WWTPs in Portugal in 2014, where median concentrations of 0.47 µg/L and 0.52 µg/L were found in influent and effluent samples, respectively (Bahlmann et al., 2014). The same study recorded significant amounts of CBZ metabolites (not analysed here), suggesting there can be underestimation of CBZ circulation in the water cycle when metabolites are not covered in the monitoring.

Despite not being detected within the limits of quantification in this study, clofibric acid is a common lipid regulator that has been detected in domestic effluents in the past three decades and is known to be a recalcitrant pharmaceutical substance and is estimated to persist in the environment for up to 21 years (Razavi et al., 2009; Ternes, 1998). Of the two studied lipid regulators, Bezafibrate was the only one successfully quantified in the WWTP effluent of both FO and FNW (Figures 3.1-3.3). Its occurrence was higher in FNW-WWTP influent within narrow ranges of 0.2-0.24 µg/L and 0.13-0.18 µg/L in FO-WWTP over summer and winter depicting no seasonality impacts (Figure 3.3). These concentration levels were slightly lower than those reported by Silva et al. (2021) for the 2016-2019 monitoring study in the Beirolas WWTP (near Lisbon) and the FNW-WWTP, at median concentrations 0.58 µg/L and 0.32 µg/L, respectively. A monitoring study on the occurrence of drugs in German WWTPs and rivers by Ternes (1998) in 1995 showed significantly higher concentrations of up to 4.6 µg/L of bezafibrate and 1.6 µg/L clofibric acid in the sewage. In the FO-WWTP influent (Figure 3.1), slightly higher concentrations were found for bezafibrate in the summers compared to winters, while relatively stable levels were detected in the FNW-WWTP for the 3 time points analysed (Figure 3.2-3.3).

Of the studied NSAIDs, in the influent raw sewage of the FO-WWTP (Figure 3.6), ibuprofen occurred at higher concentrations in the range 9-15 µg/L, which was much higher than the NSAID, naproxen, which occurred at 2.3-8 µg/L.

Diclofenac was found to have the lowest occurrence concentration of the three compounds in the sewage with an average of 1.6-2.2 µg/L. A similar trend was observed in the FNW-WWTP (Figure 3.5) and the occurrence profiles for ibuprofen, naproxen, and diclofenac in that hierarchical order were 14-19 µg/L, 1.8-8 µg/L, and 1.2-1.8 µg/L. Although it was present at low concentrations, presumably due to its resistance to treatment and high stability, diclofenac was still detected in the environmental samples in the Ria-Formosa (Figure 3.6), at 0.059 µg/L in the winter of 2023.

The relatively high abundance of ibuprofen was consistent with the findings of (Monteiro et al., 2016) that showed the highest consumption of NSAID in Portugal to be of ibuprofen.

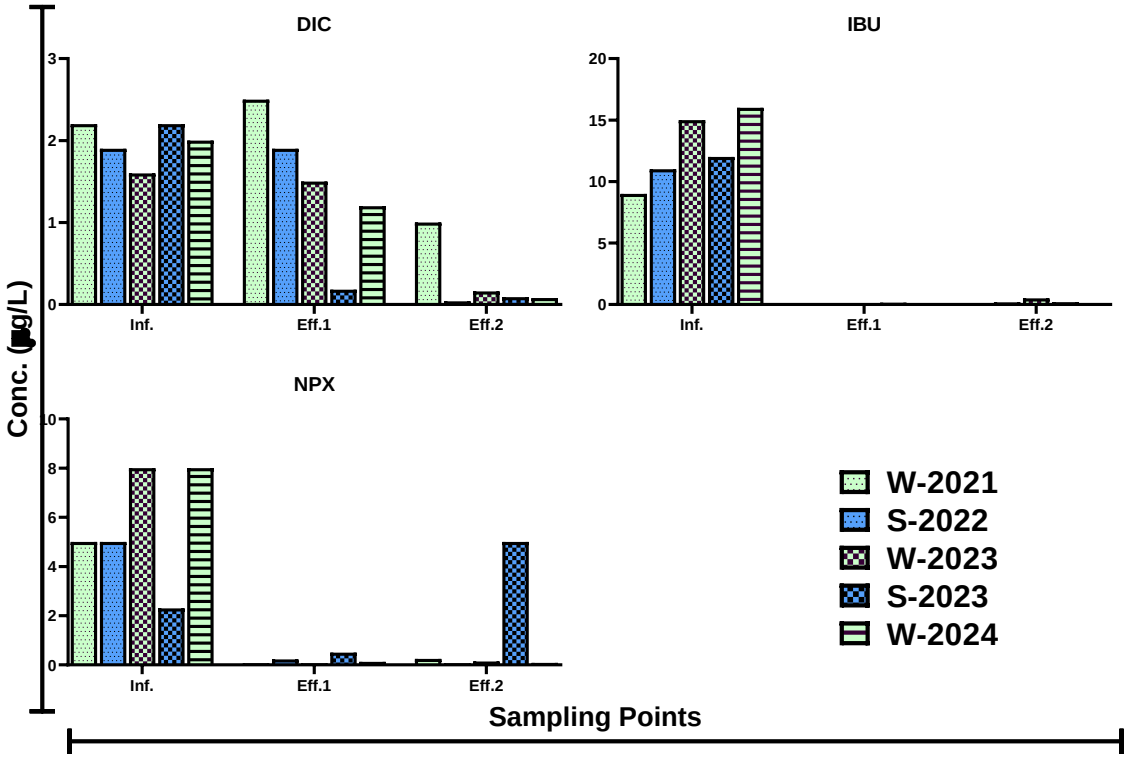

Figure 3.4: Occurrence concentrations of non-steroidal anti-inflammatory drugs (NSAIDs) diclofenac (DIC), ibuprofen (IBU) and naproxen (NPX) in the Faro-Olhão wastewater treatment plant (FO-WWTP), across seasons from 2021 to 2024 (S= summer; W= winter). Each point represents the detection by LC-MS/MS (in µg/L) in extracts from the raw sewage or influent (Inf., composite sample of 24h), the treated effluent discharged to the lagoon (Eff.1, composite sample of 24h) and the final treated effluent discharged from the lagoon to the Ria Formosa environment (Eff.2, single sample), (n = 1).

The popularity of ibuprofen is also reported in the southern hemisphere by (Padayachee, 2021) in the report on "awareness regarding non-steroidal anti-inflammatory drug-related side effects in Johannesburg, South Africa". This is also supported by the number of units sold by the Portuguese National Health System (Monteiro et al. (2016).

Concerning diclofenac, there were no major differences between the concentrations detected across seasons in the FO-WWTP and no observable seasonal pattern in both units (except for a lower value in the summer of 2023), suggesting a similar consumption across the seasons (Figure 3.6). Interestingly, diclofenac was the only NSAID with high levels also detected in the treated effluents of both units, suggesting an incomplete treatment, as discussed below.

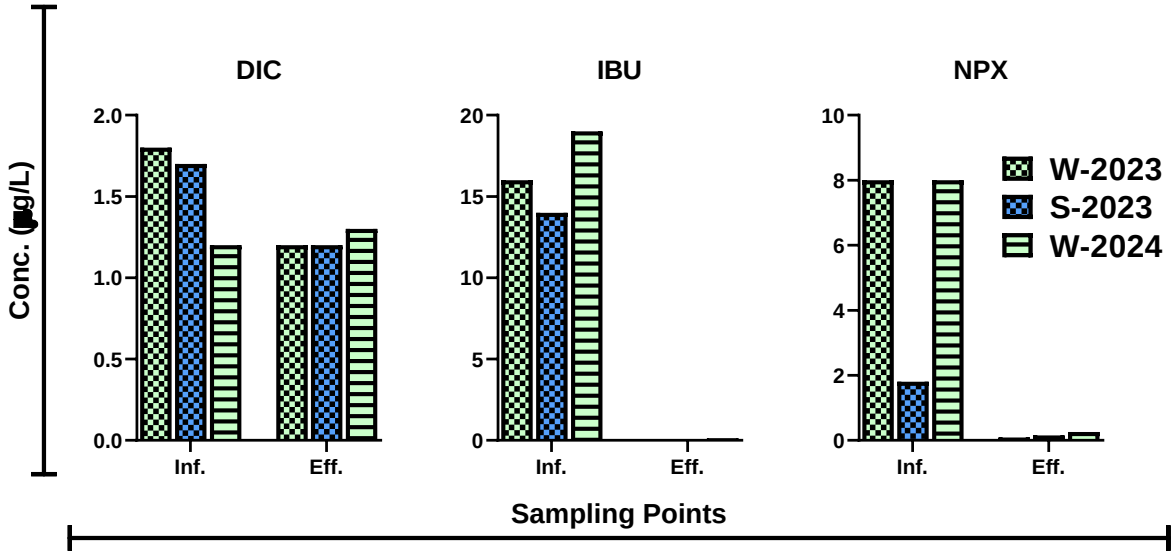

Figure 3.5: Occurrence concentrations of non-steroidal anti-inflammatory drugs (NSAIDs) diclofenac (DIC), ibuprofen (IBU) and naproxen (NPX) in the Faro-Noroeste wastewater treatment plant (FNW-WWTP) in the winter (W) and summer (S) of 2023 and winter of 2024. Each point represents the detection by LC-MS/MS (in µg/L) in extracts from the raw sewage or influent or from the treated effluent discharged to the environment (Eff.1), both sampled in composite samples representative of 24h, (n = 1).

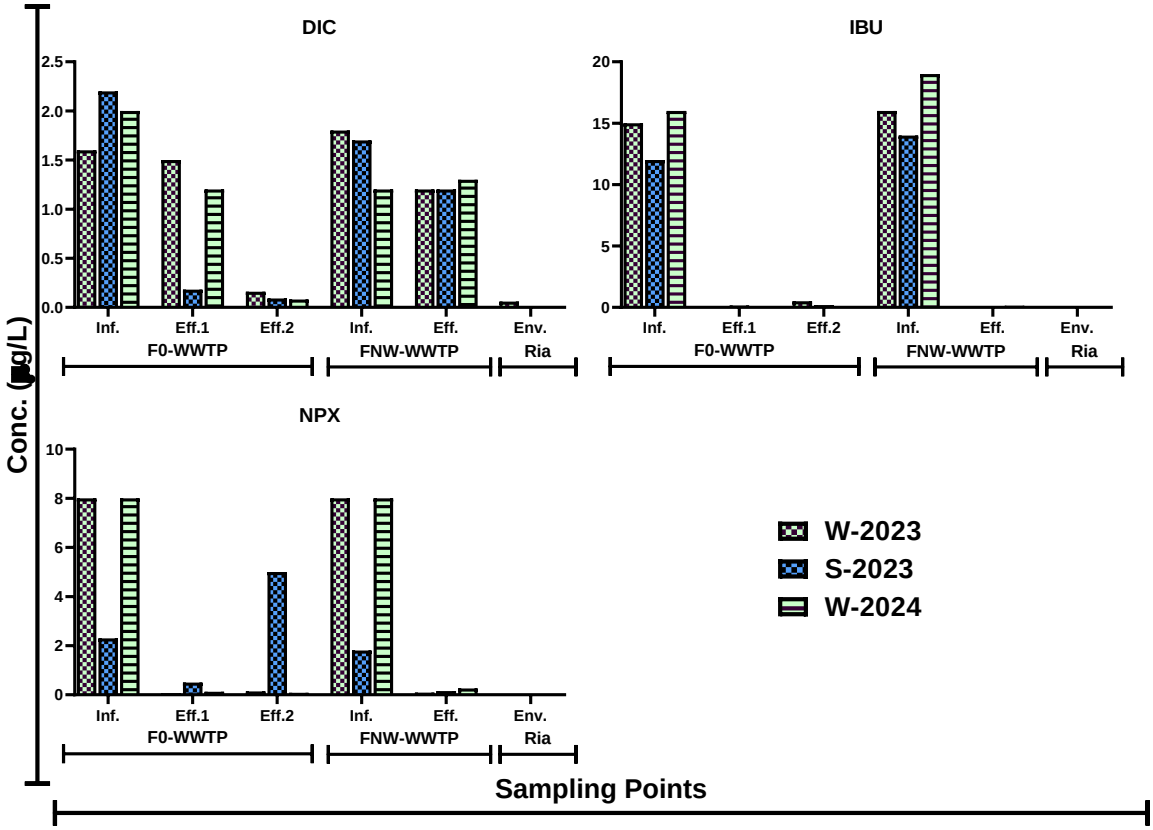

Figure 3.6: Comparison of non-steroidal anti-inflammatory drugs (NSAIDs) diclofenac (DIC), ibuprofen (IBU) and naproxen (NPX) concentrations in Faro-Olhão (FO-WWTP), Faro-Noroeste (FNW-WWTP) and in the environmental waters collected at the Ria Formosa (Ria) for winter (W) or summer (S) samples collected in 2023 and 2024. Each point represents the detection by LC-MS/MS (in µg/L) in extracts from the raw sewage or influents (Inf., composite samples of 24h), the treated effluents (Eff.1, composite sample of 24h) and, in the case of the FO-WWTP, also the final treated effluent discharged from the lagoon to the Ria Formosa (Eff.2, single sample).

The concentrations of individual antibiotics in the raw sewage influents of both treatment plants (Figure 3.9) ranged from non-detectable levels up to as high as 8 µg/L. Azithromycin was present in higher concentrations (0.3-8 µg/L) than the other antibiotics. The macrolide antibiotic clarithromycin and the sulfonamide antibiotics sulfamethoxazole and sulfapyridine were within the range 0.1 µg/L to 0.7 µg/L. Sulfadiazine and Erythromycin were not detected in the wastewater influents, with an exception of one occurrence of erythromycin in both the influent and effluents in the winter of 2021 in the FO-WWTP (Figure 3.7), at 0.22 µg/L. The occurrence of the detected antibiotics was observed to be generally higher in the winter season and increased from winter 2021 to winter 2024 (in the FO-WWTP influent, Figure 3.7) except for sulfapyridine that had a constant concentration in the sewage and a slight decline in winter 2024. The FNW-WWTP (Figure 3.8) in general received higher levels of sulfamethoxazole from winter 2023 to winter 2024, while sulfapyridine had higher concentrations in the summer 2023 compared to the winters of 2023-2024.

In agreement with Vaz-Moreira et al. (2022)'s findings comparing Portugal and Poland WWTPs, an upward concentration trend of azithromycin and clarithromycin in the influents in Poland and Portugal were observed compared to the undetectable concentrations of erythromycin, since post winter 2021 sampling campaign. This is ascribed to the growing use of new generation of broad-spectrum macrolide antibiotics, azithromycin and clarithromycin, in comparison to erythromycin which is currently less used. This results however are partly contradictory to that reported in the same study for influents in Portugal WWTPs, which could possibly be due to a temporal difference.

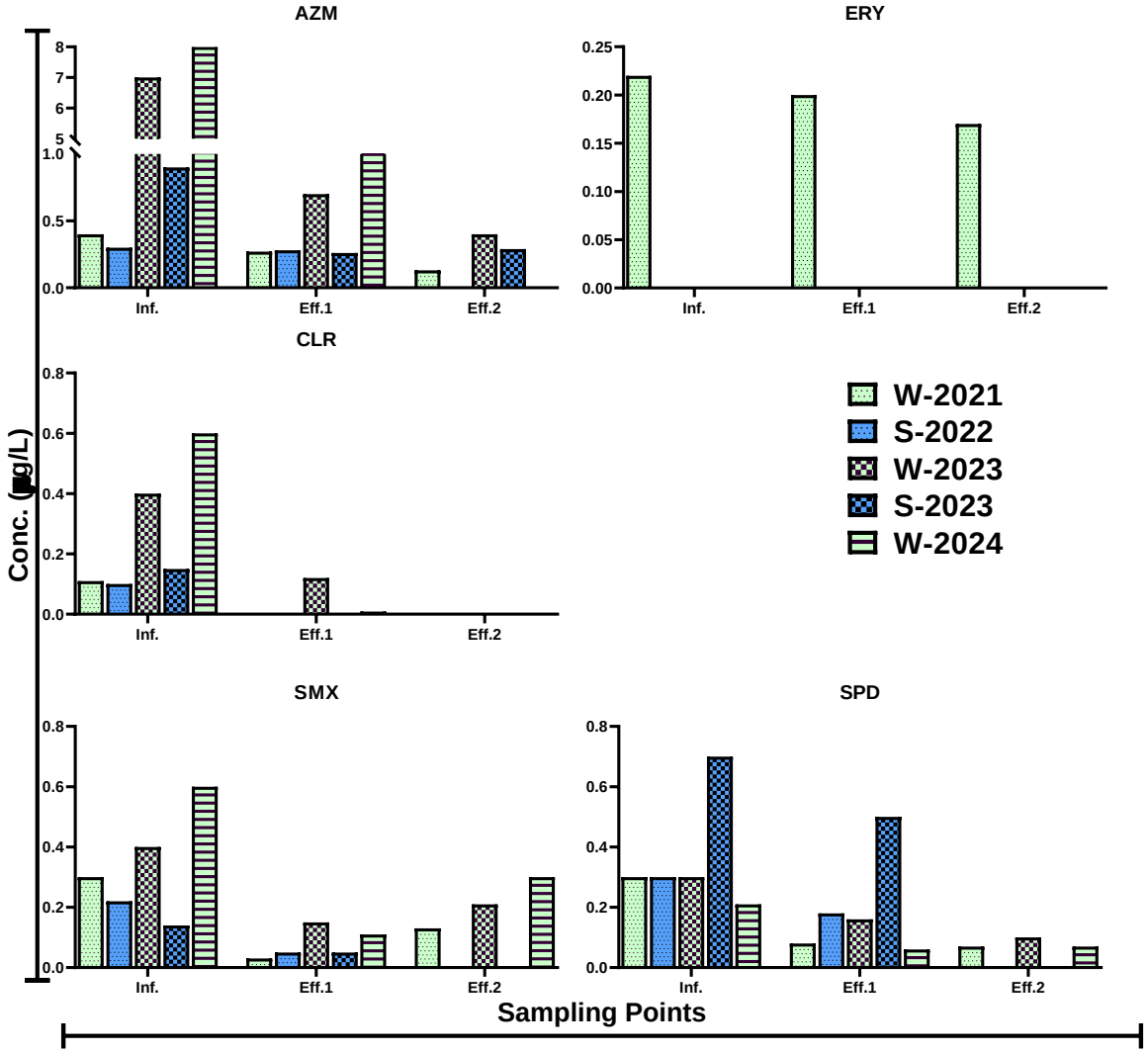

Figure 3.7: Occurrence concentrations of antibiotics azithromycin (AZM), erythromycin (ERY), clarithromycin (CLR), sulfamethoxazole (SMX), and sulfapyridine (SPD) in Faro-Olhão wastewater treatment plant (FO-WWTP), across seasons from 2021 to 2024 (S= summer; W= winter). Each point represents the detection by LC-MS/MS (in µg/L) in extracts from the raw sewage or influent (Inf., composite sample of 24h), the treated effluent discharged to the lagoon (Eff.1, composite sample of 24h) and the final treated effluent discharged from the lagoon to the Ria Formosa environment (Eff.2, single sample), (n = 1).

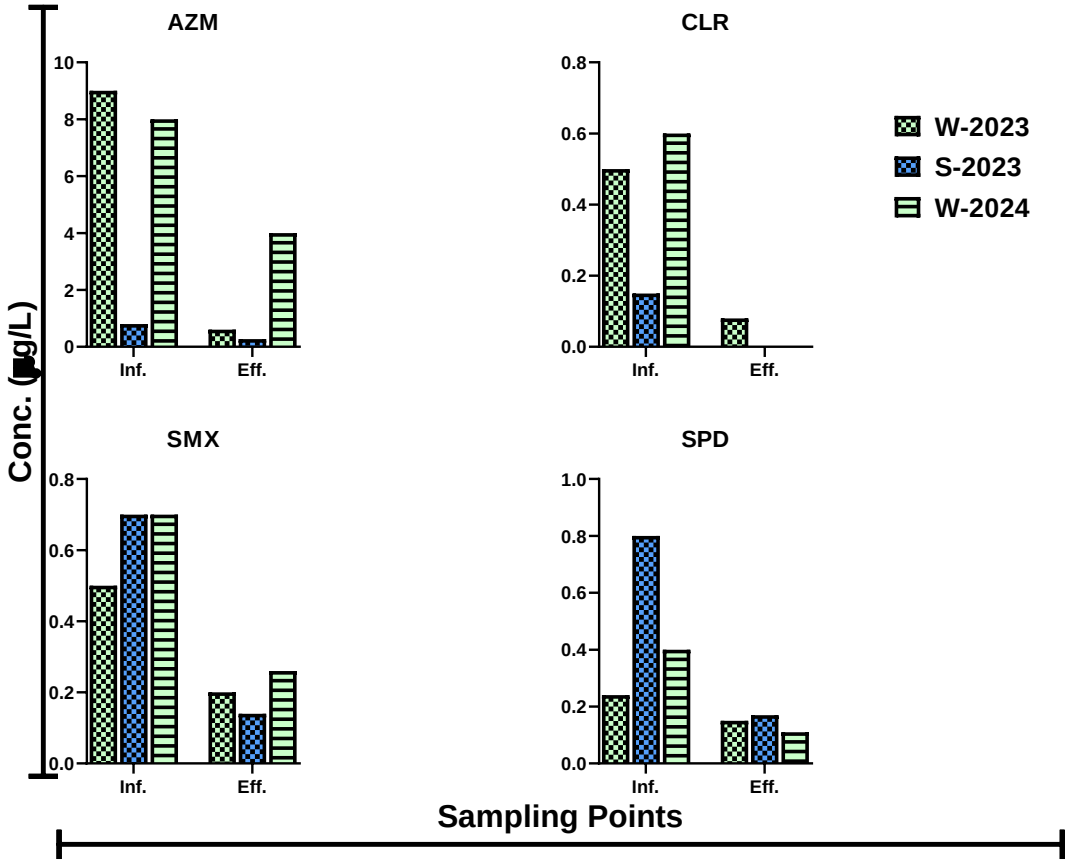

Figure 3.8: Occurrence concentrations of antibiotics azithromycin (AZM), erythromycin (ERY), clarithromycin (CLR), sulfamethoxazole (SMX), and sulfapyridine (SPD) in Faro-Noroeste wastewater treatment plant (FNW-WWTP) in the winter (W) and summer (S) of 2023 and winter of 2024. Each point represents the detection by LC-MS/MS (in µg/L) in extracts from the raw sewage or influent or from the treated effluent discharged to the environment (Eff.1), both sampled in composite samples representative of 24h.

Due to its potential anti-viral and anti-inflammatory properties, azithromycin was used in the treatment of COVID-19 pandemic as was useful in previous epidemics such as the severe acute respiratory syndrome (SARS) and the Middle East respiratory syndrome (MERS) (Arabi et al., 2019; Khoshnood et al., 2022). The increasing use of azithromycin and clarithromycin during the COVID-19 pandemic may have led to the observed rising trend from 2021 to 2024 in the FO-WWTP and FNW-WWTP sewage and other cases reported, which marks them as good markers of antibiotic use.

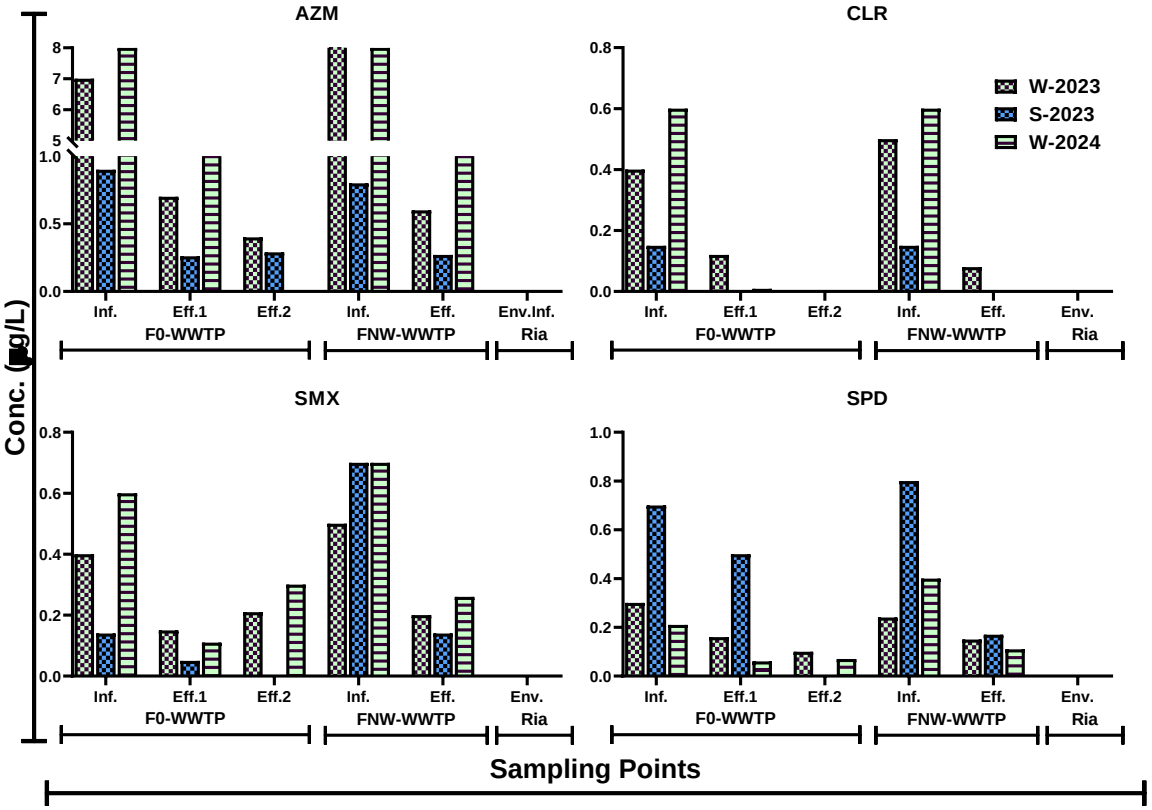

Figure 3.9: Comparison of antibiotics azithromycin (AZM), erythromycin (ERY), clarithromycin (CLR), sulfamethoxazole (SMX), and sulfapyridine (SPD) occurrence concentrations in Faro-Olhão (FO-WWTP), Faro-Noroeste (FNW-WWTP) and in the environmental waters collected at the Ria Formosa (Ria) for winter (W) or summer (S) samples collected in 2023 and 2024. Each point represents the detection by LC-MS/MS (in µg/L) in extracts from the raw sewage or influents (Inf., composite samples of 24h), the treated effluents (Eff.1, composite sample of 24h) and, in the case of the FO-WWTP, also the final treated effluent discharged from the lagoon to the Ria Formosa (Eff.2, single sample), (n = 1).

The occurrence of beta-blockers in the wastewater influents since winter 2021 to winter 2024 has generally been on a decline, looking at the longer sampling and monitoring performed in the FO-WWTP (Figure 3.10). Atenolol was the compound with the highest concentration of the three, initially at 0.6 µg/L in the winter 2021, dropping to 0.22 µg/L in summer 2023 and then showing a slight increase in the winter of 2024 to 0.32 µg/L. A similar trend was observed with metoprolol, dropping from 0.12 µg/L in the winter of 2021 to undetected levels in the summer of 2023, then with a slight reoccurrence of 0.05 µg/L in the winter of 2024. Propranolol was detected at low levels in the sewage of FO-WWTP (Figure 3.11) in the 2021 and 2022 samplings, and not detectable during the winter and summer of 2023 and was detected in the winter of 2024 at 0.07 µg/L. In the FNW-WWTP sewage it only occurred in the winter of 2023 at µg/L 0.05 concentration (Figure 3.11).

The trends of the relative abundances of the beta-blockers in the raw sewage or influents (summarized in Figure 3.12) showed atenolol to be the most abundant (0.22-0.60 µg/L) in the sewage while metoprolol and propranolol showed the lower concentration occurrences of 0-0.12 µg/L and 0-0.07 µg/L, respectively in the current study. This results were consistent with Silva et al. (2021)'s results in both the Beirolas WWTP (near Lisbon) and FNW-WWTP Algarve. However, the concentration levels were higher in the study by (C. Silva et al., 2021) in 2016-2019 showing the median concentration of FNW-WWTP, also analysed in that study, at 0.75 µg/L for atenolol, then 0.02-0.30 µg/L and 0.01-0.14 µg/L for metoprolol and propranolol, respectively.

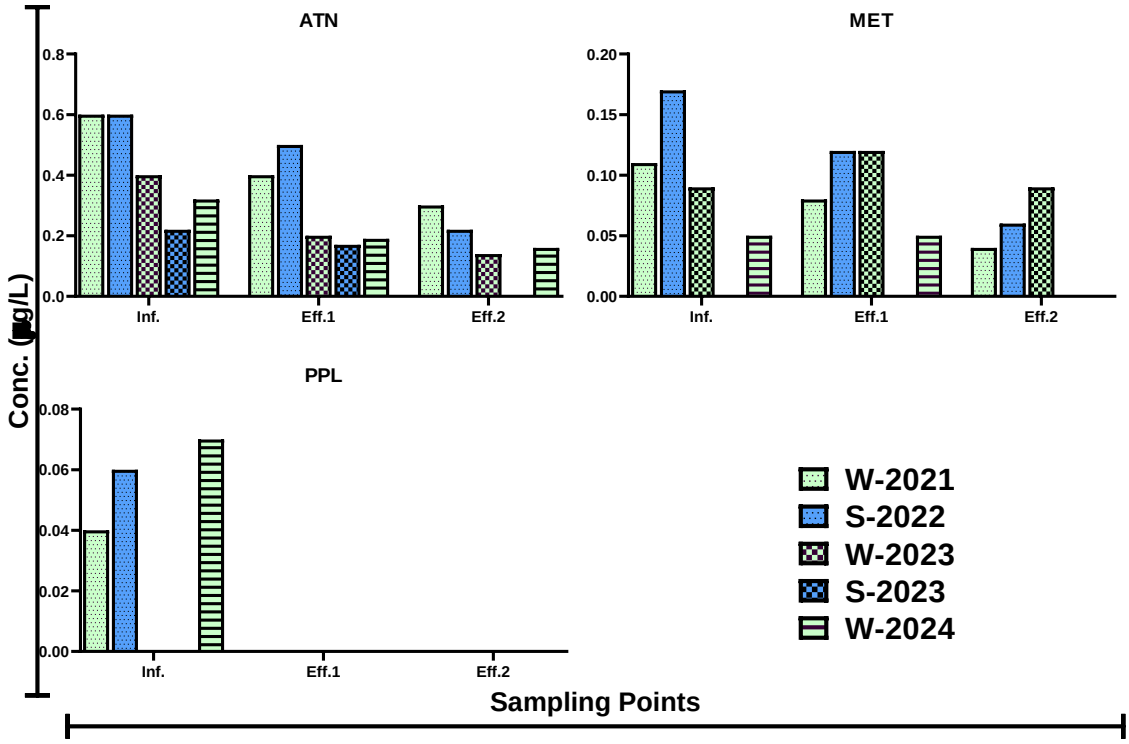

Figure 3.10: Occurrence of beta-blockers atenolol (ATN), metoprolol (MET), and propranolol (PPL) in Faro-Olhão wastewater treatment plant (FO-WWTP), across seasons from 2021 to 2024 (S= summer; W= winter). Each point represents the detection by LC-MS/MS (in µg/L) in extracts from the raw sewage or influent (Inf., composite sample of 24h), the treated effluent discharged to the lagoon (Eff.1, composite sample of 24h) and the final treated effluent discharged from the lagoon to the Ria Formosa environment (Eff.2, single sample), (n = 1).

A different pattern (Figure 3.12) was observed in the atenolol results of FNW-WWTP where the sewage concentration levels were higher (0.27-0.80 µg/L) than those of FO-WWTP, and the levels were higher in the summer than in the winter while; the inverse (lower levels in the summer) was detected in FO-WWTP for the 2023-2024 period.

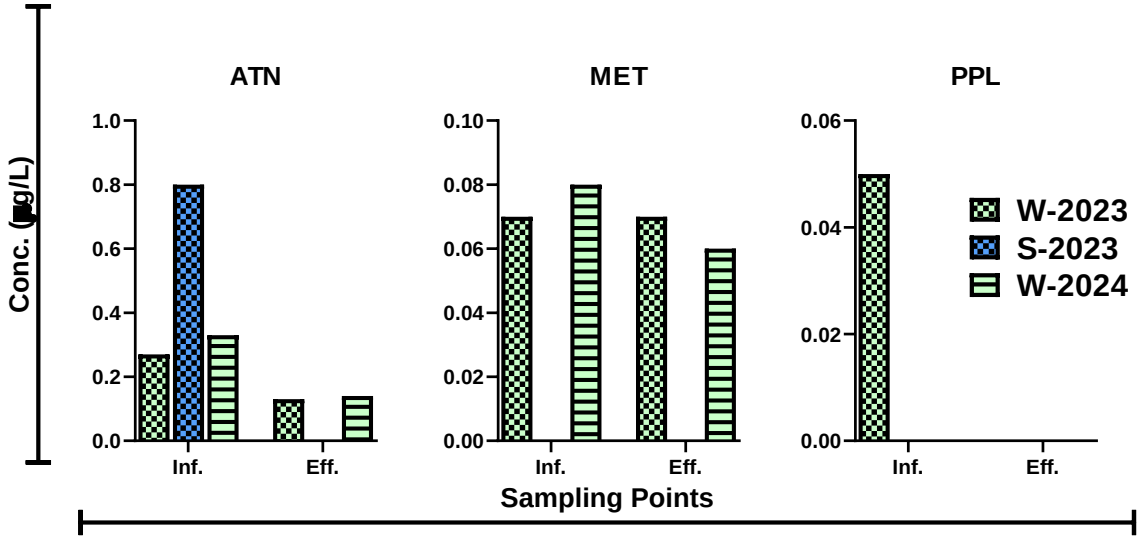

Figure 3.11: Occurrence of beta-blockers atenolol (ATN), metoprolol (MET), and propranolol (PPL) in Faro-Noroeste wastewater treatment plant (FNW-WWTP) in the winter (W) and summer (S) of 2023 and winter of 2024. Each point represents the detection by LC-MS/MS (in µg/L) in extracts from the raw sewage or influent or from the treated effluent discharged to the environment (Eff.1), both sampled in composite samples representative of 24h, (n = 1).

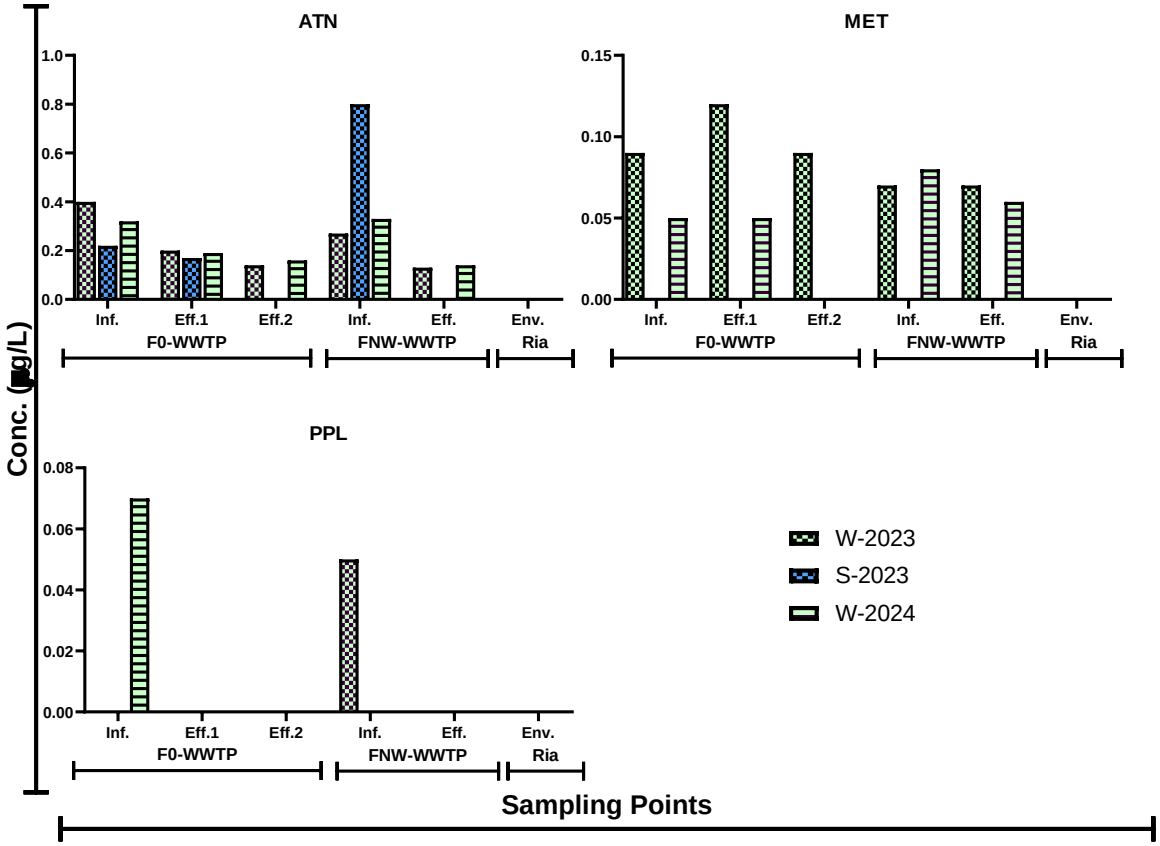

Figure 3.12: Comparison of beta-blockers atenolol (ATN), metoprolol (MET), and propranolol (PPL) occurrence concentrations in Faro-Olhão (FO-WWTP), Faro-Noroeste (FNW-WWTP) and in the environmental waters collected at the Ria Formosa (Ria) for winter (W) or summer (S) samples collected in 2023 and 2024. Each point represents the detection by LC-MS/MS (in µg/L) in extracts from the raw sewage or influents (Inf., composite samples of 24h), the treated effluents (Eff.1, composite sample of

24h) and, in the case of the FO-WWTP, also the final treated effluent discharged from the lagoon to the Ria Formosa (Eff.2, single sample), (n = 1).

Fluoxetine, an antidepressant classified of medium environmental risk was also monitored in other studies (Daughton & Ternes, 1999; Kosma et al., 2010; Verlicchi et al., 2012) with its highest concentration reaching up to 0.05 µg/L and averaging at 0.003 µg/L. Although low, these are detectable levels contrary to the current monitoring study, where fluoxetine was not detected in any sewage, effluent or environmental water (data not shown). The low concentrations of fluoxetine may be due to N-demethylation metabolism to its active metabolite norfluoxetine (Melchor-Martínez et al., 2021; Perez-Caballero et al., 2014), which was not monitored in this study. Thus, fluoxetine in wastewater may potentially exist in its metabolite state and/or in very small quantities, hence a possible complementary approach would be to develop quantification methods also for its main metabolites. In addition, fluoxetine has been reported to degrade significantly in WWTPs. However, although nanogram quantities are detected in some effluents they may induce various deleterious effects in marine organisms (Melchor-Martínez et al., 2021).

The hormonal substances (corticosteroids, sex hormones, and contraceptives) included in the monitored LC-MS/MS panel here quantified were all found to be below the quantification limits (data not shown). An exception was cortisone and testosterone, detected in untreated wastewater of the FO-WWTP in the winter of 2021 at low levels, which were 0.15 and 0.05 µg/L, respectively. These results were consistent with the results of Silva et al. (2021) from their similar study based on Beirolas WWTP in Lisbon and the FNW-WWTP, Portugal, between 2016 and 2019. These compounds are known for their occurrence and endocrine disruption potential/activity at very low quantities in the nano range, thus, their absence or not being quantifiable should not imply no impact on marine organisms. In their studies, Laganà et al. (2004) were able to quantify the endocrine disrupting compounds down to 3 ng/L in WWTPs and natural waters. Therefore, more sensitive methods of analysis should be developed and/or applied to quantify the least amounts as possible.

Since the measured concentrations are dependent on the time of sampling and sampling points (Johnson, 2010), the seasonal monitoring at selected points of treatment is an attempt to find temporal and spatial variations of pollution in WWTPs. The seasonal occurrence of the various contaminants can also provide hints on the trends of prescription, consumption and release/excretion of each PhC and give clues on the origin of contamination. This information can potentially come in handy in finding approaches for remediating pollutants. Such

compounds from whose occurrence the source and magnitude of contamination can be deciphered as well as easily evaluated are referred by Gonçalves et al., 2014 and Marasco et al., 2019 as ideal contamination markers.

The occurrence profiles of the PhCs in pre-treated wastewater will therefore enable the management of PhCs' release into the municipal wastes, and together with further source-tracking mechanisms may identify pollution point sources. In tandem with post-treatment PhCs' profiles, the treatment efficiencies of the WWTPs are made possible.

3.2. Removal efficiency (RE) comparisons of FO-WWTP (AGS) and FNW-WWTP (AS)

The efficiencies of the FO and FNW WWTPs were investigated by the comparative analysis of the corresponding wastewater samples of the untreated influent (raw sewage) against the treated effluent after the unit (Eff. In the case of FNW and Eff.1 in FO, not considering the additional lagoon retention). The calculable removal efficiencies from the 26 monitored in both WWTPs are illustrated in Figure 3.13 and Table 3. 1 by order of occurrence abundances where the boxes represent median values and whiskers represent the 5-95 percentile values. This provide the opportunity for a comparison between the AS and AGS treatment systems.

Acetaminophen, caffeine, ibuprofen, and propranolol showed the least persistence in wastewater translating to highly effective removal by the WWTPs, in both FO and FNW (Figure 3.13). Their removal efficiencies in FO-WWTP were at least 98.8% and 100% in FNW-WWTP. Interestingly, in the case of caffeine despite the efficient removal, the values remaining in the effluents and detected in environmental waters in the Ria Formosa (up to 0.5-µg/L) are still of concern.

In the case of acetaminophen, this is in alignment with the findings by Lam et al. (2004) indicating low persistence, with an average half-live of 1 day studied in outdoor filed microcosms. The next efficiently treated pollutants were clarithromycin and naproxen with over 70% removal efficiency (RE) in all sampling campaigns here analysed. Erythromycin and propranolol also showed high removal efficiencies; however, their low frequencies of occurrence or detection do not provide a definitive conclusion on their actual persistence.

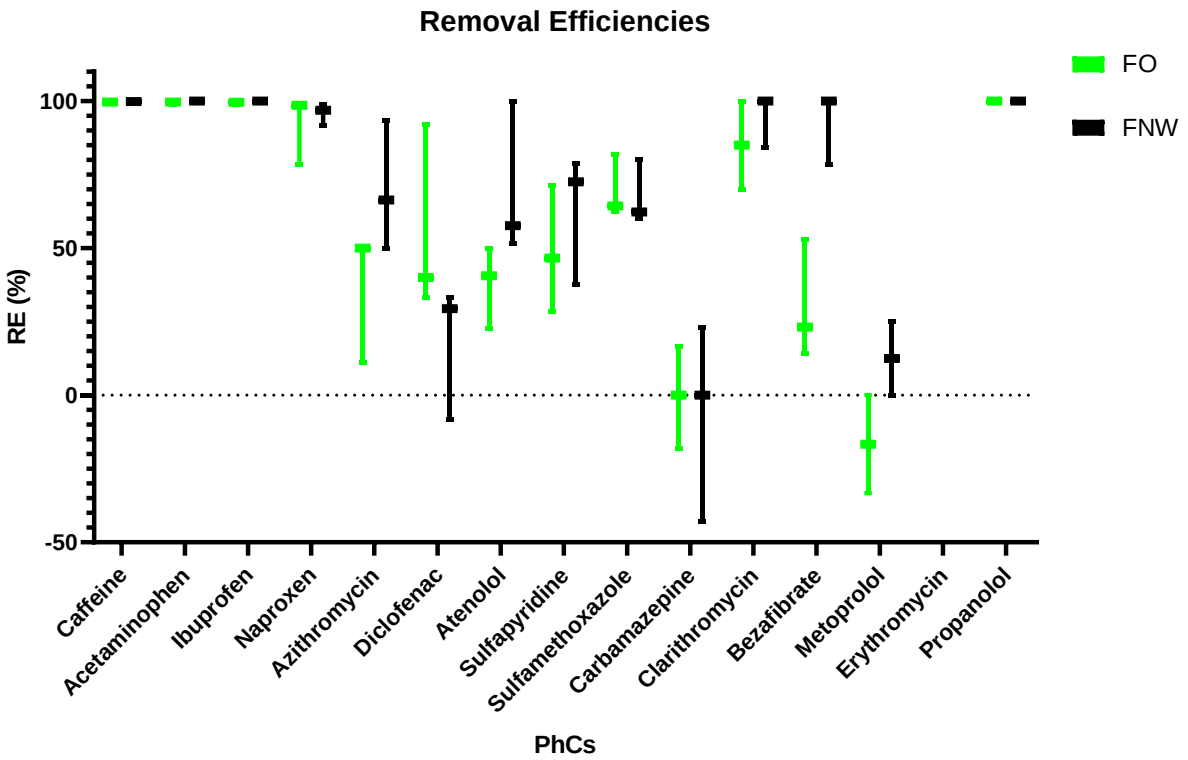

Figure 3.13: Box and whisker plots of the removal efficiencies of FO-WWTP and FNW-WWTP for the 15 detected pharmaceutical compounds (PhCs). Efficiency calculated from Inf and Eff1 valuesnot considering the additional lagoon retention The boxes represent median values and whiskers represent the 5-95 percentile values.

Evidence from multiple studies (Gaffney et al., 2017; Kim et al., 2007) indicate carbamazepine and diclofenac as some of the most recalcitrant compounds in wastewater treatment. This is confirmed in this study, as their concentrations are only slightly lowered or remain unchanged in the effluents of the WWTPs compared to those in the raw sewage influent. In some effluent samples such as carbamazepine and metoprolol in FO-WWTP (Figure 3.13), the effluent showed elevated concentration with down to -20% removal efficiency, implying an accumulation instead of removal. The behaviour of CBZ to increase in concentration rather than decrease post treatment was also recorded by Bahlmann et al. (2014) showing an average increase of 14% in WWTP effluents; this was suspected to be due to the redissolution from faeces during or after treatment (compared to the raw sewage), since the average value of CBZ excreted in faeces (12.5%) is reported to be higher than that in urine (0.8%) from the parent consumed drug. The laboratory experiments by Lam et al. (2004) also proved carbamazepine as the most persistent compound of the tested in a microcosm and persistence studies with 82 days half-life. This environmental persistence was also observed for compounds such as metoprolol with a maximum of 25% RE in both treatment plants of the current study, despite its general low occurrence concentrations. The observed recalcitrance of these compounds implies a continuous release of the pollutants into the receiving waters or environment.

The comparative study of the overall efficiencies of the two wastewater treatment technologies, activated sludge (AS) and aerobic granular sludge (AGS) indicated global better treatment efficiencies for the FNW-WWTP over the FO-WWTP as depicted in Figure 3.13 and Table 3. 1, implying a current superior treatment by the AS technology over the AGS technology comparing this units. Although AGS is referred to have several advantages such as minimal energy consumption and process design complexities, lower operational costs, water-biomass separation challenges and land area requirements than AS (Giesen et al., 2016; Nancharaiah & Sarvajith, 2019; Pronk et al., 2015), it may need more optimization of operation compared to the highly optimized and routine biological treatment of AS. In addition, the WWTP quality control is by routine performed based on other operational and analyses parameters, while the PhC removal efficiency is generally not accessed except in some sporadic projects such as the LIFE IMPETUS project (LIFE IMPETUS, 2019).

Table 3. 1: Removal efficiencies of PhCs from the influent into FO-WWTP and FNW-WWTP (Inf) and from effluent from F0-WWTP (Eff1) into the lagoon that releases effluent into the ocean (Eff2)

		FO-WWTP						FNW-WWTP		
		W-2023		S-2023		W-2024		W-2023	S-2023	W-2024
PhC Class	**PhC**	**Inf-Eff1**	**Eff1-Eff2**	**Inf-Eff1**	**Eff1-Eff2**	**Inf-Eff1**	**Eff1-Eff2**	**Inf-Eff1**	**Inf-Eff1**	**Inf-Eff1**
Analgesic	**Acetaminophen**	100	n.a.	100	100	99	100	100	100	100
Anticonvulsant	**Carbamazepine**	-20	17	-18	-12	17	25	0	23	-43
Antibiotic	**Azithromycin**	90	43	11	100	50	100	93	66	50
	Clarithromycin	70	100	100	n.a.	85	100	84	100	100
	Erythromycin	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Sulfadiazine	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Sulfamethoxazole	63	-40	64	100	82	-173	60	80	63
	Sulfapyridine	47	38	29	100	71	-17	38	79	73
Antidepressant	**Fluoxetine**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Anti-dyslipidemic	**Clofibric acid**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Bezafibrate	23	10	53	100	14	100	78	100	100
Beta-blocker	**Atenolol**	50	30	23	100	41	16	52	100	58
	Metoprolol	-33	25	n.a.	n.a.	0	100	0	n.a.	25
	Propranolol	n.a.	n.a.	n.a.	n.a.	100	n.a.	100	n.a.	n.a.
NSAID	**Diclofenac**	6	89	92	50	40	93	33	29	-8
	Ibuprofen	100	n.a.	99	-27	100	0	100	100	99
	Naproxen	99	-100	78	-900	99	33	99	92	97
Psychostimulant	**Caffeine**	96	-19	100	-8	99	-32	100	100	100
Corticosteroid	**Cortisone**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Sexual hormone	**Testosterone**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	beta-Estradiol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Estriol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Estrona	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Contraceptive	**Ethinylestradiol**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Diethylstilbestrol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Gestodene	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

After the operational treatment by the FO-WWTP, the wastewater is released into a lagoon (that was remaining from the previous unit) where it is further treated by retention / sun light exposure before being released into the receiving waters in the Ria Formosa channels. The removal efficiency of the lagoon was also studied to observe the effect it has on the treatment of the pollutants resistant to the AGS treatment.

Concerning the removal of these antibiotics from the sewage influents, most of these antibiotics were not completely treated by the WWTP, being detected in the treated effluents, but clarithromycin exhibited significant reduction following their transition in the lagoon (see Figure 3.9). This suggests further treatment by the lagoon.

The effect of the lagoon after the treatment in the FO-WWTP unit was poorly evident for atenolol (none to little differences between the concentrations in Effl.1 and 2) and only moderate for propranolol (Figure 3.12). This contrasts with a study by (Gentili & Fick, 2017) that showed up to a 99% reduction of PhCs using algae.

Most pollutants that were not thoroughly removed in FO-WWTP were further removed (attenuated PhC release) by the effect of the lagoon (Figure 3.13 and Table 3.1). The effect can be attributed to the sorption of the pollutants to the sediments or sludge in the lagoon, which we investigated by testing by LC-MS/MS one sample of on the lagoon superficial sediment in the sampling of 2022. The superficial sediment sample taken from the lagoon, near to the outlet where the final treated effluent is released to the Ria Formosa environment (same point where the lagoon water Eff.2 samples were collected) had no detectable levels of any of the pollutants under analysis. As Khetan and Collins (2007) indicated, the rest of the pollutants are possibly further removed by photo-degradation in sun-lit lagoons. This may be due to the susceptibility of these pollutants to UV light, which encourages photochemical reactions. In addition, the PhCs can have adsorb to the lagoon sediment, but then degraded over time (at least at the superficial samples taken) also due to microbial or UV actions, justifying the undetectable levels (for this single point in time), and it would be interesting to analyse deeper samples of the lagoon sediment to further investigate this hypothesis.

The effect of the UV radiation is exemplified by a study by Yuan et al. (2012) on the degradation of bezafibrate using UV / hydrogen peroxide (H_2O_2) in surface water and WWTP effluents showed that despite the accelerated degradation in the presence of H_2O_2, bezafibrate can also be degraded by ultraviolet (UV) light alone. The significant degradation of bezafibrate in the lagoon beyond the WWTP-FO system (refer to Figure 3.3) could be ascribed to the action of

UV action from sunlight and possible microbial action. This phenomenon is also supported by the evidence of the open oxidation ponds used for biological treatment in the FNW-WWTP, achieving a relatively higher removal efficiency for bezafibrate compared to the FO-WWTP which is less exposed to the sunlight UV (closed biological treatment bioreactor of AGS), of 78.2-100% vs 14.28-52.94%, respectively Figure 3.13.

The lagoon improvement of PhC removal can be argued to provide a similar or comparable effect to the open reactors of the AS system, especially the secondary treatment with more exposure to sunlight. Even though effective as a further removal of pollutants, some photodegradation products of these pharmaceutical pollutants may be more toxic than their parent API (Donner et al., 2013), so the monitoring of the main PhCs should be further complemented by the investigation of relevant metabolites.

3.3. Toxicity Studies

3.3.1. Acute toxicity of Carbamazepine and Coumestrol on *Artemia sp*.

The toxicities of these selected substances were estimated and expressed in the form of LC_{50} values, the concentration of the test substance that causes death to 50% of the test population. Here, LC_{50} values were calculated using the Quest Graph™ LC_{50} Calculator based on the Hill equation non-linear regressions and correlation graph between the concentrations of the substance and the lethality/immobilisation (%) (AAT Bioquest Inc., 2023). The graphs were constructed with the mean values of the immobilisation % with standard mean errors.

The 100 mg/L used as the highest concentration in the range-finding test was also used to serve as a limit test. Since there were still moving test animals at 100 mg/L for both tested compounds CBZ and CMT, higher maximum concentration of 1000 mg/L was used for the definitive test for carbamazepine. For coumestrol a maximum of 100 mg/L was used since it was unavailable in house in sufficient quantities.

The test organisms manifested behavioural changes and showed concentration dependant immobilisation, as illustrated in the immobilisation curves of Figure 3.14, Figure 3.15, Figure 3.16, and Figure 3.17 (for both compounds tested in either natural seawater or artificial seawater). At zero concentration (negative control) and 5 mg/L concentration of the test substances, the surviving *Artemia sp.* nauplii exhibited quick and lively movement. The rate of movement and liveliness decreased with increasing concentration and time, and so did the mobility in response to both CBZ and CMT in both natural seawater and artificial seawater assays. At high concentrations beyond 75 mg/L in CBZ contaminated seawater, most *Artemia*

sp. nauplii were immobilised, while the surviving nauplii exhibited sluggish movement characterised by slight movement of the limbs without lively swimming compared to the control and lower concentrations.

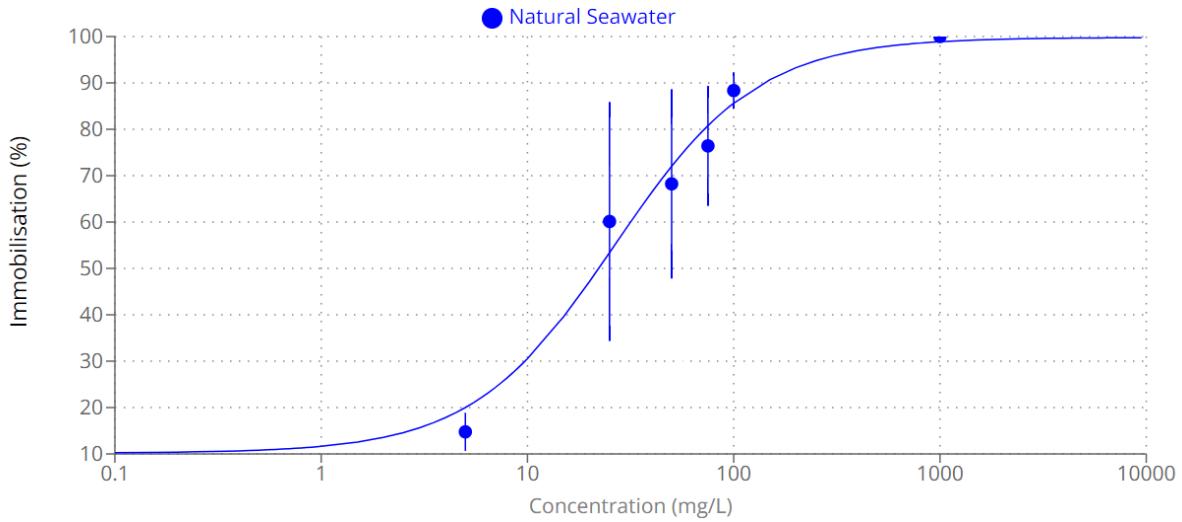

Figure 3.14: *Artemia sp.* immobilisation assay in natural seawater contaminated with carbamazepine. Graphs constructed using mean values of immobilisation (%) and presented with mean standard errors, (n = 6).

LC_{50} of CBZ in natural seawater = 26.47 mg/L

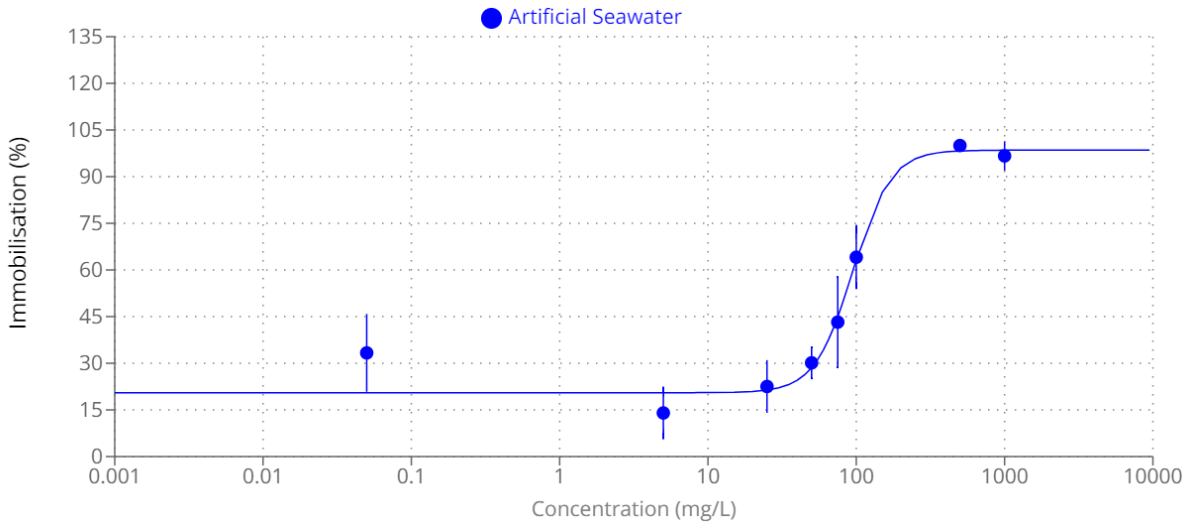

Figure 3.15: *Artemia sp.* immobilisation assay in artificial seawater contaminated with carbamazepine (CBZ). Graphs constructed using mean values of immobilisation (%) and presented with mean standard errors, (n = 6).

LC_{50} of CBZ in artificial seawater = 94.53 mg/L

In natural seawater, the LC_{50} value for CBZ was determined to be 26.47 and 5.09 mg/L for CMT while in artificial water the LC_{50} values were determined to be 94.53 and 39.87 mg/L for CBZ and CMT, respectively. These toxicities according to the European Commission (2008) toxicity classifications (refer to Table 1. 2) would indicate chronic category 2 for CMT in natural seawater, but chronic category 3 in artificial seawater. For CBZ, this toxicity is classified as category 3 in both natural seawater and in artificial seawater. For both compounds, the LC_{50} values in natural seawater assays were found to be lower than in artificial seawater, implying a higher toxicity (reaching 50% of lethality, measured based on immobilisation as defined in the methods) compared to natural seawater. This can be attributed to the presence of known and controlled concentrations of pollutants (in this case the test compounds CBZ or CMT) in the test artificial seawater compared to the respective controls. In contrast, there is no control on the presence of other pre-existing pollutants, microbes or other substances in natural seawater that could potentially contribute, interact and/or affect the toxicity of the test compounds. Thus, it appears that the complex composition of the natural seawater may contain one or more substances that are adding or potentiating the toxicity of the two test compounds, as their influence results in lower LC_{50} values for both. The LC_{50} of CBZ showed similar comparison (chronic category 3) to the results reported by Albendín et al. (2021) where the LC_{50} of CBZ on *A. salina* was determined to be 43.225 mg/L in seawater.

Phytoestrogens have reported occurrence in WWTPs and as environmental pollutants. They can also be used as PhCs in replacement therapies and as additives in aquaculture feeds (Z. hua Liu et al., 2010). Thus, their accumulation in fish poses a threat to them and animals up the trophic level.

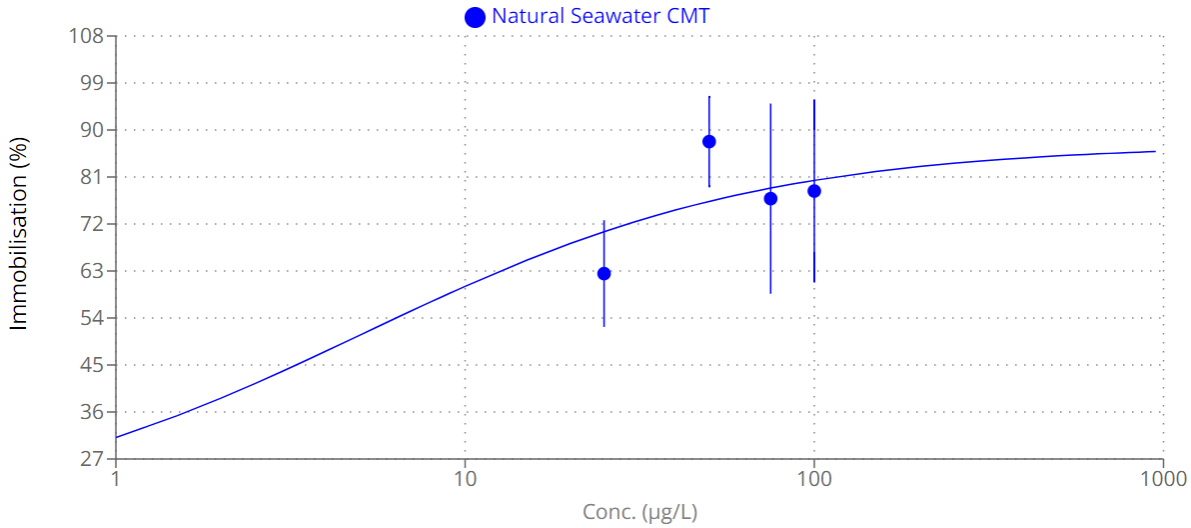

Figure 3.16: *Artemia sp.* immobilisation assay in natural seawater contaminated with coumestrol (CMT) Graphs constructed using mean values of immobilisation (%) and presented with mean standard errors, (n = 3).

LC_{50} of CMT in natural seawater = 5.09 mg/L

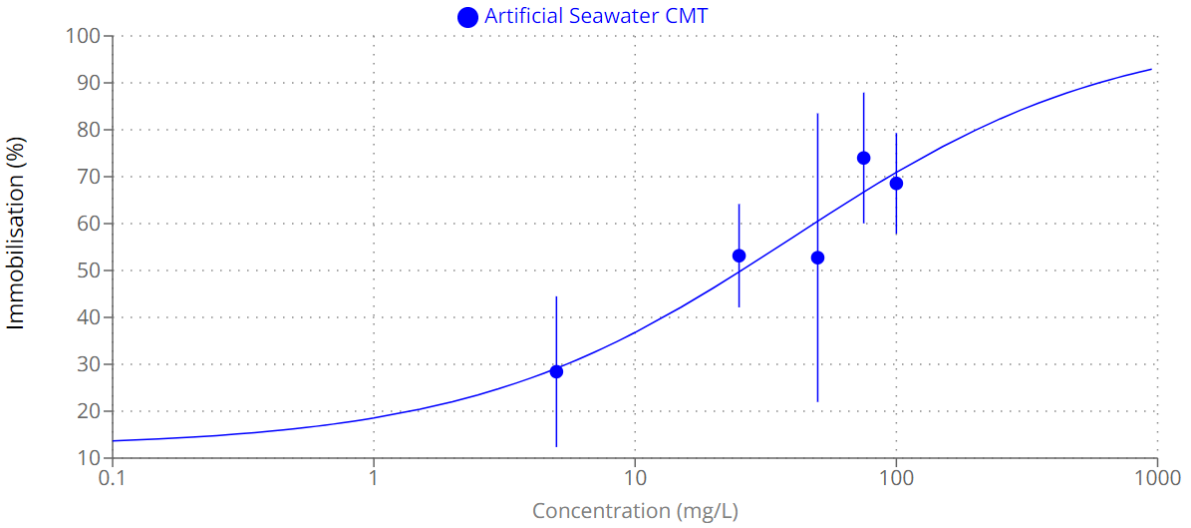

Figure 3.17: *Artemia sp.* immobilisation assay in artificial seawater contaminated with coumestrol (CMT) Graphs constructed using mean values of immobilisation (%) and presented with mean standard errors, (n = 6).

LC_{50} of CMT in artificial seawater = 39.87 mg/L

As seen in Figure 3.16 and Figure 3.17, though the best-fit line shows concentration dependence of the immobilisation, some data points deviate. The OECD guidelines state that if the mortality does not follow the concentration-response pattern, then the death of test animals may be due to mishandling or accident during transfer or an unexplained effect not related to the concentration of the test substance (OECD, 2012). The assays could be improved by performing

the test with higher number of test organism (> 10 nauplii), although technically challenging, or complemented by the use of *In vivo* imaging systems and software.

The toxicity levels of carbamazepine are determined to be higher than the quantified carbamazepine concentrations in the wastewater and released into the environment, 0.5 µg/L in both. However, as seen for diclofenac and caffeine, already existing in the environmental samples of the Ria Formosa, the continuous release of the pharmaceutical pollutants could lead to their accumulation and detectable presence in the environment. This can be confirmed and followed by seasonal monitoring programs, similar to the one performed in the MicroWaste project and reported in this thesis, and in future studies investigating the potential bioaccumulation in marine organisms such as *Artemia*, bivalves or fish, to be tested as sentinels of PhC pollution.

Despite the guideline recommending enumeration after 48 hours, the immobilisation was observed after 12 hours of incubation and after 24 hours. In addition to the concentration dependence of toxicity on the test organisms, the factor of time was also observed. In comparison to the recommended enumeration time after 24 hours of incubation, enumeration was also carried out after 24 hours. This showed higher immobilisation at 24 hours enumeration, implying higher correlation of toxicity with prolonged exposure. as a significant variable where the immobilisation was observed to be higher after 24 hours of incubation than 12 hours.

3.3.2. The combined effect of the Pharmaceutical pollutants on *Artemia sp*.

In this assay, a combination of three selected PhCs caffeine (CAF), carbamazepine (CBZ), and diclofenac (DIC) was used to test the combined effect of PhCs against their individual effect. The tested concentrations (of individual tests and mixtures) simulated the influent and effluent concentrations of the PhCs detected in our monitoring survey (section 3.1) (CAF 84 and 3.2 µg/L, CBZ 0.5 and 0.5 µg/L, and DIC 1.6 and 1.5 µg/L, respectively). These were validated with the negative and solvent controls, and the immobilisation in the control samples was found to be below 10%, thus ensuring validated results.

As illustrated in Figure 3.18, the influent concentrations generally showed higher immobilisation than the effluent concentrations, thus proving that the immobilisation of the test organism is concentration dependent. A deviation from this trend was observed on CBZ where the effluent concentration exhibited higher immobilisation on the test organism than the influent concentration though at same concentration (0.5 µg/L). CAF and DIC concentrations in the influent are substantially higher than of CBZ, and accordingly they showed a significantly

higher immobilisation effect in influent tests, whereas no significant difference was observed in effluent concentrations.

The comparison between individual compounds and their mixture exhibited probable antagonistic effect in the influent and the effluent concentrations at both 24 and 48 hour observations (Figure 3.18). The overall combined effect of the mixed PhCs was less than the sum of their individual effects on immobilisation of the test organisms.

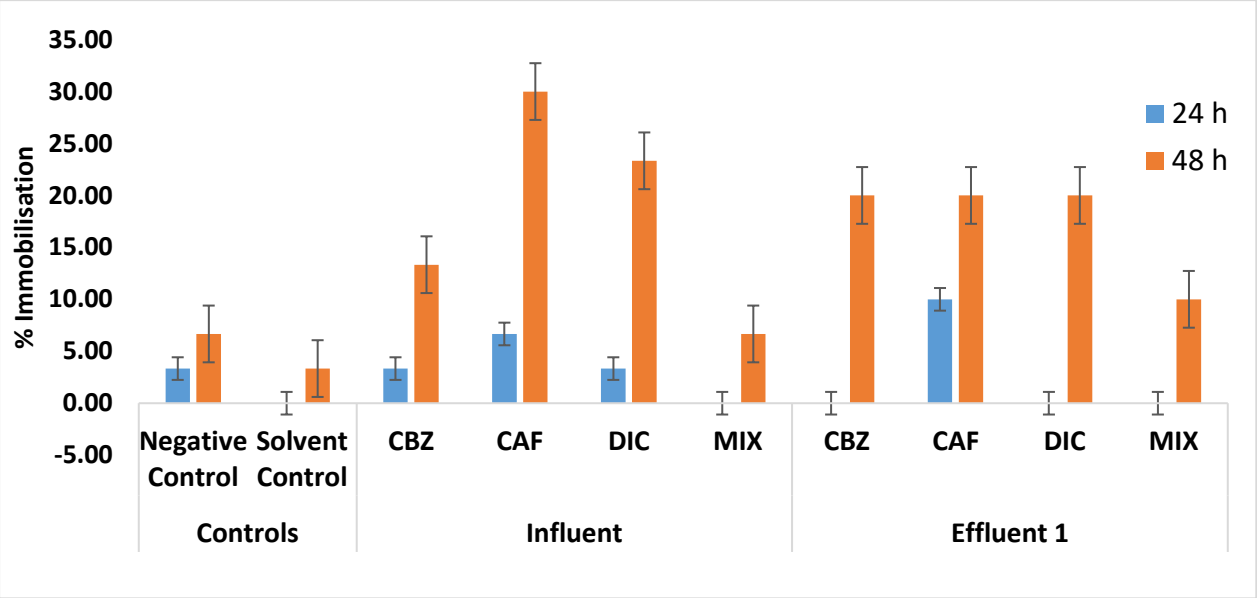

Figure 3.18: Immobilisation assay of mixtures of Pharmaceutical pollutants carbamazepine (CBZ), caffeine (CAF), diclofenac (DIC) and their mixtures (MIX). Concentrations in influent: CBZ = 0.5 µg/L, CAF = 84 µg/L, and DIC = 1.6 µg/L. Concentrations in effluent: CBZ = 0.5 µg/L, CAF = 3.2 µg/L, and DIC = 1.5 µg/L, (n = 3).

Since the proportions of the PhCs in the influent and the effluent as established from the monitoring study were altered due to the unequal treatment by the WWTPs, the study further explored the effect of these alterations on the test organisms. As observed in Figure 3.18, though the effluent mixtures led to higher lethality (measured by immobilization % as defined in the methods) compared to the influent mixtures, the difference was not statistically significant across the individual compounds and the corresponding mixtures. Thus, for the three studied PhCs, the alterations of the PhCs proportions by the treatment appear not to impact the global impacts on the test organisms. However, more experimental data would be required to confirm these preliminary findings.

3.3.3. Effect of wastewater on *Artemia sp.*

The preliminary study on the effect of wastewater on *Artemia sp.* (Figure 3.19, one single assay) showed immobilisation of approximately 17 % of the test organism population after 24 hours

when exposed to the influent original sample, which increased to half the population after 48 hour of incubation. There was no significant difference in the mortality results of the original and the dilutions of the sewage at 24 hours. However, the immobilisation in case of exposure to the treated effluent was lower in the original sample compared to the hundred-fold dilution. The wastewater treatment process was suggested to decrease the acute toxicity of the sewage as illustrated by the significantly lower mortality in the original sample of the effluent than the influent.

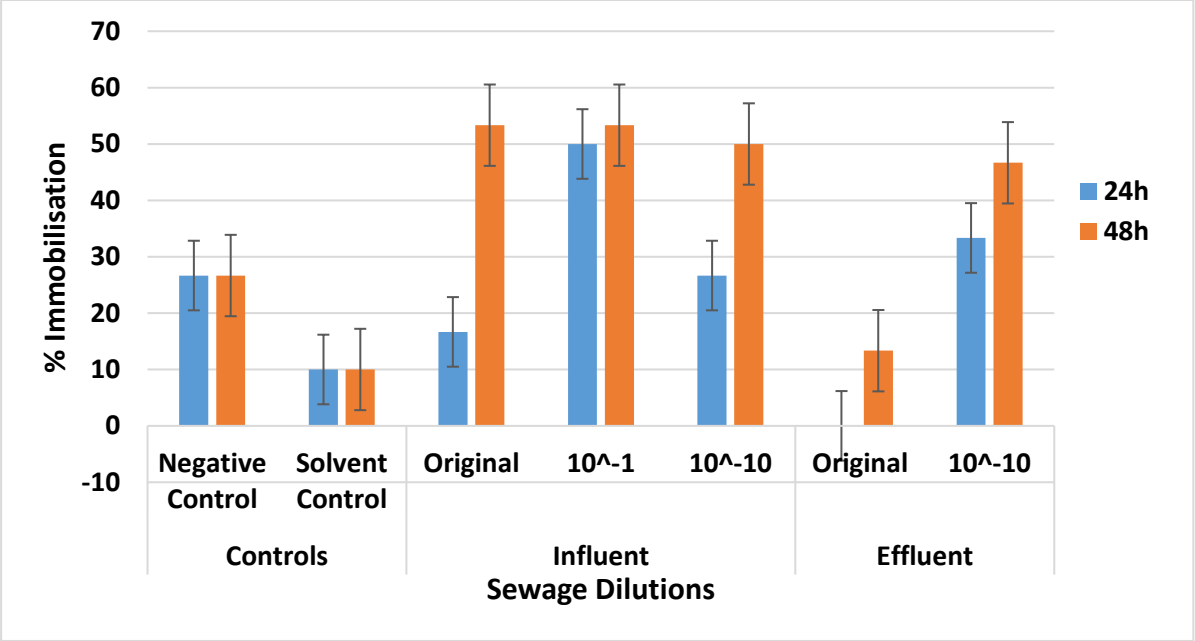

Figure 3.19: Immobilisation assay in wastewater influent (raw) and effluent (treated), (n = 3).

The comparative study between the original samples and the controls show promising features of the assay to be used as a presumptive test for water contamination in WWTPs especially where immediate analytical testing is not feasible. However, the limited dilution tests of this single assys did not give a good reflection of the effects of the different concentrations.

3.3.4. Immobilisation Recovery of *Artemia sp.*

While the prior assays aided in establishing whether the PhCs have any immobilisation effect on marine organism *Artemia sp.*, they however do not tell whether the effect is temporary or permanent. Therefore, additional qualitative experiments were carried out to observe whether the immobilised *Artemia sp.* nauplii would recover in fresh artificial seawater after 12 hours of exposure to CBZ contaminated seawater.

After an hour in fresh seawater from the 50 µg/L CBZ contaminated artificial seawater, majority of the *Artemia sp.* nauplii were still immobilised while others showed a slight movement. However, the movement did not improve after 12 hours (data not shown). From the 100 µg/L

CBZ contaminated water, no observable recovery from immobilisation was observed from the *Artemia sp.* nauplii both after one hour and after 12 hours in fresh seawater, with 100% of the naupii remaining immobilized. These tentative results indicated an irreversible damage at high concentrations of CBZ contamination. However, the small observed recovery at lower concentrations suggest that in cases of contamination causing *Artemia* immobilization, minimal reversible damage can be encountered in active marine environments where tides keep replenishing fresh seawater and diluting contaminated receiving waters.

4. Conclusion

The merits of the monitoring studies include surveying the level of contaminants that the environmental organisms and people accessing the water are exposed to, with particular susceptibility for aquatic organisms with longer exposure times. It further provides data on the seasonal occurrences of the pharmaceutical pollutants in WWTPs aimed to reflect the fluctuations in the use of pharmaceuticals. The pharmaceutical pollutants showed a wide presence in wastewater and varied retentions in the treated wastewater. The retained pollutants find their way into the environment as discharges of treated wastewater, which are controlled by regular quality control samplings and analyses to comply to the legal requirements but these do not include yet the PhC concentrations. In addition to the active pharmaceutical ingredients analysed, further studies should incorporate their degradation products since these also can pose a threat to the ecosystems. The occurrence of these contaminants in treated wastewater provides an indication of the inefficiency of the treatment system, and thereby can aid in tailor making systems that target specific contaminants of great concern.

The observed occurrence and retention behaviours of these pharmaceutical compounds warrant further studies on their potential ecological risks to the environment. Additionally, the quantification of the pharmaceutical concentrations therefore makes it possible to assess the risks posed by the pharmaceutical pollutants to aquatic organisms.

The *Artemia sp.* immobilisation assays were tentatively proven as a promising tool for preliminary assessment of toxicity. Recalcitrant pharmaceutical carbamazepine was determined to have a relatively lower toxicity compared to phytoestrogen coumestrol. Both compounds were determined to fall in the chronic categories 2-3 depending on the medium of seawater they were cultured. The study could benefit from incorporating the phytoestrogens in future water monitoring programs to help in the development of future regulations, due to their observed higher potential risk to the aquatic environment than some of the pharmaceutical pollutants. The study further supported that the pharmaceutical mixtures of the three compounds that mimic wastewater compositions gave an observable difference to their individual effect. It was further suggested that there is no additive effect of the three pharmaceuticals on their immobilisation effect on *Artemia sp.* nauplii, rather they may exhibit chemical antagonism.

This study provides investigative data to support regulations of wastewater effluents and efforts of wastewater treatment improvement for alleviation of pharmaceutical pollution. It will also

contribute to the hazard and risk assessment exercises aimed to inform the development of regulations on the release of pharmaceutical residues into the environment.

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APPENDIX 1

Table 1: *Artemia sp.* immobilisation assay data for natural seawater contaminated with carbamazepine (CBZ) with mean values of immobilisation (%) and mean standard errors, (n = 6)

Chemical substance	Conc. (mg/L)	Immobilisation/lethality (%)						Mean	SD	SEM (Std. error)	LC50
CBZ: Average Natural seawater	0.00	10.00	30.00	10.00	6.67	5.00	16.67	13.06	8.41	3.43	26.47
	5.00	14.28	20.00	10.00	14.28	20.00	10.00	14.76	4.10	1.67	
	25.00	50.00	40.00	60.00	25.00	85.71	100.00	60.12	25.77	10.52	
	50.00	70.00	70.00	30.00	72.73	66.67	100.00	68.23	20.41	8.33	
	75.00	70.00	81.82	60.00	66.67	80.00	100.00	76.42	12.94	5.28	
	100.00	90.00	88.89	80.00	92.31	88.89	90.00	88.35	3.90	1.59	
	1000.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00	

Table 2: *Artemia sp.* immobilisation assay data for artificial seawater contaminated with carbamazepine (CBZ) with mean values of immobilisation (%) and mean standard errors, (n = 6)

Chemical Substance	Conc. (mg/L)	Immobilisation/lethality (%)						Mean	SD	SEM (Std. error)	LC50 (mg/L)
CBZ: Average Artificial seawater	0.00	20.00	25.00	10.00	10.00	10.00	0.00	12.50	8.04	3.28	94.53
	0.05				30.00	50.00	20.00	33.33	12.47	5.09	
	5.00	20.00	9.09	25.00	0.00	10.00	20.00	14.02	8.46	3.45	
	25.00	20.00	37.50	22.22	10.00	18.18	27.27	22.53	8.46	3.45	
	50.00	30.00	33.33	37.50	20.00	30.00	30.00	30.14	5.28	2.16	
	75.00	70.00	37.50	40.00	27.27	30.00	54.55	43.22	14.82	6.05	
	100.00	70.00	54.55	80.00	70.00	60.00	50.00	64.09	10.24	4.18	
	500.00	100.00	100.00	100.00					0.00	0.00	
	1000.00	90.00	100.00	100.00	90.00	100.00	100.00	96.67	4.71	1.92	

Table 3: *Artemia sp.* immobilisation assay replicates data for natural seawater contaminated with coumestol (CMT) with mean values of immobilisation (%) and mean standard errors, (n = 3)

Chemical Substance	Conc. (mg/L)	Immobilisation/lethality (%)			Mean	SD	SEM (Std. error)	LC50 (mg/L)
CMT: Average Natural seawater	0.00	20.00	18.19	7.69	15.29	5.43	2.22	5.09
	25.00	50.00	75.00	62.50	62.50	10.21	4.17	
	50.00	83.33	80.00	100.00	87.78	8.75	3.57	
	75.00	100.00	55.56	75.00	76.85	18.19	7.43	
	100.00	77.78	100.00	57.14	78.31	17.50	7.14	

Table 1: *Artemia sp.* immobilisation assay replicates data for artificial seawater contaminated with coumestol (CMT) with mean values of immobilisation (%) and mean standard errors, (n = 6)

Chemical Substance	Conc. (mg/L)	Immobilisation/lethality (%)						Mean	SD	SEM (Std. error)	LC50 (mg/L)
CMT: Average Artificial seawater	0.00	20.00	25.00	10.00	10.00	10.00	0.00	12.50	8.04	3.28	39.87
	5.00	58.33	40.00	22.22	20.00	10.00	20.00	28.43	16.06	6.56	
	25.00	50.00	66.66	66.66	55.56	40.00	40.00	53.15	11.00	4.49	
	50.00	0.00	87.50	88.89	40.00	40.00	60.00	52.73	30.75	12.55	
	75.00	70.00	81.82	88.89	80.00	45.45	77.78	73.99	13.93	5.69	
	100.00	80.00	50.00	71.43	80.00	60.00	70.00	68.57	10.72	4.38	

APPENDIX 2

List of Corrections:

Request 1: Page 16 incorrectly states that caffeine has a molecular weight of 194 g/mol (molar mass)

Correction: "molecular weight" replaced with "molar mass" in text.

Request 2: On page 31, in point 2.1 Sample collection and preservation, sampling was not carried out by EPAL sampling technicians.

Correction: Clarity provided on the sampling by replacing

"Samples were collected by EPAL (Empresa Portuguesa das Águas Livres, SA) employees into clean 1 L amber glass bottles and stored in ice-cooled sample transport boxes and transferred refrigerated to EPAL in Lisbon, using the internal transport of the Águas de Portugal group to which both companies belong."

With

"In collaboration with the team of regular samplings from Águas do Algarve (AdA, group "Águas do Algarve"), the CCMAR researchers collected the raw sewage, effluents or environmental samples above specified into clean 1 L amber glass bottles, which were stored in ice-cooled sample transport boxes. These samples were then sent by AdA for delivery in Lisboa at EPAL (Empresa Portuguesa das Águas Livres SA), using the internal transport of the Águas de Portugal group to which both companies belong.".

Request 3: On page 32, in point 2.2 Reagents and Materials, the antibiotics azithromycin and clarithromycin are not mentioned.

Correction: Antibiotics azithromycin and clarithromycin added to the list of reagents with their supplier details.

Request 4: On page 32, in point 2.2 Reagents and Materials, the concentrations of the individual stock solutions of 1 mg/L and 2 mg/L are not correct, since it then appears that the concentration of the combined solution is 6 mg/L.

Correction: The reagent concentration sentence rephrased to represent the actual concentrations from

"Individual stock solutions in methanol (1 mg/L) were prepared for each compound, except

ibuprofen where 2 mg/L was prepared. A joint stock solution of the standards was prepared by mixing all compounds, and the hormones with concentration 6 mg/L."

to,

"Individual stock solutions were prepared in methanol for each compound and a joint standard solution was prepared by diluting each individual standard solution in methanol to have a concentration of 2 mg/L for ibuprofen, 6 mg/L for the hormones and 1 mg/L for the rest of the compounds."

Request 5: Then it is incorrectly stated that the standard addition method is carried out with UP water. In reality, this analysis is carried out using matrix-adjusted calibration, in which the calibration curve is carried out on the sample itself to minimise the effects of the matrix on the instrumental analysis by mass spectrometry.

Correction: Correction made by replacing sentence that states,

"The working solutions for the calibration and standard addition curves were prepared in ultra-purified water produced using Milli-Q, Academic A-10 model from Millipore (France)."

With,

"All working solutions for the calibration and standard addition curves were prepared in adjusted matrix (wastewater) to compensate for matrix effects that may influence the mass spectrometry results."

Requests 6 & 7:

6) On pages 33 and 34, in point 2.3.2 LC-MS/MS, the methodology for confirming the identity of compounds in a sample by means of chromatographic retention time and the use of 2 MRM transitions for each compound in the MS is not indicated. It is important to indicate the values of the MRM transitions of each compound, which allow the identification of these compounds in a sample and guarantee the high selectivity of the test method.

7) Section 2.3.2 LC-MS/MS, pages 33 and 34, should mention the method's limits of quantification for the compounds analysed, as this information is extremely useful for assessing the sensitivity of the method.

Corrections: Table 2.1 added in 2.3.2 with the requested information, limits of quantification of the method, the retention times for the specific PhC chromatography peaks, to confirm the results obtained were positive.

Requests 8 & 9:

8) Page 52 states that fluoxetine was not detected in the samples during the monitoring campaigns, probably due to the fact that it undergoes degradation processes, but that improving the sensitivity of the analysis method should also be considered.

9) However, the limit of quantification for fluoxetine is 0.07 ug/L, which is quite acceptable for this type of wastewater sample. For example, hormones have a LOQ of 0.4 ug/L. The compound with the lowest LOQ is, for example, acetaminophen with 0.03 ug/L

Correction: Information about improving the sensitivity of the analysis method deleted. Initial sentence,

"Thus, fluoxetine in wastewater may potentially exist in its metabolite state and/or in very small quantities, hence it should be measured in both states and an improvement on the analytical method sensitivity should be considered"

corrected sentence,

"Thus, fluoxetine in wastewater may potentially exist in its metabolite state and/or in very small quantities, hence a possible complementary approach would be to develop quantification methods also for its main metabolites."

Request 10: On page 41, it refers to the levels of diclofenac found in the Ria Formosa, mentioning Figure 3.4. It's actually Figure 3.6

Correction: Reference to a correct figure made, replacing "Figure 3.4." with "Figure 3.6.".

Additional corrections:

1. Page 57, replacement of "H2O2" with "H_2O_2" for a correct chemical formula of hydrogen peroxide.
2. Correction of axis labelling from (_g/L) to (µg/L) in Figures 3.3, 3.5, 3.7, and 3.8.