André Monteiro Pais Teixeira Pereira

RISK ASSESSMENT OF HUMAN PHARMACEUTICALS IN THE PORTUGUESE AQUATIC ENVIRONMENT

Doctoral Thesis in Pharmaceutical Sciences, specialization in Bromatology and Hidrology, supervised by Professor Angelina Lopes Simões Pena, Professor Celeste de Matos Lino and Professor Maria Leonor Martinho Ferreira Meisel and presented to the Faculty of Pharmacy of the University of Coimbra

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Risk assessment of human pharmaceuticals in the Portuguese aquatic environment

Avaliação do risco de medicamentos de uso humano no meio aquático Português

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This work was conducted at the Laboratory of Bromatology and Farmacognosy of the Faculty of Pharmacy of the University of Coimbra, supervised by Professor Angelina Lopes Simões Pena, Professor Celeste de Matos Lino and Professor Maria Leonor Martinho Ferreira Meisel.

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COMPETE
PROGRAMA OPERACIONAL FACTORES DE COMPETITIVIDADE

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QUADRO DE REFERÊNCIA ESTRATÉGICO NACIONAL

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Acknowledgments

After graduating in the Faculty of Pharmacy of the University of Coimbra in 2004, like most of my colleagues I registered as a pharmacist. However, I soon realized that this did not completely fulfil me and, in 2006, I found myself coming back to University of Coimbra and enrolled in a Masters in Public Health (Faculty of Medicine of the University of Coimbra). I developed my Master Thesis in the Laboratory of Bromatology and Hidrology in the Faculty of Pharmacy of the University of Coimbra where I reencountered Professor Angelina Pena and Professor Celeste Lino, who were my supervisors. Somehow, I felt challenged studying and working on the laboratory pushing my own boundaries, something I had not felt working in the pharmacy and it was exactly what I was looking for. After my Master Thesis, I had the opportunity to apply to an investigation scholarship, and enrolled fulltime in my PhD, leaving behind my practice as a community pharmacist.

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Abstract

Potential risks associated with releases of pharmaceuticals into the environment have become an increasingly important issue in environmental health. This concern has been driven by the widespread detection of pharmaceuticals in all aquatic environmental compartments, including wastewater and surface waters. Human pharmaceuticals are emergent contaminants that are continuously introduced in the environment and wastewaters are regarded as the main route of entry. Albeit detected in trace amounts, they are of concern since they are designed to perform a biological effect and can promote deleterious consequences at low concentrations in aquatic biota.

There is little knowledge on pharmaceuticals environmental occurrence, fate and exposure in the Portuguese aquatic environment, important issues for a proper risk assessment that must be tackled to meet the Water Framework Directive (WFD) of the European Union (EU). Therefore, the aim of the present work was to evaluate the occurrence, fate and environmental risk assessment (ERA) of human pharmaceuticals in the Portuguese aquatic compartment, selected from the most prescribed and chronically consumed.

Samples from 15 wastewater treatment plants (WWTPs) influents (WWIs) and effluents (WWEs), from five different Portuguese regions were collected during four sampling campaigns and were assessed through solid phase extraction (SPE) and liquid chromatography coupled to tandem mass detection (LC-MS*n*). A contamination mapping, encompassing temporal and spatial variation, and the ERA of the presence of pharmaceuticals in wastewaters were accomplished. Additionally, based on WWTPs measured data, the most impacted surface waters were selected to set monitoring stations, as required by the Directive 2013/39/EU.

To further evaluate the influence of WWEs, temporal variations and the impact of surface waters flow rates in pharmaceutical concentrations, surface waters from the most vulnerable areas were collected from 20 sites, upstream and downstream the selected WWTPs, during two sampling campaigns, and were assessed through SPE followed by LC-MS*n*. Moreover the ERA was performed providing the risk characterization for the Portuguese surface waters.

The results obtained showed that pharmaceuticals are ubiquitous in Portuguese WWTPs, with WWIs presenting higher concentration (up to 150 μg L^{-1}) than WWEs (up to 33 μg L^{-1}). Temporal and geographical variations were detected, with winter season and Alentejo and Algarve regions presenting higher contamination levels. Additionally, risk quotients (RQs) higher than one and up to 469 were observed for seven pharmaceuticals in WWEs, posing possible risk to the aquatic biota. Based on these results, the rivers Mondego, Tagus, Ave,

Trancão, Fervença and Xarrama were selected as surface waters monitoring stations, since they were expected to present higher concentrations.

Additionally, based on the previous obtained data, suggestions were made to improve the European Medicines Agency (EMA) Guideline on ERA. This includes changing some parameters in the predicted environmental concentrations (PECs) calculation, such as the default value of the penetration factor (Fpen) from 0.01 to 0.04, adding a safety factor of 10 and account for national consumption and excretion data (using worst-case scenario). This would enable a more accurate ERA, strengthening the protection of the environment against pharmaceutical contamination.

Finally, surface waters evaluation showed 27.8% of contamination, with an increase in frequency and concentration levels downstream WWTPs, during summer and in smaller rivers. This proved that WWTPs are a major source of pharmaceuticals contamination in surface waters and that the river flow rates significantly influence the pharmaceuticals concentration in this water compartment. In drought periods, flow rates may decrease at least ten times comparing to the lowest value observed at the time of our sampling campaigns, and, consequently, the concentrations of pharmaceuticals could increase in the same proportion. When using these data to perform the ERA, RQs higher than 0.1 would be observed for all of the 11 detected pharmaceuticals and, from these, 5 should present RQs higher than one, posing the aquatic biota at risk.

Overall, these results present a global picture of the pharmaceuticals contamination and ERA of the Portuguese aquatic environment, an important input for setting prioritizing measures and sustainable strategies, to minimize their impact in the aquatic environment.

Keywords:
Environmental contaminants; pharmaceuticals; environmental risk assessment; wastewater treatment plants; surface waters.

Resumo

O problema dos potenciais riscos associados à disseminação de fármacos no meio ambiente tem vindo a adquirir uma importância crescente no âmbito da saúde ambiental. Esta preocupação tem sido impulsionada pela detecção generalizadade fármacos em todos os compartimentos aquáticos, incluindo águas residuais e águas de superfície. Os fármacos para uso humano são contaminantes emergentes, continuamente introduzidos no meio aquático, sendo as águas residuais consideradas a sua principal via de contaminação. Embora detectados em concentrações residuais, representam motivo de preocupação, uma vez que são desenvolvidos para produzir um efeito biológico e mesmo em concentrações baixas possuem aptidão para promover efeitos deletérios em organismos aquáticos.

A escassez de dados sobre a ocorrência, destino e exposição a fármacos no ambiente aquático português impossibilita uma correcta avaliação do risco para cumprimento da Directiva-Quadro da Água (WFD). Assim, o objectivo do presente trabalho foi avaliar a ocorrência, o destino e o risco ambiental de fármacos para uso humano no meio aquático português, seleccionados entre os mais prescritos e consumidos cronicamente.

Primeiramente, ao longo de quatro períodos de amostragem, foram recolhidas amostras de afluentes e efluentes em 15 estações de tratamento de águas residuais (ETARs) provenientes de cinco regiões portuguesas, as quais foram avaliadas através de extracção em fase sólida (SPE) e cromatografia líquida acoplada a detecção por massa (LC-MS*n*). Foi elaborado um mapa de contaminação, contemplando variações temporais e geográficas, e foi avaliado o risco ambiental relativo à presença de fármacos em águas residuais. Adicionalmente, com base nos dados obtidos nas ETARs, foram seleccionadas as águas de superfície potencialmente mais contaminadas para estabelecer estações de monitorização, de acordo com a Directiva 2013/39/UE.

Para confirmar a influência dos efluentes das ETARs e para verificar as variações temporais e o impacto do caudal das águas de superfície nas concentrações dos fármacos, foram analisadas águas de superfície dos 20 locais mais vulneráveis, a montante e a jusante das ETARs seleccionadas, durante dois períodos de amostragem, e foram avaliadas através de SPE seguida de LC-MS*n*. Foi ainda realizada a avaliação de risco ambiental (ERA), caracterizando o risco para as águas de superfície portuguesas.

Os resultados alcançados mostraram que os fármacos estão omnipresentes nas ETARs portuguesas, com os afluentes a apresentar uma concentração mais elevada (até 150 μg L^{-1}) do

que os efluentes (até 33 μg L^{-1}). Foram detectadas variações temporais e geográficas, com o inverno e as regiões do Alentejo e Algarve a apresentar níveis de contaminação mais elevados. Foram ainda observados quocientes de risco (RQ) superiores a um e até 469 para sete fármacos nos efluentes das ETARs, o que representa um risco potencial para os organismos aquáticos. Com base nestes resultados, foram seleccionados como estações de monitorização de águas de superfície os rios Mondego, Tejo, Ave, Trancão, Fervença e Xarrama, uma vez que seria expectável que estes apresentassem as concentrações mais elevadas.

Com base nos dados obtidos, foram feitas sugestões para melhorar a *guideline* da Agência Europeia do Medicamento (EMA) sobre a ERA, tendo sido proposto alterar alguns parâmetros no cálculo das concentrações ambientais previstas (PECs), tais como o valor padrão do fator de penetração (Fpen) de 0.01 para 0.04, adicionar um factor de segurança de 10 e incluir os dados de consumo nacional e de excreção humana. As alterações propostas permitiriam uma ERA mais precisa, reforçando a protecção do ambiente contra a contaminação por fármacos.

Por fim, analisadas as águas de superfície, estas apresentaram 27,8% de contaminação, tendo-se verificado um aumento da frequência e dos níveis de concentração a jusante das ETARs, durante o verão e em rios com menor caudal. Isto demostrou que as ETARs são uma importante fonte de contaminação de fármacos em águas de superfície e que o caudal dos rios influencia significativamente a sua concentração neste compartimento aquático. Com efeito, em períodos de seca, os caudais podem ser pelo menos dez vezes menores do que o menor caudal observado nos dias de amostragem, pelo que, consequentemente, aquela concentração pode aumentar na mesma proporção. Por conseguinte, com base neste pressuposto, num período de seca, seriam observados RQs superiores a 0,1 para os 11 fármacos detectados e, entre estes, 5 apresentariam RQs superiores a um, colocando os organismos aquáticos em risco.

Em termos globais, os resultados obtidos apresentam um quadro geral da contaminação por fármacos e respectiva ERA no meio aquático português, pelo que representam um importante contributo para a definição de medidas prioritárias e estratégias sustentáveis com vista à minimização do seu impacto no meio aquático.

Palavras-chave:

Contaminantes ambientais; fármacos; avaliação de risco ambiental; estações de tratamento de águas residuais; águas de superfície.

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List of Abbreviations

4-OH-DIC – 4′-Hydroxydiclofenac

4-PARA – 4-aminophenol

ADI – Admissible daily intake

ALP – Alprazolam

Antib – Antibiotics

Antiepi – Antiepileptics

Anti-inf – Anti-inflammatories and or analgesics

Anx – Anxiolytics

AZI – Azithromycin

BEZ – Bezafibrate

BOD – Biochemical oxygen demand

CAR – Carbamazepine

CID – Collision induced dissociation

CIP – Ciprofloxacin

CIT – Citalopram

COD – Chemical oxygen demand

CLA – Clarithromycin

DDD – Defined daily dose

DIC – Diclofenac

DOSEai – Maximum daily dose

E1 – Estrone

E2 – 17β-estradiol

EC50 – Effective concentration

EE2 – 17α-ethinylestradiol

EMA – European Medicines Agency

ERA – Environmental risk assessment

ERY – Erythromycin

ESC – Escitalopram

ESI – Electrospray ionization

EU – European Union

FLU – Fluoxetine

Fpen – Penetration factor

GEM – Gemfibrozil

Horm – Hormones

HRT – Hydraulic retention time

IBU – Ibuprofen

INFARMED – National Authority of Medicines and Health Products

LC50 – Lethal concentration

LC-MS*n* – Liquid chromatography coupled to tandem mass detection

Lip reg – Lipid regulators

LOEC – Lowest observed effect concentration

log D_{ow} – pH-dependent n-octanol-water distribution ratio

log K_{oc} – Soil organic carbon-water partitioning coefficient

log K_{ow} – Octanol-water partitioning coefficient

LOR – Lorazepam

LTD – Lowest daily therapeutic dose

MDL – Method detection limit

ME – Matrix effects

MEC – Measured environmental concentration

MQL – Method quantification limit

MRM – Multiple reaction monitoring

NAP – Naproxen

N-CIT – Desmethylcitalopram

NOEC – No observed effect concentration

Nor-FLU – Norfluoxetine

Nor-SER – Desmethylsertraline

OECD – Organization for Economic Co-operation and Development

PAR – Paroxetine

PARA – Paracetamol

PBT – Persistence bioaccumulation and toxicity

PEC – Predicted environmental concentration

pKa – Acid dissociation constant

PNEC – Predicted no-effect concentration

POD – Point of departure

RQ – Risk quotient

RSD – Relative standard deviation

SER – Sertraline

SIM – Simvastatin

SPE – Solid phase extraction

SRT – Solid retention time

SSRI – Selective serotonin re-uptake inhibitor

TSS – Total suspended solids

UF – Uncertainty factor

UV – Ultraviolet radiation

WASTEWinhab – Amount of wastewater produced per inhabitant per day

WFD – Water Framework Directive

WWE – Wastewater effluent

WWI – Wastewater influent

WWTP – Wastewater treatment plant

ZOL – Zolpidem

List of Publications

1- SILVA L.J.G., **PEREIRA A.M.P.T.**, MEISEL L.M., LINO C.M., PENA A.. A one-year follow-up analysis of antidepressants in Portuguese wastewaters: occurrence and fate, seasonal influence and risk assessment. *Science of the Total Environment*, 490, 279-287, **2014** (DOI: 10.1016/j.scitotenv.2014.04.131).

2- PEREIRA A.M.P.T., SILVA L.J.G., MEISEL L.M., LINO C.M., PENA A.. Environmental impact of pharmaceuticals from Portuguese wastewaters: geographical and seasonal occurrence, removal and risk assessment. *Environmental Research*, 136, 108–119, **2015** (DOI: 10.1016/j.envres.2014.09.041).

3- SILVA L.J.G., **PEREIRA A.M.P.T.**, MEISEL L.M., LINO C.M., PENA A.. Reviewing the serotonin reuptake inhibitors (SSRIs) footprint in the aquatic biota: uptake, bioaccumulation and ecotoxicology. *Environmental Pollution*, 197, 127-143, **2015** (DOI: 10.1016/j.envpol.2014.12.002).

4- PEREIRA A.M.P.T., SILVA L.J.G., LINO C.M., MEISEL L.M., PENA A.. Assessing environmental risk of pharmaceuticals in Portugal: an approach for the selection of the Portuguese monitoring stations in line with Directive 2013/39/EU. *Chemosphere*, 144, 2507- 2515, **2016** (DOI: 10.1016/j.chemosphere.2015.10.100).

5- PEREIRA A.M.P.T., SILVA L.J.G., LINO C.M., MEISEL L.M., PENA A.. A critical evaluation of different parameters for estimating pharmaceutical exposure seeking an improved environmental risk assessment. Submitted to *Chemosphere.*

6- PEREIRA A.M.P.T., SILVA L.J.G., LARANJEIRO C.S.M., MEISEL L.M., LINO C.M., PENA A.. Human pharmaceuticals in Portuguese rivers: the impact of water scarcity in the environmental risk. Submitted to *Science of the Total Environment.*

Objectives

The challenge and key driving force of this thesis was to assess the presence of pharmaceuticals in Portuguese wastewater treatment plants (WWTPs), as well as in the most impacted surface waters. The geographical/national contamination patterns and seasonal influence to assess vulnerable areas were also evaluated. Furthermore, an important outcome of this thesis was the evaluation of the potential ecotoxicological risk posed by these pharmaceuticals to different aquatic organisms, allowing a better perception of the environmental risk in the Portuguese context.

In order to do so, a strategy that encloses the following three main goals was established:

1. Perform regional/national contamination maps of the selected pharmaceuticals in 15 representative WWTPs, influents and effluent samples, in Portugal, in order to assess the most impacted areas due to human action. Data on their levels, seasonal and regional influence, and WWTPs removal efficiency were provided.

2. In line with the Directive 2013/39/EU and based on real data measured on WWTPs, the most impacted surface waters in Portugal were identified and evaluated for the presence of the selected pharmaceuticals.

3. Characterization of the environmental risk of the selected pharmaceuticals, based on the predicted no effect concentrations (PNECs), predicted environmental concentrations (PECs) and measured environmental concentrations (MECs), was performed. Their ratios were calculated in order to detect any substantial difference between the predicted and real environmental concentrations thus, improving, if necessary, the calculation of the PEC. Their comparison with the PNECs evaluated the risk posed by these pharmaceuticals to the different trophic levels. The results highlighted the possible environmental risk for each substance.

This Portuguese surveillance model may contribute to establish a sustainable strategy to minimize the environmental risk of these pharmaceuticals.

Thesis Organization

The present thesis includes all the work developed under the scope of the doctoral project. It was divided in four parts encompassing a total of six chapters that enclose 6 scientific articles, of which 4 are already published and 2 are submitted, all to international peer-review journals (see page xi Table of contents).

For all the articles, the original structures were maintained in agreement with the journal guidelines where they were published or submitted. On the other hand, references, numbers of figures, tables and equations were standardized and numbered consecutively throughout the thesis, not maintaining the original format of the publications. Moreover, one part of the publication of Chapter V was included in Chapter I, since the subject matter was also a part of the theoretical background.

In **Part A, Chapter I** a theoretical background is given, which summarizes the published data, reported in the scientific literature, regarding the sources and fate of pharmaceuticals in the environment, their occurrence, toxicity and environmental risk assessment (ERA). This state-of-the-art review clarifies and emphasizes all the main issues regarding the presence of pharmaceuticals in the aquatic environment.

The experimental work developed during this doctoral project is present in **Part B**, divided in five chapters, which correspond to five scientific articles. In **Chapter II**, **III** and **IV**, several pharmaceuticals, belonging to different therapeutic groups, were studied in wastewater treatment plants (WWTPs) influents (WWIs) and effluents (WWEs), since these facilities are the major source of contamination of pharmaceuticals in the aquatic environment. This allowed to identify differences in contamination patterns regarding each pharmaceutical, each therapeutical group, temporal and geographical variations, removal efficiencies, to identify the receiving surface waters more impacted by the effluents discharges and to perform the ERA.

After evaluating the data obtained in wastewaters and observing the legislation regarding the presence of pharmaceuticals in the aquatic environment, especially the Guideline on the environmental risk assessment of medicinal products for human use, a critical evaluation of this guideline (**Chapter V**) was undertaken, raising awareness to this subject by suggesting improvements to minimize the possible environmental risk.

In **Chapter VI**, the surface waters belonging to the most impacted rivers from WWEs were assessed regarding the presence of pharmaceuticals. Influence of WWTPs and flow rates on pharmaceuticals frequencies and concentrations were evaluated together with the ERA.

In **Part C**, a general discussion is presented, including the main conclusion and achievements of the work as well as future prospects.
Finally, in **Part D**, all the references used throughout the thesis are listed.

Part A – Theoretical background

Chapter I – Theoretical background

I1. Introduction

Human pharmaceuticals, presenting different characteristics and, consequently, producing different environmental exposure profiles, represent a group of widely used chemicals that contaminate the aquatic environment. Albeit in trace amounts, they are of concern since they are designed to perform a biological effect. Moreover, given their continuous introduction into the environment, their impact, both as stressors and as agents of change, is of great importance [1].

The environmental impact of medicinal products has been recognized worldwide, and as its use cannot be avoided, a sound risk assessment of their presence in the environment is a key issue that must be tackled to meet the European Union (EU) Water Framework Directive (WFD) [2]. The potential for negative ecotoxicological effects, even at sublethal concentrations, in the aquatic environment has been of concern since the issue was first brought to attention in 1985 [3]. Nonetheless, the ecotoxicological risks associated to the ubiquitous occurrence of pharmaceuticals in aquatic ecosystems are far from being fully known [4].

The main source of pharmaceuticals residues in the aquatic environment is human excretion, and consequently, the widespread presence of pharmaceuticals in environmental samples is most likely to occur from wastewater treatment plants (WWTPs), which incompletely remove these compounds. Pharmaceuticals are then released into the environment as parent compounds, metabolites, as well as transformation products [5], leading to the contamination of surface waters, seawaters, groundwater and even some drinking waters, already identified by new analytical methodologies which allowed the detection at low ng L^{-1} [6–13].

Although no legal limits have been established in water, six pharmaceuticals and one metabolite became part of the WFD watch list established by the Directive 2013/39/EU and the recent Commission Implementing Decision from the EU 2015/495. This list is dynamic, changing with the awareness on the persistence in the water cycle and its validity in time is limited. Therefore, identifying and prioritizing new pharmaceuticals are important goals to be accomplished for future updates in order to minimize the aquatic environmental contamination by pharmaceuticals [14]. Also, as a part of the strategy implemented by the Directive

2013/39/EU, all member states shall monitor the substances in the watch list at the selected surface waters representative monitoring stations.

According to the European Medicines Agency (EMA) legislation, and since 2006, before a pharmaceutical obtains a marketing authorisation approval, it must be demonstrated that it poses no risk to the environment through an environmental risk assessment (ERA). ERA compares the predicted environmental concentrations (PECs), with the predicted no effect concentrations (PNECs) of three trophic levels of aquatic organisms [15,16]. Therefore, high-quality monitoring data, to assess the validity of PECs, along with data on ecotoxicological and toxicological effects are crucial to perform the ERA, which associates the presence of pharmaceuticals with their impact on the aquatic mesocosm and human health, supporting the selection of possible new priority substances to be monitored [1,17,18].

In Portugal, heavy contamination pressures from extensive urban activities characterize the main rivers that might lead to high aquatic contamination levels and consequent environmental and human exposure. Although the concentrations of pharmaceuticals in influents (WWIs) and effluents (WWEs) of WWTPs and surface waters are routinely monitored in many countries, only in recent years there has been an increase in the number of studies concerning the occurrence of pharmaceuticals in the Portuguese aquatic environment [19–23]. However, most of these studies are primarily focused on a small number of targeted compounds in localized areas. Therefore, there is a knowledge gap which demands a comprehensive and systematic evaluation of pharmaceuticals, its metabolites and transformation products in the Portuguese aquatic environment.

Thus, a systematic and nationwide monitoring programme is necessary, in order to provide a clear insight on pharmaceuticals contamination of the water compartment, embracing, not only several parent compounds, but also, metabolites and transformations products belonging to different therapeutic groups, including: the anxiolytics and hypnotics, further referred only as anxiolytics, alprazolam (ALP), lorazepam (LOR) and zolpidem (ZOL); the antibiotics azithromycin (AZI), ciprofloxacin (CIP), clarithromycin (CLA) and erythromycin (ERY); the lipid regulators bezafibrate (BEZ), gemfibrozil (GEM) and simvastatin (SIM); the antiepileptic carbamazepine (CAR); the selective serotonin re-uptake inhibitors (SSRIs) citalopram (CIT) and its main metabolite desmethylcitalopram (N-CIT), escitalopram (ESC), fluoxetine (FLU) and its main metabolite norfluoxetine (Nor-FLU), paroxetine (PAR), sertraline (SER) and its main metabolite desmethylsertraline (Nor-SER); the anti-inflammatories and/or analgesics, further referred only as anti-inflammatories, diclofenac (DIC) and its main metabolite 4-hydroxydiclofenac (4-OH-DIC), ibuprofen (IBU), naproxen (NAP), paracetamol (PARA) and

its transformation product 4-aminophenol (4-PARA); and the hormones 17β-estradiol (E2) and its main metabolite estrone (E1) and 17α-ethinylestradiol (EE2). The pharmaceuticals in study, key representatives of major classes of pharmaceuticals, were selected based on their high consumption, pharmacokinetics, physicochemical properties, persistence, previous studies on the occurrence on WWTPs and surface waters, and their potential toxicological impact, both on humans and on the aquatic environment [14,24–26]. This monitoring would provide a more realistic water quality assessment in Portugal contributing for a more integrative approach to rank and prioritize pharmaceuticals, based on an integrated assessment of ERA and exposure of surface water.

In a larger vision of future water resource management sustainability, with the escalating population growth and intensified agricultural and industrial activity, water scarcity will be a reality [27,28]. Therefore, there will be the need for water/wastewater recycling and the contamination of water resources by pharmaceuticals gains yet another perspective, since a good ecological status is currently achieved in only 43% of the reported freshwater bodies [29]. *"Water is not a commercial product like any other but, rather, a heritage which must be protected, defended and treated as such"*, the claim by the EU WFD contrasts with a poor ecological status in many European rivers and lakes. In addition, and despite the enormous efforts, the picture that emerges regarding ecological and chemical status is still incomplete, fragmented and with contradictory assessments of the situation. Therefore, it is important to obtain a better understanding of the regional and global context, concerning the environmental risk posed by pharmaceuticals in the aquatic environment.

I2. Sources and fate of pharmaceuticals in the environment

I2.1. Sources

Pharmaceuticals are widely consumed throughout the world and can reach the aquatic environment, primarily through human excretion or by direct disposal of unused or expired drugs in toilets, being WWTPs considered the primary sources of these contaminants into the water bodies (Figure 1) [24,30]. Although they are administered within healthcare facilities, namely, hospitals, nursing, assisted living and independent living healthcare facilities, its contribution to the input of pharmaceuticals into the municipal WWTPs is quite low since these

facilities typically make a small contribution to the overall load [6,31,32]. The hospital contribution to the total load of pharmaceuticals in municipal WWTPs is for most compounds under 10% and usually, even below 3% [12]. However, wastewaters from drug production can be a potentially source of pharmaceuticals in certain locations, namely in major production areas for the global bulk drug market [9]. Finally, veterinary medicines can also enter the environment, however, their environmental exposure routes and fate differ from human pharmaceuticals [25,33].

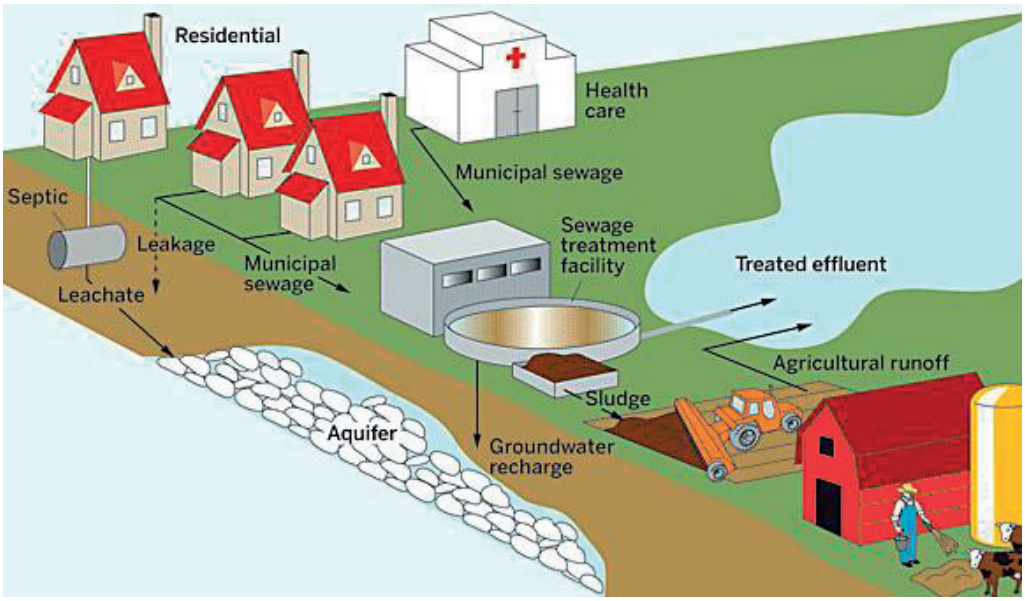


Figure 1. Primary sources and aquatic contamination of pharmaceuticals.
(Adapted from http://www.eusem.com/main/CE/SIP_C3_bg)

Thus, these drugs, their metabolites and/or transformation products may enter the environment via WWTPs effluents or by land application of biosolids, originating from WWTPs sludges, which through runoff or leaching can enter the aquatic environment, surface or groundwaters [3]. It is important to highlight that the EU banned disposal of sewage sludge at sea in 1998, and since then, its application rate to land has risen significantly [34].

I2.2. Consumption patterns

The presence of pharmaceuticals in the environment generally correlates well with the amount used in human medicine. Therefore, these data can be used to identify pharmaceuticals that may pose a risk to the environment [35]. An accurate estimate of the extent of drug exposure in a

population is difficult in most countries, as precise consumption data are often lacking. In addition, the statistics frequently cover prescription drugs only and do not include over-the-counter medicines or hospital use of pharmaceuticals [36].

Nevertheless, for several reasons, consumption of pharmaceuticals is expected to increase and thus increasing the burden of their presence in the environment. First, as the number of older people is rising, with frequent therapeutic regimes of five or more medicines, the extensive use of pharmaceuticals will also increase. In addition, with a rise in living standards and with a decrease in pharmaceuticals price, their usage will escalate throughout the world [12].

Bearing in mind the available data on antidepressants (Figure 2 (A)) and lipid regulators (Figure 2 (B)) provided by the Organization for economic co-operation and development (OECD), in defined daily dose (DDD), which is calculated per 1000 inhabitants per day, the increased consumption from 2000 to 2013 is clear [37]. Although Portugal is below the OECD average on economic indicators, the consumption of antidepressants and lipid regulators was above the OECD average, as seen in both charts. In fact, in 2013, Portugal was the third country with the highest consumption of antidepressants, being SSRIs the most representative of this therapeutic group. These figures may indicate the same trend for other therapeutic groups [37,38].

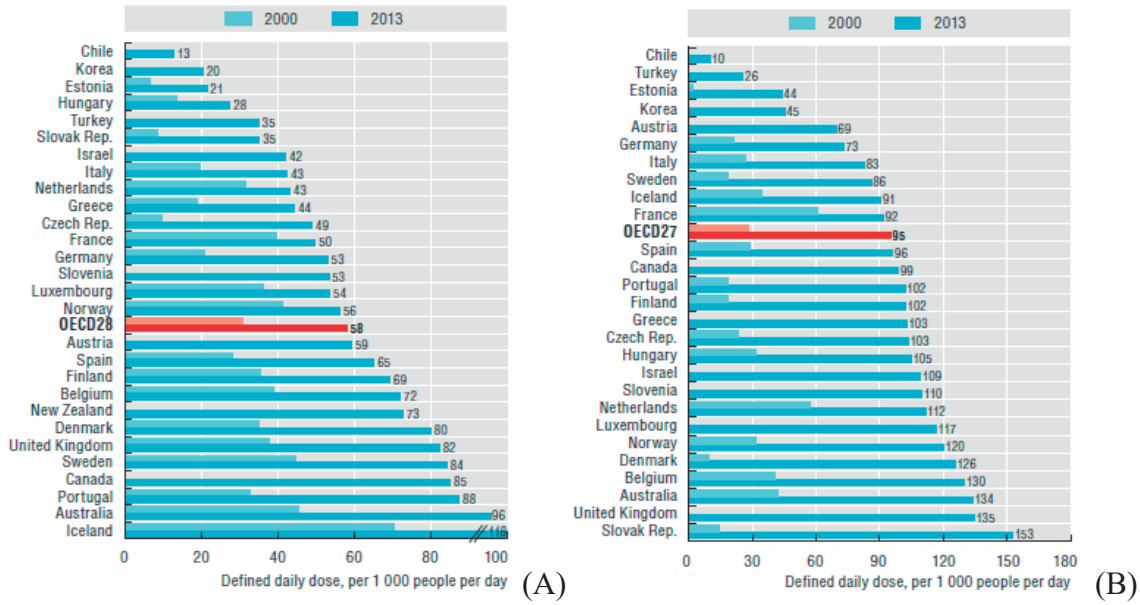


Figure 2. Pharmaceutical consumption data for antidepressants (A) and for lipid regulators (B) (OECD).

However, the correlation between consumption data and environmental contamination is related to the amount consumed per year (kg y^{-1}), which may not correspond to an higher DDD, that varies widely between pharmaceuticals. For example, in 2000, approximately 100 million women worldwide were current users of combined hormonal contraceptives, however, since the DDD is very low for hormones, this will not correlate with the amount sold in kg [38].

When observing the pharmaceuticals consumption data on other European countries (Table 1), namely the amount consumed per year, we can realize that the amount used in Switzerland and Sweden is lower than the rest of the countries. This is explained by the fact that they have a significant lower population when compared to the other countries referred in Table 1 (Germany, France, Italy and Spain).

Besides the differences in population, different patterns are also observed between countries, even within each therapeutic group, however some trends are clear regarding the global consumption of therapeutic groups. Anti-inflammatories are clearly the group with higher consumption (in kg), being PARA the pharmaceutical with the highest consumption. This group is followed by the antiepileptic CAR with particular high values in Germany. Antibiotics and lipid regulators have similar consumption patterns, nonetheless, these groups have great variations within them, showing distinct trends in different countries. Anxiolytics, SSRIs and hormones, in decreasing order, were the therapeutic groups with lowest consumption.

Table 1. International consumption of the selected pharmaceuticals.

Therapeutic group	Pharmaceutical	DDD 1000 inh^{-1} d^{-1}	mg inh^{-1} y^{-1}	kg y^{-1}	Year	Country	Reference
Anx	ALP	17.64[a]	6.4[a]	302[a]	2010	Spain	[33]
		NA	2.9	178	2004	France	
	LOR	19.67[a]	17.9	844	2010	Spain	[33]
		NA	9.6	585	2004	France	
		13.3	NA	709	2010	Italy	[8]
Antib	AZI	0.9[a]	98.6	4634[a]	2010	Spain	[33]
		NA	67.1	4073	2004	France	
		NA	NA	13870	2010	Italy	[39]
		1.3	NA	13870	2010	Italy	[8]
	CIP	1.1[a]	401.5	18870[a]	2010	Spain	[33]
		NA	200.7	12186	2004	France	
		NA	NA	21672	2010	Italy	[39]
		1.0	NA	21672	2010	Italy	[8]
	CLA	0.6[a]	231.0	10864[a]	2010	Spain	[33]
		NA	150	12360	2010	Germany	
		NA	232.9	1700	2010	Switzerland	
		NA	276.1	16889	2010	France	
		NA	NA	64470	2010	Italy	[39]
		3.0	NA	64470	2010	Italy	[8]
	ERY	0.1[a]	NA	1716a	2010	Spain	[33]
		NA	NA	0.12	2010	Italy	[39]
Lip reg	BEZ	0.6[a]	133.0[a]	6178[a]	2010	Spain	[33]
		NA	475.2	39158	2010	Germany	
		NA	215.6	1574	2010	Switzerland	
		NA	343.4	20852	2004	France	
		NA	66.7	NA	2005	Sweden	
		NA	NA	7600	2001	Italy	[8]
	SIM	NA	282.7[a]	13340[a]	2010	Spain	[33]
		NA	114.3	6943	2004	France	
Antiepi	CAR	1.2[a]	438.0	20595	2010	Spain	[33]
		NA	1010.9	83299	2010	Germany	
		NA	857.5	6260	2010	Switzerland	
		NA	554.3	33364	2010	France	
		NA	463.0	820	2005	Sweden	
		NA	NA	31190	2010	Italy	[39]
		NA	0.61–0.98	NA	2010	Europe	[40]
		NA	NA	31190	2010	Italy	[8]
		NA	NA	88000	2001	Germany	[1]
SSRIs	ESC	0.01[a]	38.8	1824[a]	2010	Spain	[33]
		NA	0.08	4.6	2004	France	
	FLU	0.02[a]	62.0	2914[a]	2010	Spain	[33]
		NA	61.6	3740	2004	France	

Table 1. International consumption of the selected pharmaceuticals. *(continued)*

Therapeutic group	Pharmaceutical	DDD 1000 inh^{-1} d^{-1}	mg inh^{-1} y^{-1}	kg y^{-1}	Year	Country	Reference
	PAR	0.02[a]	69.4	3264[a]	2010	Spain	[33]
		NA	90.8	5515	2004	France	
	SER	0.05[a]	102.1	4800[a]	2010	Spain	[33]
		NA	102.5	6224	2004	France	
Anti-inf	DIC	7.9[a]	369.9	17395[a]	2010	Spain	[33]
		NA	953.6	78579	2010	Germany	
		NA	934.1	6819	2010	Switzerland	
		NA	370.1	22640	2010	France	
		NA	375.9	NA	2005	Sweden	
		NA	60–880	NA	2009	Europe	[40]
		4.5	NA	9602	2010	Italy	[8]
		NA	NA	345000	2001	Germany	[1]
	IBU	NA	4647.5	218527	2010	Spain	[33]
		NA	3043.6	250792	2010	Germany	
		NA	3078.2	22471	2010	Switzerland	
		NA	953.8	58353	2010	France	
		NA	NA	7864	2005	Sweden	
		NA	NA	622000	2001	Germany	[1]
	NAP	5.15[a]	1205.9	56700[a]	2010	Spain	[33]
		NA	614.7	37332	2004	France	
	PARA	NA	22667.7	1065835	2010	Spain	[33]
		NA	54389.5	3303077	2004	France	
		NA	NA	836000	2001	Germany	[1]
Horm	E2	0.894[a]		12.6[a]	2010	Spain	[33]
	EE2	1.1969[a]	0.03	1.2[a]	2010	Spain	[33]
		NA	0.58	48.2	2001	Germany	
		NA	0.54	4.0	2000	Switzerland	
		NA	0.11	NA	2005	Sweden	

Anx - anxiolytics; Antib - antibiotics; Lip reg - lipid regulators; Antiepi - antiepileptics; Anti-inf - anti-inflammatories; Horm - hormones; NA - not available.
[a]) Estimated consumption.
Data on ZOL, GEM and CIT was not possible to obtain.

To estimate the Portuguese pharmaceutical consumption in 2013, the Portuguese National Authority of Medicines and Health Products (INFARMED) provided information on pharmaceutical sales data by package, pharmaceutical form and quantitative composition, all of which enabled us to calculate the amount of the active substance for each pharmaceutical in tonnes per year. All pharmaceutical forms and administration routes were included. The collected data refer to medicines dispensed by ambulatory pharmacies and in hospitals within the Portuguese National Health Service, as well as over-the-counter medicines [25,33]. It was

assumed that the entire amount of each product was consumed and that it was evenly distributed throughout the year and throughout the Portuguese population.

This set of data, which considers pharmaceuticals distributed by Portuguese hospitals and pharmacies, showed that 343 tonnes of the selected pharmaceuticals were dispensed in 2013, with pharmaceuticals dispensed from pharmacies accounting for 98% of the total pharmaceutical consumption.

Considering the consumption by different therapeutic groups of the pharmaceuticals chosen in this study, anti-inflammatories had markedly higher values, accounting for 314 tonnes per year, were followed by antibiotics (9.4 tonnes), lipid regulators (7.0 tonnes), antiepileptics (6.6 tonnes), SSRIs (5.9 tonnes), anxiolytics (0.7 tonnes) and hormones (0.003 tonnes), translating patterns slightly different from other European countries (Figure 3 (A)). PARA and IBU stand out from the other pharmaceuticals due to consumption rates of 214 and 83 tonnes per year, respectively, which are at least seven times higher than any of the other compounds (Figure 3 (A)). Besides anti-inflammatories, antibiotics, lipid regulators and SSRIs also had significant variations in consumption within each therapeutic group, being CIP, SIM and SER the pharmaceuticals with higher values for each group, respectively. Regarding temporal variation, higher consumption rates were observed in the first (96 tonnes) and fourth (88 tonnes) quarters of the year, mainly due to the consumption of anti-inflammatories and antibiotics; the other therapeutic groups presented the same consumption pattern throughout the year (Figure 3 (B)). One should note that there are often discrepancies between pharmaceuticals sold and those actually consumed, due to delays between sales and actual use of medication. Moreover patterns of local consumption might differ from those observed on a national scale [39,40].

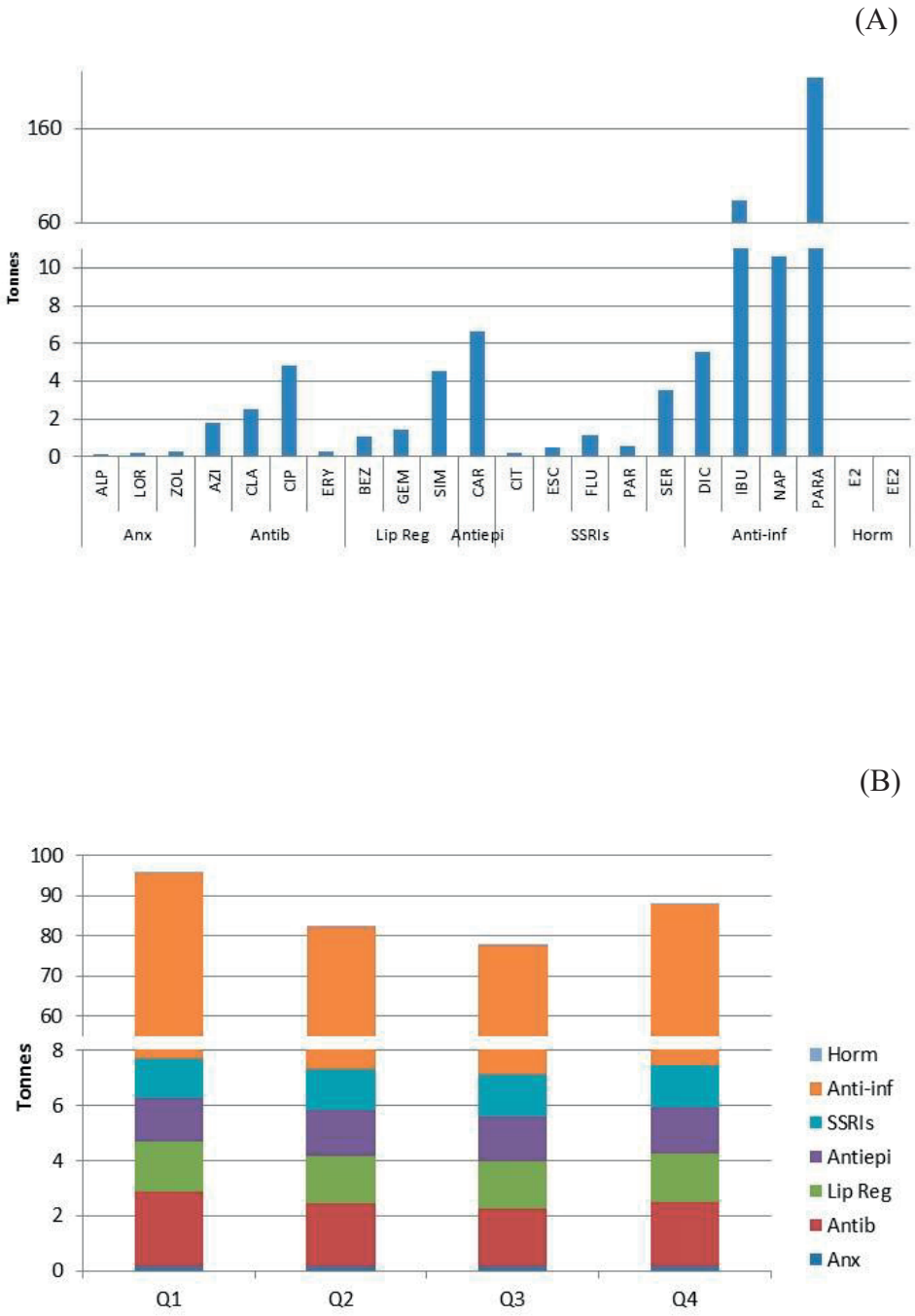


Figure 3. Portuguese consumption of pharmaceuticals (2013) by active compound (A) and for each quarter by therapeutic group (B).
(Anx - anxiolytics; Antib - antibiotics; Lip reg - lipid regulators; Antiepi - antiepileptics; Anti-inf - anti-inflammatories; Horm - hormones)

I2.3. Mechanism of action, metabolization and excretion

Pharmaceuticals have different mechanisms of action resulting in several therapeutical indications, which differ between therapeutic groups. However, within each group some variations can also occur since there are more than one class of pharmaceuticals in each group. The therapeutic group of anxiolytics include pharmaceuticals from the class of benzodiazepines like ALP and LOR which are used for numerous indications, including anxiety, insomnia, muscle relaxation, relief from spasticity caused by central nervous system pathology, and epilepsy. They act by binding to gamma-aminobutyric acid increasing its activity, reducing the excitability of neurons and promoting a calming effect on the brain [41]. Although the hypnotic ZOL is not a benzodiazepine, it also acts on gamma-aminobutyric acid, promoting a shorter effect than benzodiazepines [42].

The selected antibiotics belong to two different classes, fluoroquinolones (CIP) and macrolides (AZI, CLA and ERY), which inhibit bacterial growth. Fluoroquinolones act by inhibiting bacterial DNA synthesis and macrolides link to the bacterial ribosomes, inhibiting protein biosynthesis [43,44].

Lipid regulators drugs are used to treat dyslipidaemias, primarily raised cholesterol. Statins like SIM have the capacity to reduce the endogenous cholesterol synthesis, by inhibiting the principal enzyme involved. The fibrates (BEZ and GEM) increase the expression of some proteins in the liver, which results in a substantial decrease in plasma triglycerides and is usually associated with a moderate decrease in cholesterol concentrations [45,46].

The antiepileptic CAR has been extensively used in the treatment of epilepsy, as well as in the treatment of neuropathic pain and affective disorders, mainly due to the inhibition of sodium channel activity [47].

The SSRIs (CIT, ESC, FLU, PAR and SER) are antidepressants that, via inhibition of the serotonin reuptake mechanism, induce an increase in serotonin concentration within the central nervous system [48]. It should be noticed that CIT is a racemic mixture of *R*-citalopram and *S*-citalopram enantiomers with different potencies, but since *S*-citalopram is more potent it is also marketed as the single *S*-enantiomer formulation, ESC [49].

The anti-inflammatories DIC, IBU and NAP are non-steroids and their mechanism of action is through inhibition of cyclooxygenase (1 and 2) in periphery and central nervous system, reducing pain, inflammation but also other physiologic processes [50]. As for PARA, it acts on cyclooxygenase (2 and 3) in the central nervous system and only reduces pain and fever [51].

Finally, the hormones E1 and E2 are estrogens sex hormones, mainly female, and although they regulate the reproductive system they also act in very different endocrine systems. As pharmaceuticals, E2 is mostly used in hormone replacement therapy and EE2, a synthetic hormone more potent than E2, is primarily used in oral contraception [52,53].

According to other authors pharmacokinetic data could provide a better knowledge of the environmental fate of pharmaceuticals, especially in the water compartment [35,54].

After consumption, pharmaceuticals are metabolized and primarily excreted in urine and faeces as a mixture of the parent compound and its metabolites. The elimination in urine and/or faeces is driven by two mechanisms, Phase I and Phase II metabolites. The first one uses the hepatic metabolism and, through biochemical oxidations, reductions and hydrolysis, increases the polarity and water solubility of the metabolites. Phase II metabolites are produced by a biochemical reaction through a conjugation step (i.e. glucuronidation and sulphation), where polar groups are transferred to parent compounds or metabolites, allowing these conjugated metabolites to become enough hydrophilic and water soluble to be eliminated through urine and/or faeces [1,55,56]. These processes usually promote the loss of pharmaceutical activity of the compound. However, there are pharmaceuticals that are only active after metabolic activation by enzymatic system(s) of the parent compound (pro-drugs) to metabolite(s) [1].

To determine this pharmacokinetic feature, the proportion of the unchanged active molecule excreted in urine and/or in faeces and the proportion of the parent molecule excreted as conjugates (glucuronide and sulphate) was included, when available [57,58] (Table 2). The excretion rate, in addition to the consumption data, contributes to either a greater or lesser environmental impact and is related to the reported occurrence of the parent compound and its metabolites in the aquatic compartment [35]. Therefore, the excretion features were revised and are presented in Table 2.

Table 2. Excretion rates of the selected pharmaceuticals.

Therapeutic group	Pharmaceutical	Excretion results	References
Anx	ALP	20	[59]
	LOR	72.5	[60]
	ZOL	0.75	[61]
Antib	AZI	12	[60]
	CIP	60/83.7	[1]
		70	[8]
		70	[60]
	CLA	25	[62]
		25	[31]
	ERY	25	[54]
		10	[62]
		5	[63]
Lip reg	BEZ	72	[64]
		69	[8]
		47.5	[1]
		50	[65]
		45	[66]
	GEM	50	[67]
	SIM	12.5	[1]
		12.5	[66]
Antiepi	CAR	33	[31]
		5	[68]
		3	[34]
		3	[63]
SSRIs	CIT	23	[60]
		12/20	[69]
	ESC	9	[70]
	FLU	5/10/11	[69]
		10	[3]
	SER	0.2	[60]
		0.2	[3]
		0.2	[69]
	PAR	3	[60]
		3	[3]
		3	[69]
Anti-inf	DIC	39	[8]
		15	[1]
		15	[67]
		15	[64]
		12.5	[66]
	IBU	15	[71]
		10	[72]
		10	[65]
		5	[1]
	NAP	10	[31]
		<1	[63]
	PARA	80	[73]
		75	[60]
Horm	E2	5.6	[74]
	EE2	22/26/27/35/42/53/66/68	[75]

Anx - anxiolytics; Antib - antibiotics; Lip reg - lipid regulators; Antiepi - antiepileptics; Anti-inf - anti-inflammatories; Horm - hormones.

While several publications are available on the metabolism of pharmaceuticals, the results of these studies can vary. The observed differences are probably explained by genomically distinct metabolizing capacities, as well as differences in race, sex, age and health status of the studied subjects, which are all known to affect the route and rate of metabolism [18]. SSRIs are clearly the therapeutic group with lower excretion rates, ranging from 0.2 to 23%, whereas the other groups present higher variability. The compounds with higher excretion rates are CIP (84%), PARA (80%), LOR (73%), BEZ (72%), E2 (68%) and GEM (50%).

In the anxiolytics therapeutic group, benzodiazepines like ALP and LOR are metabolized extensively in the human body to form glucuronides which are pharmacologically inactive and are excreted through urine [76]. This leads to the high excretion rates observed for LOR (up to 73%). Since ZOL is not a benzodiazepine, its excretion is much lower (0.8%).

The antibiotics, with exception for CIP that is the pharmaceutical with higher excretion rates (84%), have rates under 25%.

Lipid regulators have elevated excretion rates, especially the fibrates (BEZ and GEM) with values above 45%, regarding the statin (SIM) lower values were found (13%).

The antiepileptic CAR is mainly metabolized in the liver, and at least 30 different metabolites have been identified. Three major metabolic pathways have been reported and it has been found to be partially excreted as glucuronide conjugates [55,56]. For this pharmaceutical there is also great variability in the excretion data, nonetheless the higher value reported is 33%.

Like for other lipophilic drugs, SSRIs undergo hepatic metabolism, in order to form more hydrophilic excretable compounds. SSRIs, following oral ingestion, are widely metabolized and the primary metabolites released are generally N-desmethyl products [49,77] that, in some cases, retain pharmacologic activity [55]. FLU is metabolized to the active metabolite, Nor-FLU, where the antidepressant effect is as potent as the parent pharmaceutical [78]. On the contrary, the N-desmethylated metabolites of CIT and SER, N-CIT and Nor-SER, although still retaining their pharmacological activity, are less potent than the parent compounds [3,49]. Although some discrepancies can be observed in the excretion rates, low values are observed for all SSRIs (up to 23%). While the excretion of FLU ranges between 5 and 11%, the values reported for the excretion of both Nor-FLU and FLU N-glucuronide are of 10 to 20%, respectively (after oral ingestion of FLU) [79]. For CIT and its enantiomer, the excretion rates are between 9 and 23%, much higher than the ones reported for SER and PAR (0.2 and 3%, respectively).

Concerning the anti-inflammatories, DIC is mainly metabolized to its hydroxylated 4-OH-DIC, and further conjugated and eliminated, mostly through glucuronides, with an excretion rate up

to 39% (DIC) [55,80]. The 4-OH-DIC has lower activity than the parent compound, however, it has been shown to have 30% of the anti-inflammatory and antipyretic activity of DIC [80]. After oral administration, IBU and NAP have similar low rates of excretion from 1 to 15%. Conversely, PARA has the higher rates (up to 80%), mainly because the principal elimination mechanisms for PARA are through glucuronidation and sulphation and not Phase I metabolization processes [81].

Regarding hormones, besides the excretion due to pharmacological consumption of E2 and EE2, both natural hormones E1 and E2 (E2 is converted reversibly to E1) are released naturally through urine, and the rates vary throughout the women fertile cycle, with averages of 11.7 (550 for pregnant women) and 3.2 (393 for pregnant women) μg/day, respectively. Males also excrete these hormones but in lower quantities: 1.3 and 0.9 μg/day for E1 and E2, respectively [75,82]. Metabolization of both E2 and EE2 occurs through hepatic hydroxylation and by glucuronidation and sulphation, being mainly eliminated in urine [55,83]. For E2, the main metabolite is E1 with 21.1% excretion in urine [74]. However, there are only a few studies on excretion and only one, for EE2, included the conjugates (glucuronide and sulphate) with excretion rates up to 68%. Observing these values, probably the presented excretion ratios for E2 (5.6%) are underestimated [75].

I2.4. Physicochemical properties and fate

I2.4.1. Physicochemical properties

The fate and persistence of the excreted pharmaceuticals and/or metabolites in the aquatic environment depend upon their physicochemical properties and the chemical and biological characteristics of the receiving water compartment. Several important chemical measurements of the pharmaceuticals in study, such as, pKa (acid dissociation constant), log K_{ow} (octanol-water partitioning coefficient), log D_{ow} (the pH-dependent n-octanol-water distribution ratio), log Koc (soil organic carbon-water partitioning coefficient) and solubility, are presented in Table 3. These features can provide strong evidence of the ionization state of the compounds, their hydrophobicity, and can help determining whether they will partition into water, biosolids, sediment and/or biological media [3,84].

Some authors defend that the log K_{ow} and log K_{oc} approaches are excessive restrictive models of pharmaceuticals distribution in environment. In complex natural water and wastewater samples, partitioning due to hydrophobicity/lipophilicity is not the only physicochemical force

of attraction operating between molecules. Electrostatic interactions, chemical bounding and non-specific forces between ionized molecules and dissolved organic matter are neglected through exclusive log K_{ow} and K_{oc} approaches. Some studies have illustrated that water pH could play an important role in the interactions between organic matter and pH depending pharmaceuticals, since there is a great variability between these compounds as regard to their pKa (4.0-18.3) [1]. Therefore, the log D_{ow} and log K_{oc} values presented in Table 3 are specific for pH 7.4, value close to the ones usually observed in the water compartments (wastewater and surface water) [34,84,85].

With a log D_{ow} superior to 1, the likelihood of predominance of the chemical in the aqueous phase decreases logarithmically, whereas below a log D_{ow} of −1, the likelihood of predominance of the chemical in the aqueous phase increases logarithmically. Therefore, compounds having log D_{ow} values between −1 to +1 could be anticipated to be distributed in both the water and organic phases [84].

As seen in Table 3 the physicochemical properties of pharmaceuticals show a high variability. For example, the log D_{ow} ranges from -2.23 to 4.6, the log K_{oc} varies between 0 and 3.88 and even solubility goes from 0.1 to 101 200 (mg L^{-1}). These variations are not only observed between different therapeutic groups, but also within each group, since, as previously referred, this pharmaceuticals grouping does not correspond to similar chemical structures and there are more than one class per group. This can be seen especially for antibiotics, lipid regulators and anti-inflammatories, where greater fluctuations in these parameters are reported.

Anxiolytics are one of the therapeutic groups with higher log D_{ow} (3.06) and log K_{oc} (3.01) values, indicating lipophilicity and possible higher concentrations in soils and sediments, however, like most of the selected pharmaceuticals they have relatively high water solubilities. As for antibiotics, since this therapeutic group includes two separate classes, slightly different behaviours are observed. Macrolides (AZI, ERY and CLA) have higher log D_{ow} and log K_{oc}, and therefore they have lower water solubility (217-514 mg L^{-1}) than the fluoroquinolone (CIP) (1350 mg L^{-1}). Nonetheless, antibiotics are the therapeutic group with higher water solubilities. Regarding lipid regulators, for fibrates lower log D_{ow} and log K_{oc} are observed when compared to SIM (4.6 and 3.88 for log D_{ow} and log K_{oc}, respectively), being the latest more lipophilic and presenting the highest sorption comparing to the other pharmaceuticals.

Table 3. Physicochemical properties of the selected pharmaceuticals (adapted from Chemspider, Drugbank, Pubchem and ECOSARv1.11).

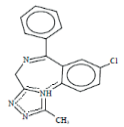
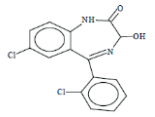
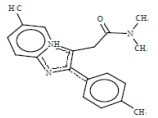
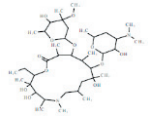
Therapeutic group/Compound	Cas Number	Structural Formula	Molecular Weight	pKa (DB)	log K_{ow}	log D_{ow} (pH 7.4)	log K_{oc} (pH 7.4)	Solubility (mg L^{-1})
Anx			307.40-321.16	5.1-18.3	2.41-3.87	2.49-3.06	2.73-3.01	16.6-32.4
ALP	28981-97-7	 $C_{17}H_{13}ClN_4$	308.77	5.1/18.3	3.87	2.63	2.81	32.4
LOR	846-49-1	 $C_{15}H_{10}Cl_2N_2O_2$	321.16	10.6	2.41	2.49	2.73	16.6
ZOL	82626-48-0	 $C_{19}H_{21}N_3O$	307.40	5.65	3.85	3.06	3.01	31.3
Antib			331.35-749.00	5.76-12.46	0.01-3.24	-2.23-1.69	0-2.33	217-1350
AZI	83905-01-5	 $C_{38}H_{72}N_2O_{12}$	749.00	9.57/12.43	3.24	1.36	1.24	514

Table 3. Physicochemical properties of the selected pharmaceuticals (adapted from Chemspider, Drugbank, Pubchem and ECOSARv1.11). *(continued)*

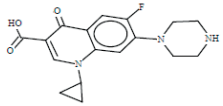
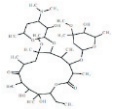
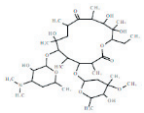
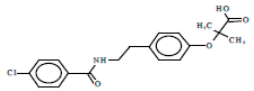
Therapeutic group/Compound	Cas Number	Structural Formula	Molecular Weight	pKa (DB)	log K_{ow}	log D_{ow} (pH 7.4)	log K_{oc} (pH 7.4)	Solubility (mg L^{-1})
CIP	85721-33-1	 $C_{17}H_{18}FN_3O_3$	331.35	5.76/8.68	0.01	-2.23	0	1350
CLA	81103-11-9	 $C_{38}H_{69}NO_{13}$	747.97	8.38/12.46	3.16	0.67	2.33	217
ERY	114-07-8	 $C_{37}H_{67}NO_{13}$	733.95	8.38/12.44	3.06	1.69	1.96	459
Lip reg			250.34-418.58	3.83-14.91	4.25-5.19	-0.11-4.60	0-3.88	1.5-27.8
BEZ	41859-67-0	 $C_{19}H_{20}ClN_1O_4$	361.83	3.83	4.25	-0.11	0	1.5

Table 3. Physicochemical properties of the selected pharmaceuticals (adapted from Chemspider, Drugbank, Pubchem and ECOSARv1.11). *(continued)*

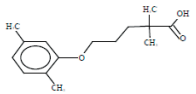
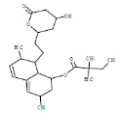
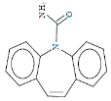
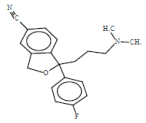
Therapeutic group/Compound	Cas Number	Structural Formula	Molecular Weight	pKa (DB)	log K_{ow}	log D_{ow} (pH 7.4)	log K_{oc} (pH 7.4)	Solubility (mg L^{-1})
GEM	25812-30-0	 $C_{15}H_{22}O_3$	250.34	4.42	4.77	1.58	1.04	27.8
SIM	79902-63-9	 $C_{25}H_{38}O_5$	418.58	14.91	5.19	4.60	3.88	12.2
Antiepi								
CAR	298-46-4	 $C_{15}H_{12}N_2O$	236.28	15.96	2.45	2.28	2.62	152
SSRIs			291.06-329.14	9.05-10.5	1.22-4.82	0.5-3.14	0.37-2.16	0.1-58.8
CIT	59729-33-8	 $C_{20}H_{21}FN_2O$	324.16	9.78	1.39	1.27	1.10	58.8

Table 3. Physicochemical properties of the selected pharmaceuticals (adapted from Chemspider, Drugbank, Pubchem and ECOSARv1.11). *(continued)*

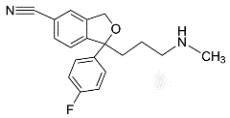
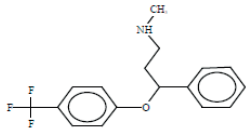
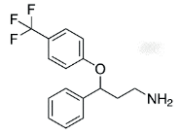
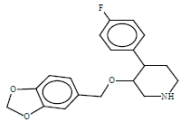
Therapeutic group/Compound	Cas Number	Structural Formula	Molecular Weight	pKa (DB)	log K_{ow}	log D_{ow} (pH 7.4)	log K_{oc} (pH 7.4)	Solubility (mg L^{-1})
N-CIT (metabolite)	62498-67-3	 $C_{19}H_{19}FN_2O$	310.15	10.50	3.53	0.50	0.37	57.0
ESC								
FLU	54910-89-3	 $C_{17}H_{18}F_3NO$	309.13	9.80	1.22	1.75	1.18	1.7
Nor-FLU (metabolite)	83891-03-6	 $C_{16}H_{16}F_3NO$	295.12	9.05	4.18	2.23	1.84	35.7
PAR	61869-08-7	 $C_{19}H_{20}FNO_3$	329.14	10.30	1.37	1.46	1.16	35.3

Table 3. Physicochemical properties of the selected pharmaceuticals (adapted from Chemspider, Drugbank, Pubchem and ECOSARv1.11). *(continued)*

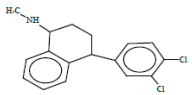
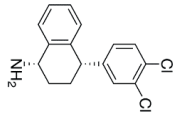
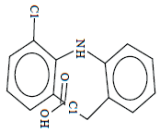
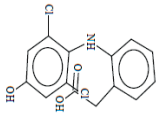
Therapeutic group/Compound	Cas Number	Structural Formula	Molecular Weight	pKa (DB)	log K_{ow}	log D_{ow} (pH 7.4)	log K_{oc} (pH 7.4)	Solubility (mg L^{-1})
SER	87857-41-8 79617-96-2	 $C_{17}H_{17}Cl_2N$	305.07	9.85	1.37	3.14	2.16	0.1
Nor-SER (metabolite)	87857-41-8	 $C_{16}H_{15}Cl_2N$	291.06	9.41	4.82	2.83	2.13	10.6
Anti-inf			109.13-312.15	4.0-10.46	0.24-4.02	0.16-1.37	0.29-1.60	4.5-101 200
DIC	15307-86-5	 $C_{14}H_{11}Cl_2NO_2$	296.15	4.00	4.02	1.37	0.71	4.5
4-OH-DIC (metabolite)	64118-84-9	 $C_{14}H_{11}Cl_2NO_3$	312.15	-	3.18	1.18	0.59	17.9

Table 3. Physicochemical properties of the selected pharmaceuticals (adapted from Chemspider, Drugbank, Pubchem and ECOSARv1.11). *(continued)*

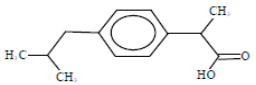
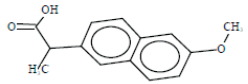
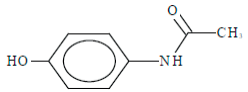
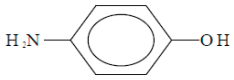
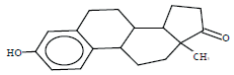
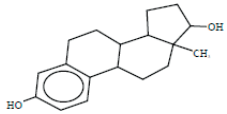
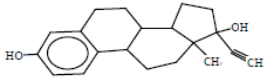
Therapeutic group/Compound	Cas Number	Structural Formula	Molecular Weight	pKa (DB)	log K_{ow}	log D_{ow} (pH 7.4)	log K_{oc} (pH 7.4)	Solubility (mg L^{-1})
IBU	15687-27-1	 $C_{13}H_{18}O_2$	206.29	4.90	3.80	0.45	0.29	68.4
NAP	22204-53-1	 $C_{14}H_{14}O_3$	230.3	4.20	3.50	0.45	0.47	51.1
PARA	103-90-2	 $C_8H_9NO_2$	151.17	9.50	0.27	0.40	1.60	4150
4-PARA (transformation product)	123-30-8	 C_6H_7NO	109.13	5.48/10.46	0.24	0.16	1.46	101 200
Horm			270.37-296.41	10.33	3.43-4.12	3.38-3.87	3.21-3.48	3.9-21.3
E1 (natural hormone/metabolite)	53-16-7	 $C_{18}H_{22}O_2$	270.37	10.33	3.43	3.38	3.21	3.9

Table 3. Physicochemical properties of the selected pharmaceuticals (adapted from Chemspider, Drugbank, Pubchem and ECOSARv1.11). *(continued)*

Therapeutic group/Compound	Cas Number	Structural Formula	Molecular Weight	pKa (DB)	log K_{ow}	log D_{ow} (pH 7.4)	log K_{oc} (pH 7.4)	Solubility (mg L^{-1})
E2	50-28-2	 $C_{18}H_{24}O_2$	272.39	10.33	3.94	3.62	3.34	21.3
EE2	57-63-6	 $C_{20}H_{24}O_2$	296.41	10.33	4.12	3.87	3.48	6.8

Anx - anxiolytics; Antib - antibiotics; Lip reg - lipid regulators; Antiepi - antiepileptics; Anti-inf - anti-inflammatories; Horm - hormones.

The antiepileptic CAR presents both high lipophilic and sorption properties with a log D_{ow} of 2.28 and a log K_{oc} of 2.62 and also possesses a high solubility of 152 mg L^{-1}.
Regarding SSRIs, although they belong to the same class, they have some variability in log D_{ow} (1.27-3.14), log K_{oc} (1.10-2.16) and solubility (0.1-58.8 mg L^{-1}), mainly due to SER being the most lipophilic. Their metabolites are usually more water soluble than the parent form (Nor-FLU and Nor-SER), however lower log D_{ow} and log K_{oc} are not always observed (Nor-FLU). Nonetheless, compared to the other therapeutic groups, SSRIs present high sorption coefficients to soils and sediments [3].
Anti-inflammatories are the therapeutic group with lower log D_{ow} and log K_{oc}, being more hydrophilic than all the other pharmaceuticals, however, with the exception of the PARA and its transformation product, 4-PARA, they have lower solubilities than the antibiotics. Once again, the metabolite and transformation product (4-OH-DIC and 4-PARA, respectively) have higher solubilities than the parent forms.
Finally, the hormones are the most uniform group, with the lowest variation between each compound. Moreover, they present the highest average log D_{ow} (3.62) and log K_{oc} (3.34), being expected to be more lipophilic and bound to soil and sediments.
In summary, although pharmaceuticals present different physicochemical properties, some are expected to be more lipophilic and others to sorb to soils and sediments, they all have relatively high water solubility, having the potential to contaminate the aquatic environment [86].

I2.4.2. Fate in wastewater treatment plants

After excretion, pharmaceuticals are transported to WWTPs through the sewer system and no significant removal occurs during transport in sewer pipes to WWTPs [87]. As hotspots of aquatic contamination, WWTPs play an important role in the life cycle of pharmaceuticals, since many are incompletely removed by conventional treatment processes, and behave as persistent organic micropollutants [88].
The removal of pharmaceuticals in WWTPs is a complex phenomenon with many plausible mechanisms, additionally, these facilities are generally not equipped to deal with complex pharmaceuticals, as they were built and upgraded with the principal aim of removing easily or moderately biodegradable carbon, nitrogen and phosphorus compounds and microbiological organisms [24,89]. The main mechanisms involved in the removal of pharmaceuticals by WWTPs are filtration, biodegradation (e.g., oxidation, hydrolysis, demethylation, cleavage of glucuronide conjugates), sorption to sludge or particulate matter (by hydrophobic or

electrostatic interactions) and chemical oxidation. Loss by volatilization can be considered as negligible [90–92].

WWTPs employ a primary, a secondary and an optional tertiary treatment process, being the last one always associated with a high treatment cost. During primary treatment, physical removal of solids is achieved through a sieve, regularly followed by coagulation-flocculation processes for the removal of particulate matter, as well as colloids and some dissolved substances, however this process is ineffective for the elimination of pharmaceuticals [93]. In the secondary treatment, usually with activated sludges, pharmaceuticals are subjected to a range of processes including dispersion, dilution, partition, biodegradation and abiotic transformation, being biodegradation and sorption to solids the main removal pathways of pharmaceuticals during this biological treatment. Afterwards, some WWTPs possess tertiary treatment like advanced oxidation processes, ultraviolet radiation (UV) or ozonation [93,94]. Most of the WWTPs in northern Europe comprise tertiary wastewater treatment, however, in other countries they are less frequent [24].

Besides the type of wastewater treatment, WWTPs efficiency in removing pharmaceuticals is influenced by operational and environmental conditions, namely: the hydraulic retention time (HRT) (high HRT allows reactions like biodegradation and sorption mechanisms to occur), solid retention time (SRT) (which controls the size and diversity of the microbial community and higher SRT will facilitate the build-up of slowly growing bacteria enhancing removal), environmental temperature (since higher temperatures reflect superior removal efficiencies), and pH conditions (effecting on the degradation kinetics of the compounds) [55,89,93,95,96].

As previously mentioned in section I2.4.1., the physicochemical characteristics of the pharmaceuticals also affect their removal in WWTPs. Since a significant part of the removal process is through sorption or biodegradation in sludge, the ability to interact with solid particles plays a major role. Thus, compounds with low sorption coefficients tend to remain in the aqueous phase, favouring their mobility through the WWTPs and into the receiving waters [97,98]. Independently of their physicochemical characteristics, some authors state that the portion of some pharmaceuticals in the treated sludge is negligible (<20%) when compared to the aqueous fraction for NAP, DIC, BEZ, GEM, LOR and CAR, although higher sorption removals were noted for selected compounds (AZI, CIP, IBU, PAR and PARA) [34,96].

Generally, during secondary treatment, compounds with log D_{ow} higher than 3, which indicates high sorption potential, tend to be removed through sorption onto sewage sludge, while compounds with log D_{ow} between 1.5 and 3 are removed mainly by biodegradation. The remaining pharmaceuticals with log D_{ow} inferior to 1.5 tend to remain dissolved [55,91,93,99].

Therefore, it is expected that the removal efficiency of substances with higher log D_{ow} are more influenced by SRT, while compounds with low log D_{ow} are more influenced by HRT [89]. During the secondary treatment, besides sorption to sludges, another removal mechanism is through microbial degradation, where nitrifiers are the most important group. This mechanism has been described has the main removal pathway for polar acidic pharmaceuticals, however, they are also sensitive to inhibitors, and some pharmaceuticals can have this effect on these microorganism [80,100].

Currently, besides the conventional treatments, new methodologies have been applied as tertiary treatments with higher removal efficiencies, but some of these new methods have high construction, maintenance and energy costs associated [88]. Advanced oxidation processes, that includes UV, ozone, hydrogen peroxide, among others, can also be used. UV treatment has been shown to partially remove some pharmaceuticals, however it does not completely eliminate them [54,68,101,102]. Ozonation alone promotes the partial oxidation of pharmaceuticals, and to overcome this drawback, this process has been combined with heterogeneous catalysts or membrane technologies, such as, nanoparticles of titanium dioxide, a known photocatalyst [14,88,93]. Adsorption by activated carbon is another methodology that proved to be effective in removing pharmaceuticals, with powdered activated carbon and granular activated carbon widely used in these adsorption processes. Generally, efficient removals are obtained when the compounds have non-polar characteristics as well as matching pore size/shape requirements. The main advantage of using activated carbon to remove pharmaceuticals is that it does not generate toxic or pharmacologically active products [93,103]. More recently, the growing trend of improving sustainability and reducing energy demand in WWTPs has encouraged alternative methods, such as, algae ponds for secondary effluent polishing, with promising results [34].

As previously referred, metabolization in the human body can lead to elimination of pharmaceuticals conjugates. However, these Phase II metabolites can be converted back into the parent compound, especially in WWTPs, being infrequently found in surface waters. One of the mechanisms used is the action of a β-glucuronidase enzyme produced by *E. coli*, capable of deconjugating the β-glucuronated pharmaceuticals excreted by the human body, resulting in the release of the active pharmaceutical into the wastewater [34,55,76,80,104]. On the other hand, the WWTPs processes responsible for pharmaceuticals elimination do not commonly lead to their complete mineralization, instead, breakdown products can emerge, which can also be toxic to the environment. In general, there is still a knowledge gap concerning the generation of metabolites and transformation products of known contaminants, which can potentially be

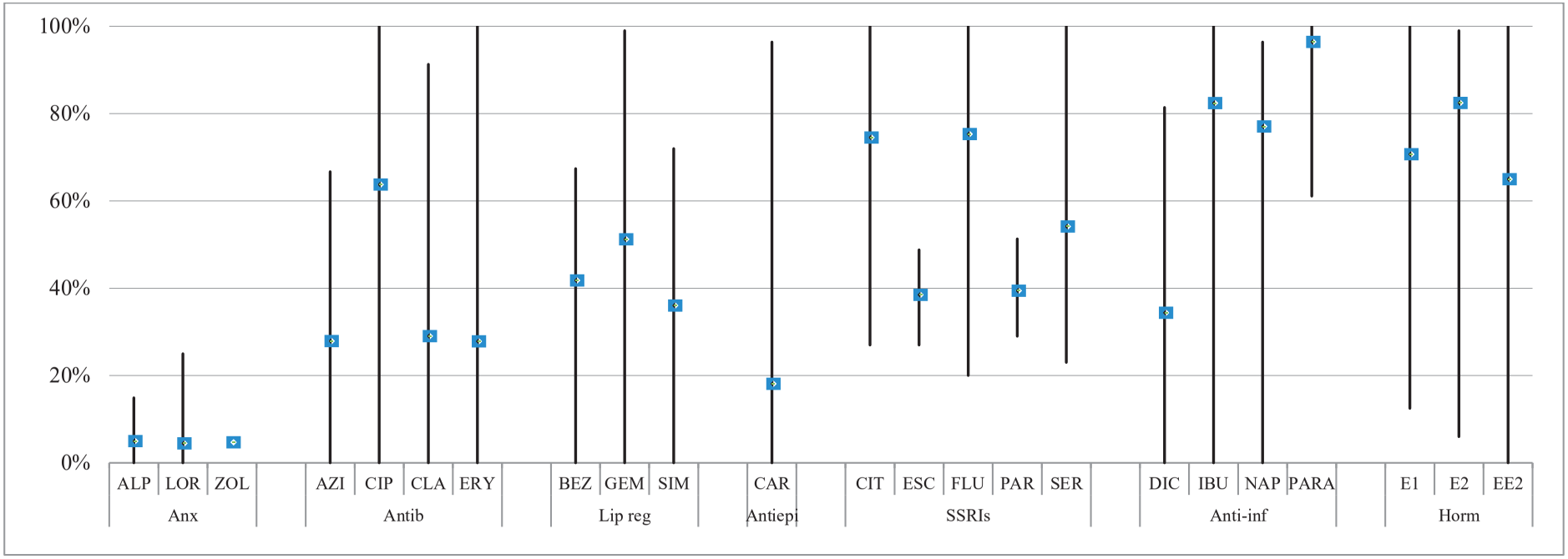
as hazardous, or even more, than the parent compounds, and can be present in different aquatic bodies at higher concentration than parent compounds [100,105–107].

Naturally, the type of treatment can affect not only the removal efficiencies but also the metabolites and transformation products generated. Mutagenic and toxic properties have been found for the reaction products of advanced oxidation processes [12]. For example, ozonation can release toxic oxidation by-products and it should not be applied without an appropriate barrier for these compounds [108]. Chlorination, another disinfection method used in WWTPs, can produce chlorinated compounds from pharmaceuticals (PARA, GEM and NAP), and their formation will result in the discharge of a mixture of unknown toxicity into the environment [109]. Additionally, the photocatalyst titanium dioxide may exert ecotoxicological effects on aquatic microorganisms and, therefore, must be retained at the WWTPs, avoiding potential aquatic pollution [88].

This supports the need for the evaluation of metabolites and transformation products, and the further development of new treatment techniques to achieve complete mineralization of emerging contaminants [100,106]. Besides the fact that some of the new treatments, like advanced oxidation processes, can originate toxic transformation products, they have higher efficiencies when compared to traditional treatments [88,93,110,111].

Data from 52 publications were collected and removal efficiencies of the selected pharmaceuticals are summarized in Figure 4. One should note that, although we are comparing the fate of pharmaceuticals in WWTPs, there are some countries with inadequate wastewater and collection infrastructures, or even functional WWTPs. For example, in Ghana and India only 7.9 and 30.7% of the wastewaters are treated, which anticipates that the presence of pharmaceuticals in the aquatic environment in these countries should represent an even bigger problem [112].

Figure 4. Minimum, maximum and average removal efficiencies in WWTPs (%).



Anx - anxiolytics; Antib - antibiotics; Lip reg - lipid regulators; Antiepi - antiepileptics; Anti-inf - anti-inflammatories; Horm - hormones. [6,8,20,23,24,56,63,67,71,72,75,77,82,89–93,96,98,99,102,110,113–141]

Although, as mentioned, some studies indicate that physicochemical properties set the efficiency of removal of pharmaceuticals in WWTPs, the literature review performed showed that the target compounds present very different removal rates, ranging between negative and high removal rates, and no obvious pattern in behaviour was observed, even within the same therapeutic group, implying that factors other than compound-specific properties affect removal efficiency [72,96]. Negative values for some compounds have been reported and may reflect deconjugation of metabolites during the treatment process, or changes in the adsorption to particles during treatment [142]. Generally, what becomes evident is that the elimination of most pharmaceuticals is incomplete and it is not exclusively related neither to the physicochemical properties, nor to the type of treatment processes. Additionally, most pharmaceuticals have always one report that shows no removal [23,24,96,99].

Concerning the removal efficiencies of each therapeutic group, anxiolytics present the lowest average, having a small variation due to their similar physicochemical properties, with values ranging from 0 to 25%. Although their log D_{ow} (from 2.49 to 3.06), higher than most of the selected pharmaceuticals, predicted large sorption to sludge and higher removal rates, this was not observed in real removal data.

As for antibiotics, the range observed in the removal efficiencies was from 0 to 100%, similar to anti-inflammatories and hormones. The average removal rates for AZI, CLA and ERY (macrolides) are near 30%, whereas CIP presented higher removal rates (64%). Despite the lower log D_{ow} for CIP (-2.23) sorption to sludges has been suggested as the primary removal mechanism for fluoroquinolones, whereas for macrolides limited sorption to sludge is observed [119,141,143].

Although the therapeutic group of lipid regulators encloses a statin (SIM) and fibrates (BEZ and GEM) and their removals vary between 0 and 99%, their averages are similar, ranging from 36 to 51%, being also found in sludges [38].

For CAR, although presenting a lower log D_{ow} (2.28) than anxiolytics and a wide range of removal efficiencies, it is one the most persistent compounds and is averagely reduced by only 18.1% [144,145]. This pharmaceutical is very resistant to wastewater treatments since it has low biological degradation and sorption, and has only higher removal rates with the use of advanced treatments such as ozonation together with the usage of the photocatalyst titanium dioxide [143,144].

Regarding SSRIs, even though they all belong to the same group, the average removal efficiencies range from 39 to 75%, with ESC, PAR and SER presenting lower values, below 55%, when compared to CIT and FLU that present higher removal rate, 75%.

The most investigated therapeutic group in WWTPs are anti-inflammatories, and despite their high variability, average removal rates are above 77% and up to 96% (PARA), with the exception for DIC (34%) [93,144]. Excluding DIC, anti-inflammatories undergo sorption to sludges and biological and photolytic degradation [38,80,93,105,146]. As for DIC, sorption to sludge and biodegradability have been reported but to a lower extent, translating into low elimination rates during wastewater treatment, moreover, a low removal efficiency of 4-OH-DIC has been reported in WWTPs [80]. Advanced oxidation processes are described as highly efficient for DIC removal since it rapidly decomposes by direct photo-oxidation, indicating that this pathway is one of its main degradation mechanisms. However, ozonation alone is not completely effective, but the O_3/H_2O_2 system shows high efficacy [14,144]. On the other hand, PARA which has the higher removal rate, during wastewater treatment, can generate different transformation products, being 4-PARA identified as the main one, and its presence in wastewater samples was already reported. However, there are other possible sources, since it is also widely used in industrial applications and is a known transformation product from pesticides. Furthermore, 4-PARA was also described as the primary degradation product of PARA during storage [81].

Hormones are the therapeutic group with higher log Dow, and high average removal efficiency, which ranges from 65 to 82%. This low variation was expected, since the molecules have similar physicochemical properties [93]. Although most hormone conjugates are degraded in the WWTPs, some are still observed in WWEs representing less than 33% of the parent compound (E1 and E2), which can be reconverted back into the parent compound in the environment [55,147]. It is also possible that E2 can be converted in E1 in the WWTPs, possibly explaining the higher removal rate for this pharmaceutical [75]. Once again, advanced oxidation processes are described as highly efficient processes in hormone removal [14].

As observed, the WWTPs are unable to completely remove the pharmaceuticals, and through direct discharge of WWEs in surface water, or by land application of WWTPs sludge, or through leaching, these facilities are the major sources of pharmaceuticals in the environment [34,63,90,148,149].

Optimization of wastewater treatment still remains a task of high priority. Biological treatment is commonly unable to remove pharmaceuticals, however, its efficacy can be improved under favourable conditions. Although advanced treatment technologies, such as membrane and advanced oxidation processes, have been promising for pharmaceuticals removal, high operation costs and formation of degradation products still remain an issue [93].

I2.4.3. Fate in surface waters

Since WWTPs are not able to completely remove pharmaceuticals, they are disseminated through their WWEs and sludges, mostly, into surface waters. In the aquatic environment, the fate and concentration of pharmaceuticals can be reliant on the receiving water body flow rate, partitioning to sediments, biological entities and consequent degradation, uptake by biota, volatilization, photodegradation, or transformation through other abiotic mechanisms such as hydrolysis (Figure 5) [34,85,143,150].

When WWEs reach the surface waters, the dilution effect varies significantly due to different flows in different rivers, however this effect can be relatively low, especially in arid or semi-arid regions due to water scarcity, like some Iberian rivers, where other processes gain relative importance [151,152]. Although multiple biotic and abiotic routes could transform pharmaceuticals once they reach the surface water, the predominant pathways to remove pharmaceuticals are photodegradation and sorption [88,151].

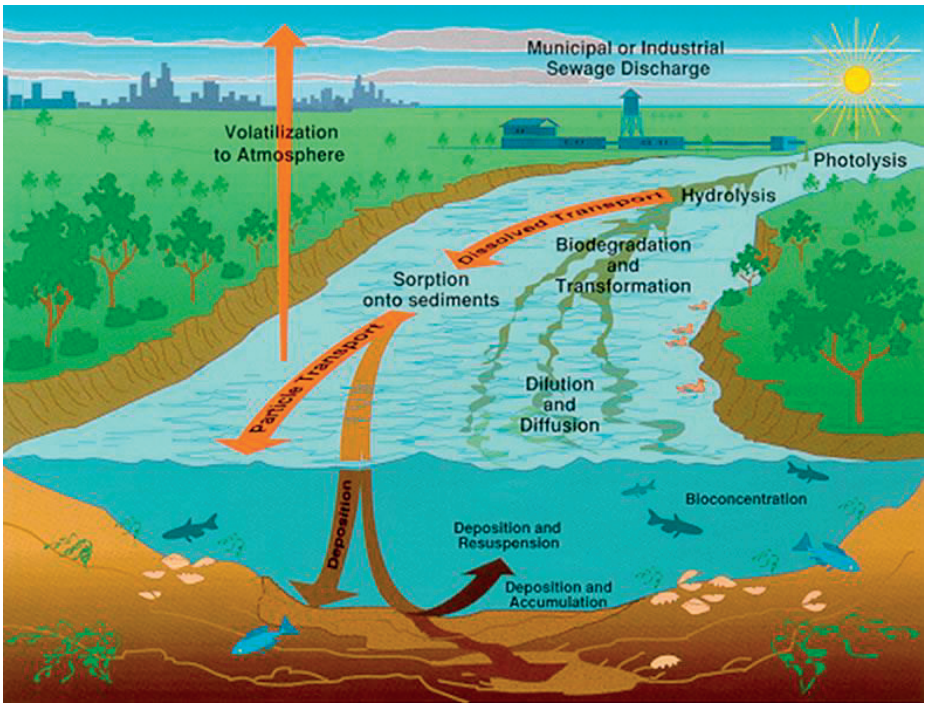


Figure 5. Fate of pharmaceuticals in surface waters.
(Adapted from http://toxics.usgs.gov/regional/emc/transport_fate.html)

The fate of different pharmaceuticals has already been studied in surface waters by several authors using estimates of mass loading, dilution and in-stream attenuation, here understood as

the reduction of the concentration of pharmaceuticals along the river segment by processes different from dilution [3,85,107,149,151].

Overall, it is expected that the log D_{ow} of a given compound influences its in-stream attenuation, in the case of hydrophobic compounds (with higher log D_{ow}), sorption to suspended particles and sediments is a dominant process leading to in-stream attenuation, by reducing the concentration in the aqueous phase along the river segment [85]. In this way, these compounds become less exposed to other biotic (biotransformation) and abiotic (photolysis, volatilization) transformation processes and therefore, become less affected by the variation of environmental conditions between river segments. Therefore, it is expected that compounds with low log D_{ow} show not only more differences in attenuation rates between sites, but also more temporal differences (i.e., seasonal and day–night) within each site [151]. This sorption mechanism in the aquatic environment represents an important sink for pharmaceuticals as it has been suggested that strong pharmaceutical interactions may act as a long-term storage of pharmaceuticals that will increase their persistence, while their bioavailability in the environment is reduced, being recalcitrant to microbial degradation [3,38]. In fact, the sediments could be a source of contaminants in downstream river segments if resuspension of fine-grained bedded sediments occurs, for instance, during seasonal increases in flow rate or during flood events [151]. Moreover, the activity of benthic invertebrate in sediments can result in an increased desorption, leading to improved bioavailability in the water compartment [34]. Additionally, sorption to colloids can also provide an important sink for the pharmaceuticals in the aquatic environment, increasing their persistence while reducing their bioavailability. In general, sorption may result in a biased risk estimation [12].

Higher levels of attenuation were observed in the smallest rivers due to shallow depth, low turbidity, and sandy sediments, that make photolysis in the water column and biotransformation relatively efficient [107]. However, in-stream attenuation is highly variable among pharmaceuticals and different rivers. As already referred, in complex natural waters, electrostatic interactions, chemical bounding and non-specific forces between ionized molecules and dissolved organic matter can also occur, meaning that we cannot generalize the attenuation of a compound based on its physicochemical properties alone [107,151]. However, the different log D_{ow} of pharmaceuticals influence the variability of rates among rivers, likely due to its effect on sorption to sediments and suspended particles, and therefore influencing the balance between the different attenuation mechanisms (biotransformation, photolysis and sorption) [151].

The attenuation of pharmaceuticals was evaluated in surface water in Spain where the total concentration of pharmaceuticals (CLA, DIC, IBU, BEZ, GEM, CAR, CIT) decreased about 40% in less than 5 km, although the number of compounds detected only decreased 13% [85]. Studies also reported that GEM is a quite persistent compound in surface water with half-lives ranging from 70 to 288 days [146]. As for CIP, photodegradation is reported to be the main mechanism of attenuation [100]. However, for CAR there are reports evaluated in a Swedish lake, where no attenuation was observed and with an estimated half-life of 780-5700 days [107]. This was also supported by other studies that revealed that CAR and IBU were stable against sunlight, while PARA suffers moderate photodegradation, and DIC was rapidly photodegraded in surface water [100,153]. Accordingly, another study noticed that no biodegradation of IBU was observed in a sterile river, but in river water and using microbial biofilms, biodegradation occurred in a few hours, evidencing that although its transformation is a complex process, microorganisms play an important role in IBU degradation [146]. Concerning SSRIs, which have high sorption coefficients, they have proven to be persistent compounds, and FLU demonstrated that was far more resistant to photolysis than the other SSRIs, with a half-life of 122 days [3].

Besides the presence of the parent compounds in surface waters, sulphate conjugates of E1 and E2 have already been observed. Although these conjugates no longer possess a significant biological activity, they can act as precursor steroid reservoirs that might be converted into free estrogens [82,147]. Even though the synthetic hormone EE2 has lower solubility than E2, it is also considerably more persistent in the aquatic environment, with an estimated half-life in surface water between 1.5 and 17 days [154].

In addition to the parent compounds, some studies also addressed the contribution of WWTPs for pharmaceuticals transformation products in surface waters and confirmed that these facilities were a major source of contamination to the recipients [85,107].

In summary, on one hand, the emissions from WWEs vary widely because of differences in regional usage of the compounds and efficiency of WWTPs. On the other hand, the processes that drive in-stream attenuation (i.e., biotransformation, photolysis, sorption, volatilization) depend on the different pharmaceutical characteristics, as well as on a series of physicochemical and biological parameters of the river, such as river flow rate, temperature, the vertical hydrological exchange between surface and subsurface compartments, turbidity, dissolved oxygen concentration, biofilm biomass and pH [151]. The magnitude of the measured attenuation rates urges scientists to consider them as important as dilution, when aiming to predict concentrations in freshwater ecosystems. Since pharmaceuticals are continuously

introduced in surface waters and are not completely removed, they eventually will reach both groundwater and seawater, contaminating all aquatic compartments [107].

I3. Occurrence

Along with advances in analytical instruments and techniques, trace levels of various pharmaceuticals and their metabolites have been detected in the aquatic compartment since the latter half of the 1970s [153]. Recent developments have made liquid chromatography-tandem coupled with tandem mass spectroscopy (LC-MS*n*) the analytical instrumentation of choice for pharmaceuticals identification and quantification [155]. Since low concentrations are usually observed in surface water, passive sampling is an alternative approach to determine their presence, and polar organic chemical integrative samplers have been successfully used, providing a slightly better picture of the pharmaceuticals in surface water. Nonetheless, concentrations are difficult to obtain with these methodologies, since there is no control on the amount of water that passes through these systems [84,107].

A literature review on worldwide monitoring programmes in recent years, presented in Figures 6-9, clearly reveals the ubiquitous distribution of pharmaceuticals in different aquatic environment compartments, including WWIs, WWEs and surface waters, with concentrations up to mg L^{-1} [153,156]. Usually, this occurrence is related to the gross domestic product per capita of each country, and is presented as the shape of an inverted-U, i.e. pollution worsens as the economy of countries starts to grow (increased consumption of pharmaceuticals) and then it improves when countries reach a higher stage of economic growth (improved WWTPs) [112].

I3.1. Wastewater

I3.1.1. Wastewater influents

Figure 6 summarizes the average and maximum concentrations of the targeted pharmaceuticals in the WWIs across the world, collected from 62 references. These concentrations are likely to be influenced by both consumption data and excretion rates.

All investigated pharmaceuticals were frequently detected in WWIs, with CLA, CIT and α-E2 (E2 isomer) presenting 100% frequency. As for the different therapeutic groups, antiepileptics and anti-inflammatories were the ones with higher frequencies, above 87%, followed by lipid regulators and hormones (74%). Anxiolytics were the group with lower values (24%), much

different from the other groups. The highest average concentration was observed in the anti-inflammatories group, with an average of 11 μg L^{-1} and with statistical difference for all of the other therapeutic groups, being the maximum individual concentration observed for IBU (700 μg L^{-1}) [89]. Antibiotics, lipid regulators and the antiepileptics had average concentrations around 500 ng L^{-1}, followed by the other groups with means under 70 ng L^{-1}.

Although anxiolytics were the group with the lower frequency and average, ALP had concentrations up to 4.7 μg L^{-1}. Additionally, the highest frequency and average belonged to LOR, with 26% and 35 ng L^{-1}, respectively [157]. These results are in line with data already mentioned, such as the low consumption and low excretion rates observed for this therapeutic group. The anxiolytic with the highest excretion rates and consumption is LOR, which is reflected on the occurrence reported.

Antibiotics were the most homogenous group, with average concentrations ranging from 260 to 810 ng L^{-1} and with all frequencies above 53%. Although some discrepancies in excretion rates, with higher values for CIP, both CIP and CLA have higher consumptions, being this pattern observed in the occurrence data.

Lipid regulators occurrence data was comparable to that of antibiotics, mostly because of similar consumption and excretion rates. Within this group, we can observe that the one with the highest consumption in Portugal and Spain, SIM, had the lowest frequency and average concentration in WWIs. This can be due to a significant difference in excretion data, where BEZ have clearly higher rates than SIM, with excretion values up to 72 and 12.5 %, respectively [1,64]. Therefore, it is shown that a pharmaceutical with low consumption can reach relatively high frequencies and average concentration in WWIs (89% and 782 ng L^{-1}, respectively).

The antiepileptic CAR with excretion rates up to 33%, and whose consumption is only surpassed by anti-inflammatories, had a frequency of 89% and concentrations up to 22 μg L^{-1} [31,122].

Like anxiolytics, SSRIs also had low consumption and excretion rates, which reflected also in low concentrations in the WWIs, with an average concentration of 51 ng L^{-1}. However, this group presented some peculiarities, being one of them, SER. This SSRI has the highest consumption in European countries, including in Portugal. Nonetheless, due to its very low excretion rate (0.2%), this compound and its metabolite (Nor-SER) present lower average concentrations than CIT and FLU [60]. On the other hand, despite the low consumption data for CIT, its higher excretion rate explains the fact that this SSRI and its metabolite (N-CIT) are the ones with the highest concentrations within this therapeutic group, followed by FLU and its metabolite (Nor-FLU), that also present higher excretion rates (up to 11%) [69].

As referred, anti-inflammatories were the group with higher concentrations in WWIs, not only due to their high consumption but also to significant excretion rates (up to 80%), with average concentrations of 0.6, 3.0, 5.6 and 41.3 μg L^{-1} for DIC, NAP, IBU and PARA, respectively [73].

In the hormones group, although the lower excretion rates observed for E2, its higher consumption (2.5 kg y^{-1}) when compared to EE2 (0.7 kg y^{-1}) resulted in higher concentrations even for its metabolite E1, being even present the enantiomer of E2 (α-E2) up to 10 μg L^{-1} [158]. As previously mentioned, one should also take into account that both E1 and E2 are produced in the human body and can be excreted naturally [75,82].

These data highlight that pharmaceutical compounds with low excretion rates are not necessarily present at low levels in WWIs, because this could be offset by the massive use of these compounds [93]. Additionally, it was also observed that, in general, the mean pharmaceutical concentrations could vary between 1 to 3 orders of magnitude from one sampling day or week to the next. Diurnal trends were also observed and peak concentrations were highly unpredictable [157].

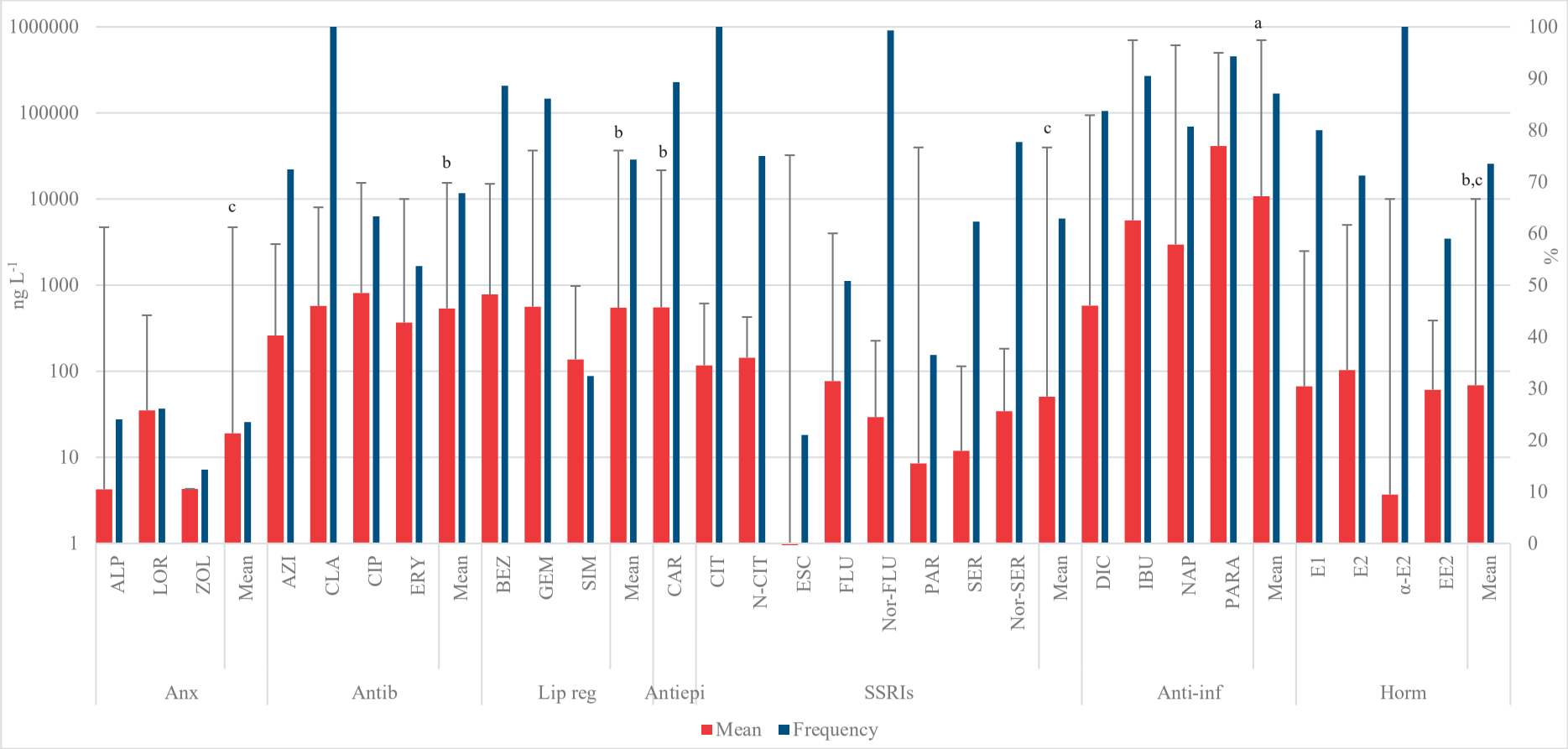


Figure 6. Occurrence of pharmaceuticals in WWIs.
(Anx - anxiolytics; Antib - antibiotics; Lip reg - lipid regulators; Antiepi - antiepileptics; Anti-inf - anti-inflammatories; Horm - hormones).
[6,20,22–24,34,39,63,67,71,72,75–77,82,89,90,93,94,97,98,105,111,113,118–120,122–126,128–130,132,133,136,139–142,148,156–173]

I3.1.2. Wastewater effluents

The first report of human pharmaceuticals in WWEs is from 1976 and subsequent studies have confirmed the presence of pharmaceuticals in this aquatic compartment [174]. After passing through WWTPs and submitted to the different treatments already discussed, it would be expected that WWEs presented lower concentrations than the influent, with a decrease proportional to the removal efficiency of the WWTP [24].

Data regarding 80 references was collected and summarized in Figure 7. In the effluents, the mean concentrations of the therapeutic groups varied from 23 ng L^{-1}, for hormones, to 562 ng L^{-1}, for anti-inflammatories, and, in general, significantly lower concentrations were found when comparing to influent samples, as showed in Figure 6. However, since concentrations in WWIs, as well as removal efficiencies, have a wide variability, the range of concentrations in WWEs is still high [89].

In general, regarding the average concentrations, anti-inflammatories were followed by antiepileptics (412 ng L^{-1}), lipid regulators (323 ng L^{-1}) and antibiotics (277 ng L^{-1}), the same pattern that in WWIs but with no statistical significance between them. The remaining three groups had lower averages, with 58, 41 and 23 ng L^{-1} for anxiolytics, SSRIs and hormones, respectively. The highest individual average concentration observed was for IBU 943 ng L^{-1}, however, the maximum concentration regarded CIP, 14 mg L^{-1}. This high value along with others that are completely offset were observed in the effluents of pharmaceutical industries and hospitals [31,32,122,175].

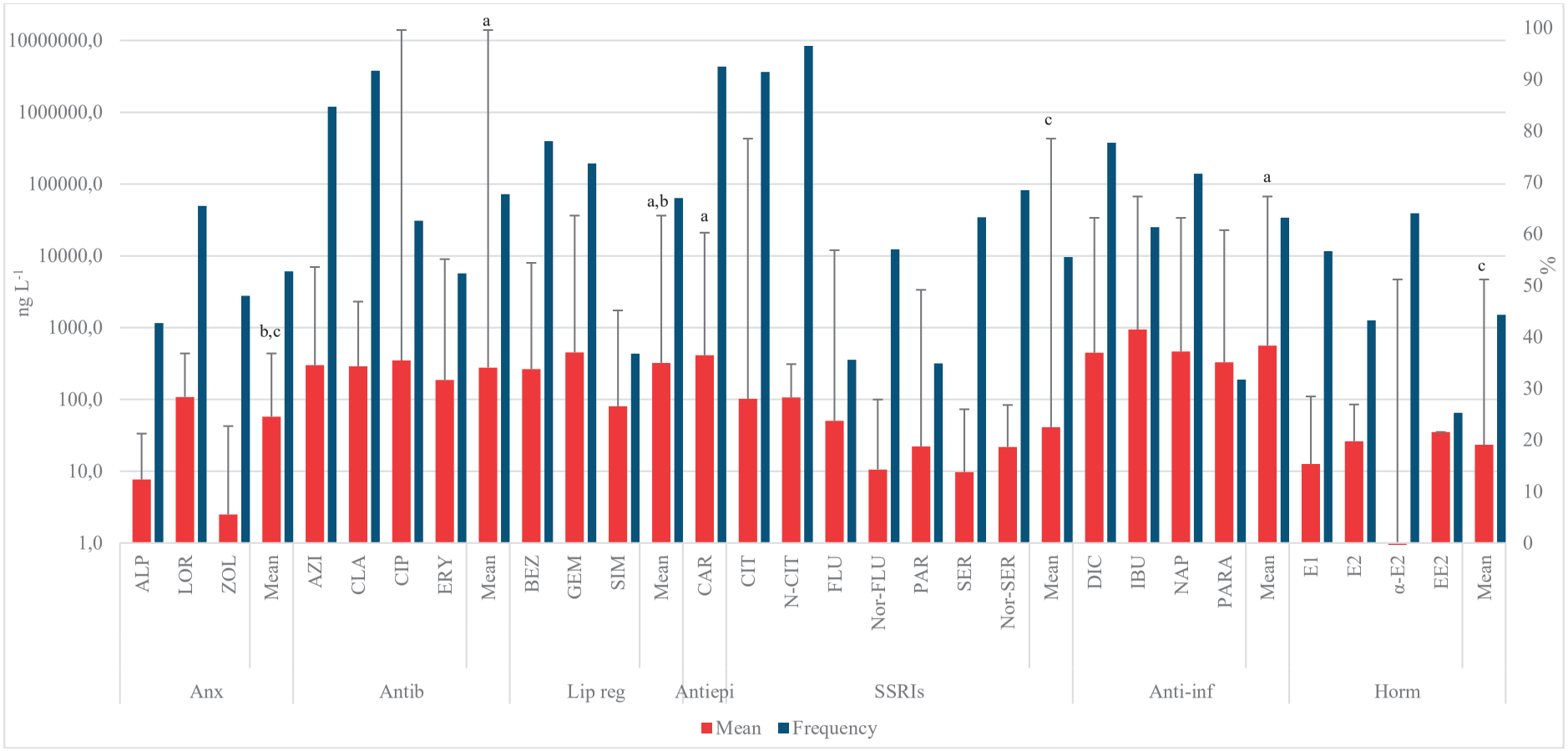


Figure 7. Occurrence of pharmaceuticals in WWEs.
(Anx - anxiolytics; Antib - antibiotics; Lip reg - lipid regulators; Antiepi - antiepileptics; Anti-inf - anti-inflammatories; Horm - hormones).
[6,8,9,11,20,22–24,34,39,63,67,71,72,75–77,82,89,90,93,94,97,98,101,105,111,113,114,118–120,122–126,128–130,132,136,138–142,145,147–149,154,156,158–174,176–185]

Anxiolytics were the only therapeutic group with higher average and individual concentrations in WWEs than in WWIs, and surpassed the average concentration of hormones and SSRIs. This is justified by the fact that anxiolytics have the lowest removal efficiencies and, in some cases, even negative values are found. This increased concentration in WWEs, is related to the transformation of metabolites and/or transformation products back into the parent compounds, during wastewater treatment [91,93]. Since all the three compounds have similar removal efficiencies, LOR, with the highest concentration in WWIs, presented again the highest values in WWEs, both average (108 ng L^{-1}) and individual (438 ng L^{-1}) levels [76].

As indicated in Figure 7, CLA was once again the antibiotic more frequently detected in WWEs (92%), and this group remained the most homogenic, with average concentrations ranging from 187 to 349 ng L^{-1}. The extremely high value found for CIP was observed in the effluent of a pharmaceutical industry [122].

Lipid regulators having removal efficiencies analogous to those observed for antibiotics, present an occurrence pattern in WWEs comparable to that of WWIs, again with SIM presenting the lowest average concentration (80 ng L^{-1}).

As regard to the antiepileptic CAR, the fact that it does not adsorb to soils and has low removal efficiencies in WWTPs results in a small average reduction from WWIs to WWEs, from 550 to 412 ng L^{-1}, respectively [186].

The therapeutic group SSRIs had also the same pattern observed in WWIs, with CIT and N-CIT presenting the higher average concentrations of 102 and 107 ng L^{-1}, respectively, and, once again, the metabolites (N-CIT, Nor-FLU and Nor-SER) concentrations were in the same range as the parent compounds [129]. The highest value regarded CIT with 430 µg L^{-1}, that was also detected in a pharmaceutical industry effluent [122].

Anti-inflammatories had one of the highest removal efficiencies, only comparable to hormones, and although they remain the therapeutic group with higher concentration average, the difference to the following groups (antiepileptics, lipid regulators and antibiotics) was significantly reduced. Within this therapeutic group, IBU presented the highest average concentration (943 ng L^{-1}), followed by NAP, DIC and PARA, with 466, 447 and 329 ng L^{-1}, respectively, meaning that PARA shifted from the second highest average concentration in WWIs to the fourth in WWEs, mainly due to the high removal average (96%) presented.

As for hormones, with average removal efficiencies above 60%, concentrations were also significantly reduced, with the highest average concentration belonging to EE2 (35 ng L^{-1}) and the lowest to α-E2 (0.4 ng L^{-1}), the highest individual value was also for α-E2 (4.7 µg L^{-1}), observed in only one study [158].

Despite these concentrations, it is possible that some conjugates, which were not evaluated, enter surface waters, where they can be reconverted back to the parent compound, increasing the pharmaceuticals contamination burden [34].
As expected, some positive correlation could be observed between the concentrations found in WWIs and in WWEs with removal efficiencies. Nonetheless, even at relatively low population densities, and low industrial and hospital activity, human pharmaceuticals are present at quantifiable levels in WWEs [174].

I3.2. Surface water

The release of WWEs into surface water, in comparison to other sources, has been considered the main cause of the presence of pharmaceuticals in this water body [63,186].
As previously discussed, following the treatment processes in WWTPs, pharmaceuticals are subjected to different degrees of natural attenuation, such as: dilution in surface water, sorption onto suspended solids and sediments, photolysis and biodegradation, which will vary depending on the characteristics of each river flow, sunlight and temperature. For example, in deeper rivers, photolysis is diminished and, with favourable hydraulic conditions, prolonged contact with bed sediments can improve biodegradation and sorption [187]. These conditions can promote a variation higher than one order of magnitude in the same sampling location, and even higher between different rivers [25]. Due to this factors, pharmaceutical compounds are expected to occur in surface waters at lower levels than in WWEs [93,107,188].
Since 1970 that the issue regarding the presence of chemicals in surface waters has been addressed by the EU. Nowadays, the chemical quality of surface waters is controlled under the WFD (Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000, establishing a framework for Community action in the field of water policy), transposed into the Portuguese legal system by the Law Nº 58/2005 of 29 December 2005 (The Water law). Within this framework, the key strategy adopted was the establishment of priority substances or groups of substances, due to their persistence, toxicity, bioaccumulation, widespread use and detection in rivers, lakes, transitional and coastal waters. Also a list of environmental quality standards have been issued for these substances, to ensure adequate protection of the aquatic environment and human health [11]. Although no pharmaceutical belongs to this list, their environmental presence in surface waters is a growing problem that must be tackled, and was addressed by the WFD, in order to minimize their aquatic environmental contamination and support future prioritization measures. Despite this awareness, legal limits have not yet been

set for pharmaceuticals in surface water, although a watch list that includes six pharmaceuticals (E2, EE2, DIC, AZI, CLA and ERY) and one metabolite (E1) has been recently established [39,189,190]. IBU has also been proposed to enter this list, however, its inclusion was rejected in January 2012 owing to a lack of sufficient evidence of significant risks to aquatic environment [12]. In the future, DIC may exit the watch list and be classified as priority substance with environmental quality standards values, ranging from 10 to 100 ng L^{-1}, since they were already proposed during the EU revision of priority substances directive, during 2012–2013 [80].

According to the Directive 2013/39/EU strategy, all member states shall monitor each substance in the watch list at selected surface waters representative monitoring stations, at least once per year. The number of monitoring stations varies within each member state, taking into account the population and area of each country, which, in the case of Portugal, regards 6 sampling locations. This monitoring was demanded to start at the 14th of September 2015 for the first watch list, or within six months after the inclusion of new substances [190]. About 40% of European water bodies still have an unknown chemical status, as not even the monitoring of the EU priority substances have been performed [27].

After reviewing 75 scientific references, as expected, with the exception of antibiotics, lower concentrations were found in surface waters than in WWEs (Figure 8). Antibiotics had the highest average concentration (1826 ng L^{-1}), even higher than in WWEs (277 ng L^{-1}), this elevated value was offset by some values in CIP and ERY, mainly the one that reported a maximum concentration of 650 µg L^{-1} for CIP, in India [9].

Anti-inflammatories presented the second highest average concentration (202 ng L^{-1}), followed by CAR and lipid regulators with an average of 150 ng L^{-1}. These four therapeutic groups had statistically significant higher average concentrations than SSRIs and hormones. Hormones, anxiolytics and SSRIs had the lowest average concentrations of 13, 7 and 3 ng L^{-1}, respectively. If we eliminate the higher values for antibiotics, we have similar patterns than in WWEs, with anti-inflammatories, antiepileptics, lipid regulators and antibiotics with higher concentrations, and, although not in the same order, hormones, anxiolytics and SSRIs with notably lower concentrations.

Regarding anxiolytics, only LOR was found in surface water, with a frequency of 16%. Both ALP and ZOL were evaluated in only one study each, but they were not detected [191]. This was the only therapeutic group, which did not present any statistical difference from all of the others.

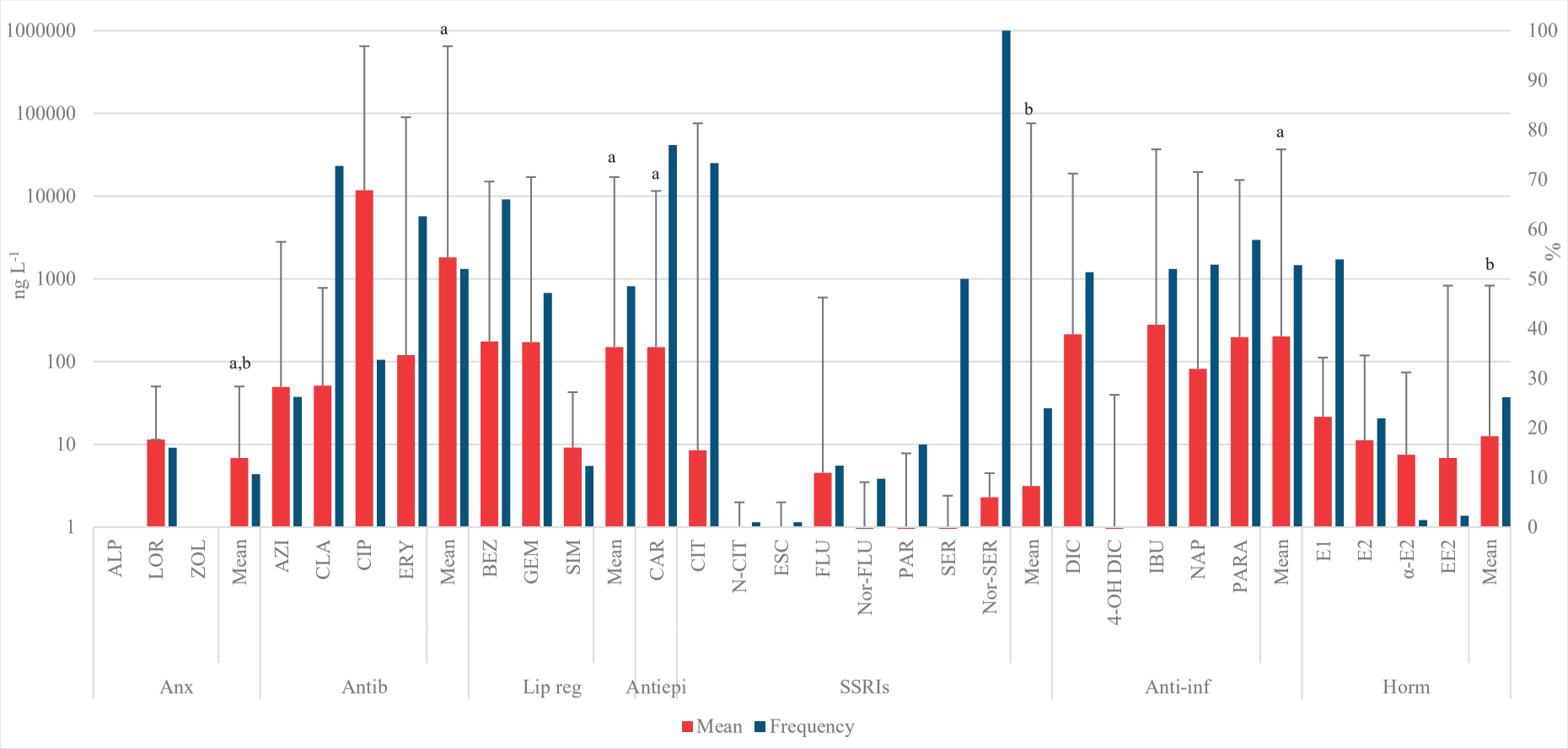


Figure 8. Occurrence of pharmaceuticals in surface waters.
(Anx - anxiolytics; Antib - antibiotics; Lip reg - lipid regulators; Antiepi - antiepileptics; Anti-inf - anti-inflammatories; Horm - hormones).
[1,8,9,34,39,62,63,77,81,82,84,85,93,105,107,112,124,132,138,141,143,145,147–149,153,154,156,163–167,169,171,172,174,175,178–180,182–188,191–217]

As above mentioned, antibiotics were the group with higher average concentration (1826 ng L^{-1}), mainly due to two extremely high average concentrations detected for CIP in surface waters near pharmaceutical industries in Pakistan (1.3 µg L^{-1}) and in India (164 µg L^{-1}), however, all the other average concentrations were below 101 ng L^{-1} [9,194]. Comparing the antibiotics concentrations with WWEs, excluding CIP, a very similar pattern was observed, with a tendency for a relative higher frequency and concentration for ERY, probably revealing a higher persistency in the environment.

Lipid regulators had an average of 50% reduction in concentration, when compared to WWEs, however SIM was the one with lower relative concentration, with an average of 9 ng L^{-1}. BEZ apparently presented higher persistence, since its frequency and average concentration, 66% and 175 ng L^{-1}, respectively, surpassed those of GEM, 47% and 172 ng L^{-1}, respectively.

As previously noted, CAR continued among the most frequently detected pharmaceutical compounds in surface waters (77%) and presented concentrations up to 12 µg L^{-1}, reflecting, as expected, the recalcitrant nature of this molecule given its high half-life [218]. In fact, it is also one of the most frequently detected pharmaceuticals in European surface waters [146].

The group with a higher reduction in average concentration (from 41 to 3 ng L^{-1}) and frequency (from 56% to 24%) from WWEs to surface waters was SSRIs. The highest concentration regarded CIT (76 µg L^{-1}), however, it was found, once again, near a pharmaceutical industry in India [9]. The metabolites suffer even a higher reduction than the parent compounds.

Anti-inflammatories presented, once again, higher concentrations when comparing with other therapeutic groups [174]. IBU remains the compound with higher average concentration (280 ng L^{-1}), however, the difference for DIC (214 ng L^{-1}) and PARA (198 ng L^{-1}) became smaller. As for NAP, it presented the lowest average concentration (82 ng L^{-1}). Looking at the frequencies, they all fall in the same range, from 51% to 58%. In this group another extremely high concentration was observed for IBU in Costa Rica, 37 µg L^{-1} [93]. Although in wastewaters no study on 4-OH-DIC was reviewed, in surface waters two studies were found and 40 ng L^{-1} was the highest concentration found for this metabolite [192]. The average concentration observed for DIC (214 ng L^{-1}) was twice the purposed value of 100 ng L^{-1} for the environmental quality standard in 2012-2013. The high values in surface waters possibly raised some issues regarding the establishment of this standard.

Within the hormones group, E1 presented higher average concentration than in WWEs. This is explained by a high average value detected in China, 180 ng L^{-1}, which increased the E1 average, whereas its frequency was slightly decreased (from 57 to 54%) [216]. Contrary to what was previously mentioned, namely that EE2 was more persistent than E2, EE2 registered a

higher decrease in average concentration and frequency (from 35 to 7 ng L^{-1}, and from 25 to 2%, respectively) than E2 (from 26 to 11 ng L^{-1} and from 43 to 22%, respectively). In surface waters, conjugates of both E1 and E2 were also found in a concentration range from a quarter to half of the parent compound [147,210].

As above mentioned, lower concentrations of pharmaceuticals were found in surface waters than in WWEs. Surface waters showed an overall trend of higher concentrations in sites influenced by the location of WWTPs [213].

I3.3. Other water bodies

As discussed earlier, the concentrations of pharmaceuticals decrease from the WWIs to WWEs and to surface waters through different mechanisms. However, data collected from 25 references showed that pharmaceuticals can reach groundwaters, seawaters and even mineral waters and drinking waters (Figure 9). Regarding groundwaters, it is important to underline that this is an important resource of water supply in the world and it is especially vulnerable to contamination, although soil provides a big inertia to propagation of the contamination and for that same reason, once contaminated, the effects can hardly ever be reverted [219].

The concentrations in remaining waters bodies should be lower than the previous ones, since they suffer attenuation mechanisms similar to surface water. Additionally, drinking water has dedicated treatment plants. However, these facilities do not completely remove pharmaceuticals and can also produce transformation products which can be toxic [153,178,198].

Although susceptible to degradation or transformation, pharmaceuticals continuous introduction into the aquatic environment confers some degree of pseudo-persistence, reaching, at extremely low concentrations, all aquatic compartments all over the world, even drinking waters [68,101]. However, it is unlikely that pharmaceuticals pose significant threats to human health at the concentrations that may occur in drinking waters [153,220].

In Figure 9 we observe that, once again, antibiotics, lipid regulators, antiepileptics and anti-inflammatories had higher frequencies and average concentrations, however, CAR stands out from the others with a higher frequency and average concentration of 42% and 67 ng L^{-1}, respectively. Groundwater and seawater were the water bodies with higher frequencies and concentrations and the highest concentration found was of 14 μg L^{-1} for CIP in groundwater [9]. No statistical significance was observed between the different therapeutic group averages.

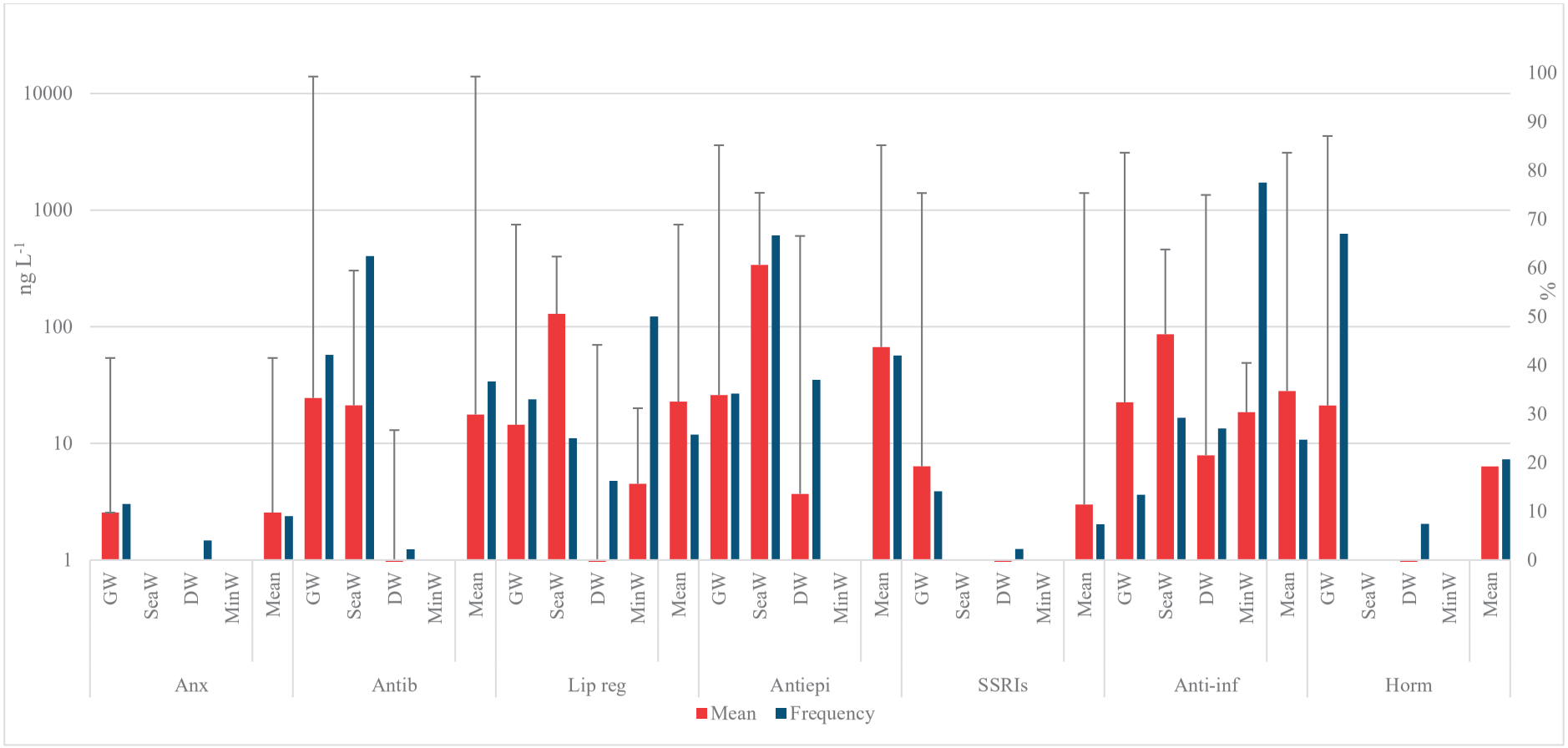


Figure 9. Occurrence of pharmaceuticals in other water bodies.
(GW - groundwaters; SeaW - seawaters; DW - drinking waters; MinW - mineral waters; Anx - anxiolytics; Antib - antibiotics; Lip reg - lipid regulators; Antiepi - antiepileptics; Anti-inf - anti-inflammatories; Horm - hormones). [1,9,82,93,101,124,145,149,153,178,183,193,198,204,207,209,211,214,217–219,221–224]

I4. Toxicity

Since pharmaceuticals are continuously introduced into the aquatic environment, they can promote toxic effects on living organisms, even when present at concentrations on the ng L^{-1} level. This potential for negative effects of pharmaceuticals even at sublethal concentrations, namely for aquatic organisms, has been of concern since the issue was first brought to attention in 1985 [3]. Therefore, their presence poses a threat to the quality of water resources [16,88].

Pharmaceuticals have a relatively clear mode of action in target organisms, and given that fish and invertebrates share more drug targets with humans, it would be expected that they would also respond to pharmaceuticals in a similar way. However, when non-target-species are exposed, unknown effects and potential risks need to be assessed. One example is the devastating impact of EE2 in the feminisation of fish [38,159,225]. Nonetheless, all the ecotoxicological risks associated to the ubiquitous occurrence of pharmaceuticals in aquatic ecosystems are far from known [4].

Sorption to sediments is one factor that influences toxicity of pharmaceuticals, although higher sorption to sediments results in an apparent reduction of bioavailability and toxicity, the activity of benthic invertebrate in sediments results in a higher exposure for these organisms [34].

Moreover, bioaccumulation (the accumulation of a substance by an organism) and biomagnification (increasing concentrations of substances in higher levels of the food chain) should also be accounted for since they can increase toxicity [38]. These parameters are also related to log $D_{ow,}$ since compounds with values higher than 3 have a tendency for bioaccumulation [38,226], which means that the ionization state can influence the toxicity of pharmaceuticals, and that the pH variability in surface water should also be taken into account [38].

Below, the ecotoxicological data in the aquatic biota is reviewed, presenting the toxicity data obtained from 116 exposure studies of three trophic levels of non-target organisms, algae (Figure 10), invertebrates (Figure 11) and fish (Figure 12). The data was divided by the different endpoints found in the literature: no observed effect concentrations (NOEC), lowest observed effect concentrations (LOEC), effective concentration (EC50) and lethal concentration (LC50). These endpoints are expected to have increasing toxicity concentrations, since they were organized from the more susceptible endpoint (NOEC) to the less one (LC50). However, each endpoint encloses various species of the same trophic level and different toxicological tests like immobilisation, growth, luminescence, reproduction, morphology, behaviour, etc. When no

experimental data was available, L(E)C50 values were estimated with ECOSAR 1.11. This program estimates data on acute toxicity through the molecule structure, sometimes underestimating toxic effects.

Although, as expected, some therapeutic groups presented higher toxicity, such as hormones, which can promote endocrine modifications, all therapeutic groups presented toxicity at low concentrations [12]. Overall, considering all trophic levels, all therapeutic groups with the exception of anxiolytics and antiepileptics, had at least one toxicity report for concentrations below 10 μg L^{-1}.

Considering the toxicity of the selected pharmaceuticals in all trophic levels, we can observe that the most sensitive one, with the lowest concentrations promoting toxic effects is fish, followed by invertebrates and algae. Naturally, for almost all pharmaceuticals, concentrations detected for chronic toxicity were lower than the ones for acute toxicity. Additionally, as already discussed earlier, chronic toxicity is the one that better represents the real exposure, since aquatic wildlife is continuously exposed to pharmaceuticals present in surface water.

The therapeutic group with higher toxicity, mainly chronic toxicity in fish and invertebrates, are hormones, which, as already referred, was an expected outcome. Additionally, the pharmaceutical that presented toxicity at the lowest concentration was EE2 at 0.1 ng L^{-1} in fish (NOEC, chronic toxicity). Also, the lowest concentration inducing toxicity observed for all the other pharmaceuticals, were mainly observed in NOEC and LOEC endpoints [227]. The highest concentrations promoting toxicity were detected in fish (LC50, acute toxicity), for CLA and ERY (1 g L^{-1}), once again, as anticipated, LC50 and EC50 data presented generally the highest concentrations [227–229].

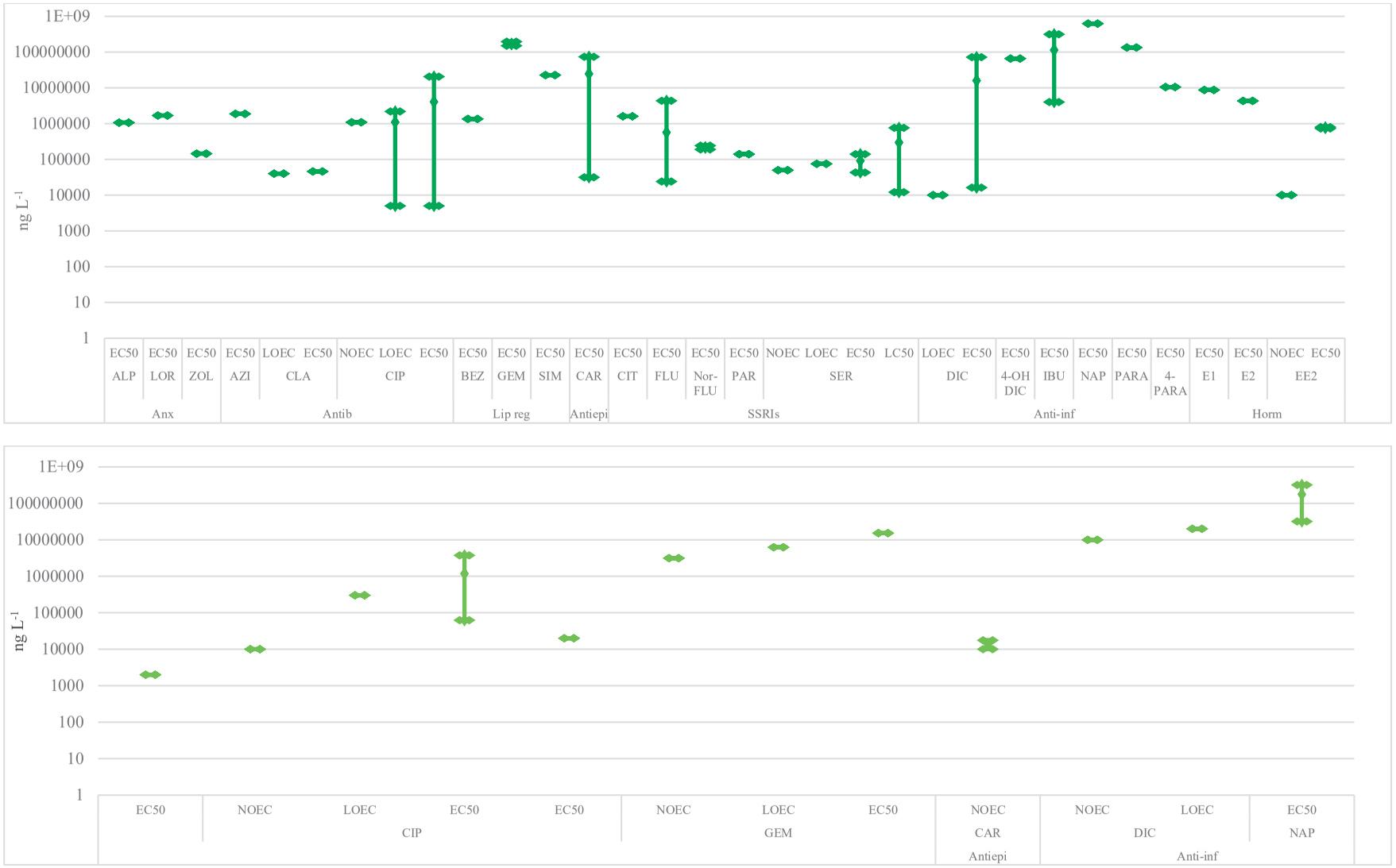


Figure 10. Acute (A) and chronic (B) toxicity data concerning algae.
(Anx - anxiolytics; Antib - antibiotics; Lip reg - lipid regulators; Antiepi – antiepileptics; Anti-inf – anti-inflammatories; Horm – hormones). [229,232–258]

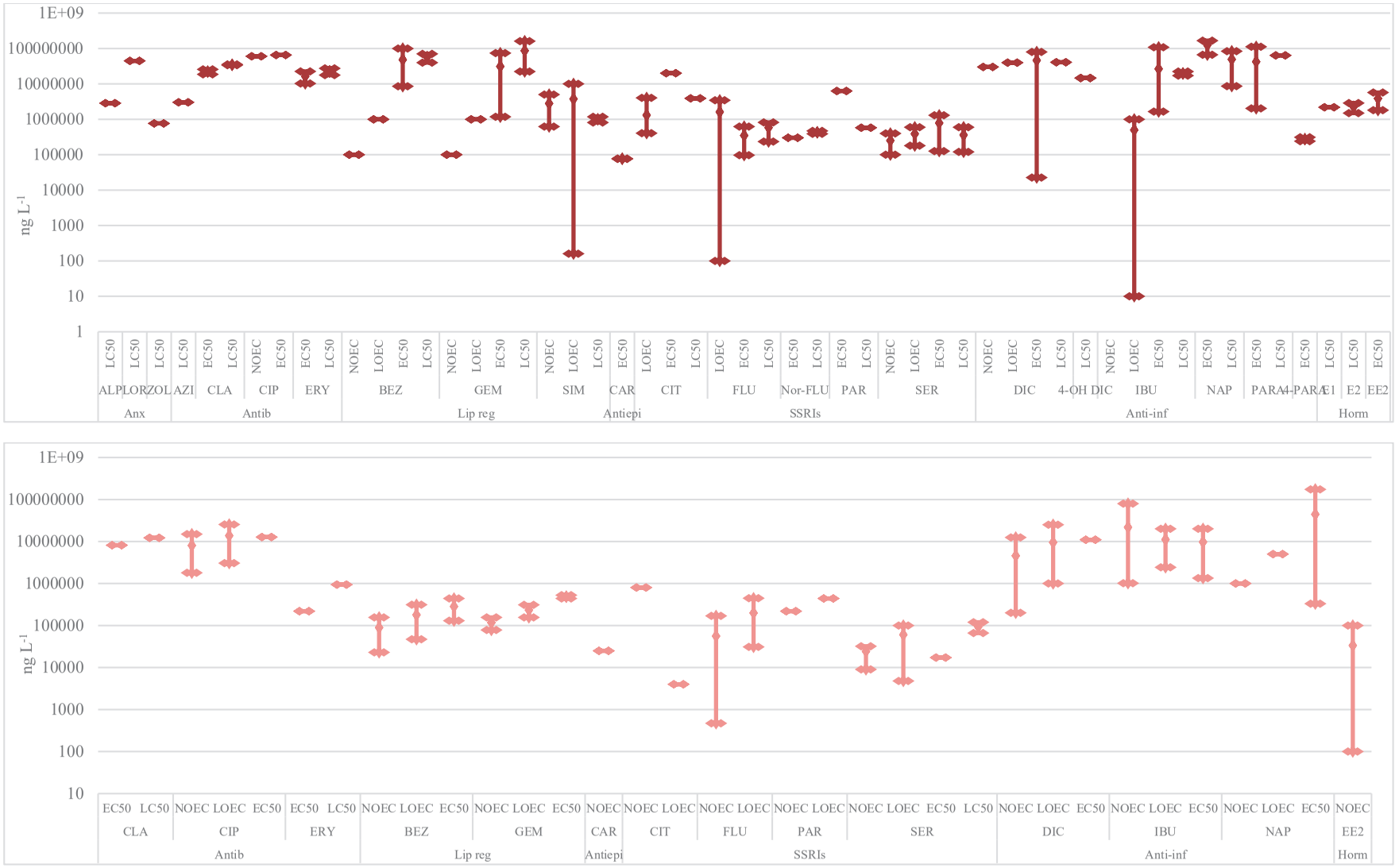
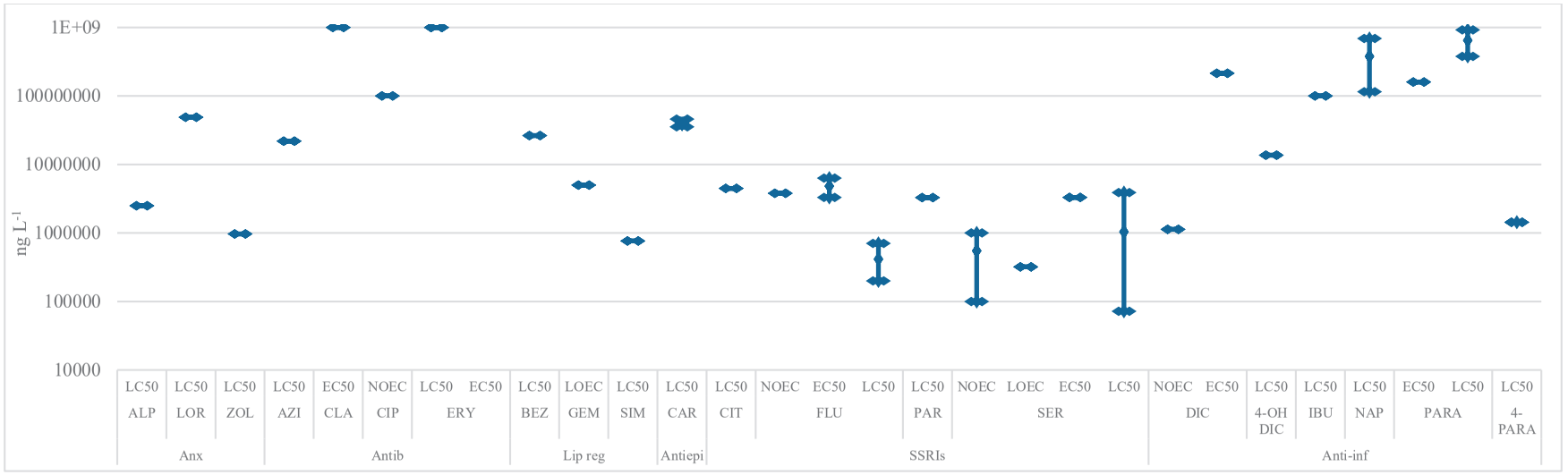
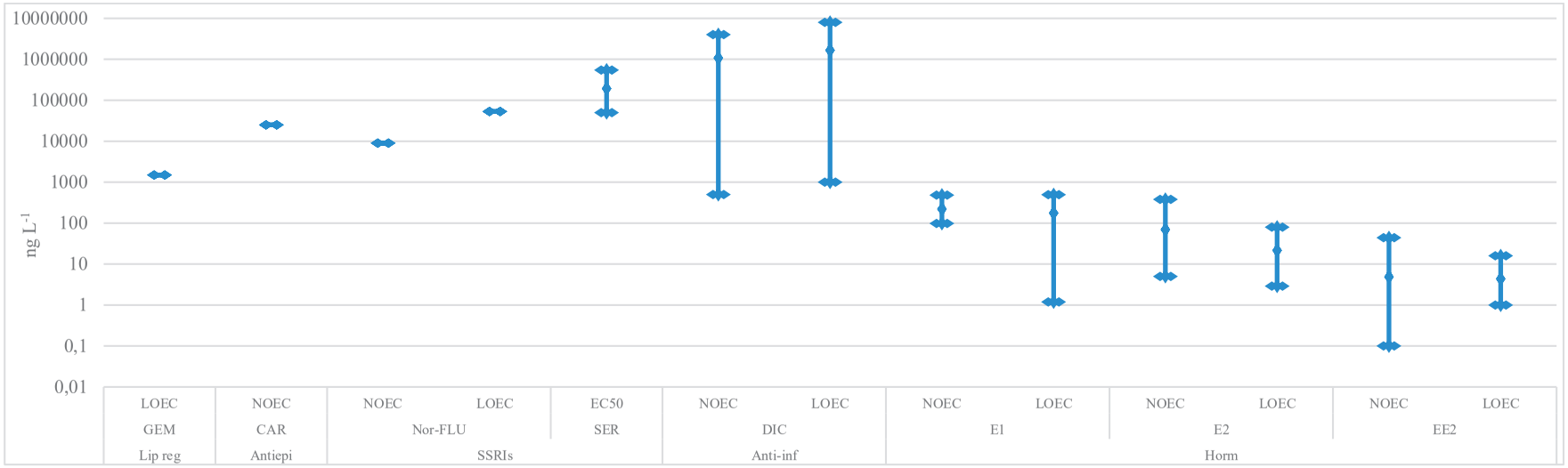


Figure 11. Acute (A) and chronic (B) toxicity data concerning invertebrates.
(Anx - anxiolytics; Antib - antibiotics; Lip reg - lipid regulators; Antiepi – antiepileptics; Anti-inf – anti-inflammatories; Horm – hormones). [79,229,233,234,239,240,243–248,251,255–257,259–288]



A)



B)

Figure 12. Acute (A) and chronic (B) toxicity data concerning fish.
(Anx - anxiolytics; Antib - antibiotics; Lip reg - lipid regulators; Antiepi – antiepileptics; Anti-inf – anti-inflammatories; Horm – hormones). [227–229,233,245,247,248,251,257,264,265,276,280,288–338]

Ecotoxicological chronic studies on pharmaceuticals are lacking and often do not produce visible results, meaning that many questions about the threat to the environment of pharmaceuticals remain unanswered. Additionally, the actual exposure scenario regards multiple pharmaceuticals, posing uncertainty regarding toxicology in long-term exposure. If many pharmaceuticals are present and share the same mode of action, then the toxicity of this mixture could be higher than if only one pharmaceutical is present, being usually considered the concept of concentration addition, although antagonistic and synergistic effects may also occur. This could result in risk underestimation, as the typical exposure is toward multicomponent chemicals [84,155,230,231].

One example of mixture effects was observed when using a mixture of anti-inflammatories (DIC, IBU and NAP). In this case, the acute toxicity was detected at concentrations where little or no effect was observed for the chemicals individually [34]. Even in mixtures with pharmaceuticals belonging to different therapeutic groups, additive and synergistic effects were reported. A mixture with E2 and FLU promoted a decrease in the reproductive success of *D. magna* more significantly than either chemical compounds alone [149]. Another example was provided by exposing *D. magna* to a mixture of CAR and a lipid lowering agent, which exhibited stronger effects during immobilization tests than the single compounds at the same concentration [34].

Taking into account mixture effects, some research has already been developed focusing on toxic effects, and not on specific pharmaceuticals. This was already used to evaluate WWTPs removal efficiencies, by evaluating and comparing the toxicity (androgenecity, cytotoxicity, anti-estrogenicity and *L. variegatus* decrease in reproduction and biomass) both in WWIs and WWEs [339,340].

Additionally to the active compounds of pharmaceuticals, excipients and additives are also present in medicines, that may contain endocrine disrupting chemical excipients and additives [12].

The measured concentrations of some of the selected pharmaceuticals reported for surface water all over the world surpassed the concentrations here described for toxicity, what suggests that the aquatic biota could be vulnerable to the presence of pharmaceuticals in their environment, and that toxic effects are expected to occur with unexpected outcomes.

It is unlikely that pharmaceuticals present in drinking water may pose a risk to the human health through chronic exposure, however, the toxicological implications are not clear [211]. Furthermore, studies have shown that infants may have difficulty in metabolizing drugs therefore, being more vulnerable to the toxic effects of these compounds [38].

As referred, many pharmaceuticals have the potential for bioaccumulation and biomagnification, and chronic effects on ecosystems cannot be ignored for animals at the higher end of the food web [185]. Thus, the health hazard of human exposure by ingestion of contaminated foods should also be taken into account [38].

I4.1. Anxiolytics

No ecotoxicological data was found in literature for ALP, LOR and ZOL, and for that reason, all the results for this therapeutic group were obtained from ECOSAR 1.11 [159]. In decreasing order, the more toxic was ZOL, followed by ALP and LOR. The trophic level with the lowest reported concentrations producing toxicity was algae (from 0.144 to 1.683 mg L^{-1}), followed by invertebrates (from 0.764 to 44.712 mg L^{-1}) and fish (from 0.967 to 49.008 mg L^{-1}).

I4.2. Antibiotics

Observing the acute toxicity for antibiotics, since there is little data on chronic endpoints, and taking into account that data on AZI was obtained from ECOSAR1.11, the pattern for the three trophic levels was similar for all antibiotics, with algae being more susceptible at lower concentrations (from 5 to 21 mg L^{-1}), followed by invertebrates (from 3 to 65 mg L^{-1}) and fish (from 22 to 1000 mg L^{-1}). If we compare each antibiotic, concerning invertebrates, it can be observed that CLA and CIP presented similar results, but when compared with ERY, lower concentrations (220 μg L^{-1}) of this antibiotic can produce the same toxic effects, in this case growth inhibition [229].

In this therapeutic class, in addition to direct toxicological risks, concern has been raised about the potential for the antibiotic residues in water, since they are typically found in the aquatic environment at sub-therapeutic concentrations, promoting the emergence of resistant bacteria and subsequent development of more resistant and virulent pathogens [341]. These bacterial resistances, through horizontal gene transfer, may end up in human pathogens, raising questions on human health and the stability of the ecosystem [9,141,176,194,342].

This emergence of bacterial resistance presents one of the major emerging threats to human health and is by far the highest risk for humans of having medicinal products residues in the environment [343]. Furthermore, historical evidence appears to indicate that in the aquatic environment resistance might be acquired faster than in the terrestrial environment [344].

Corroborating the effects on bacteria, changes in biomass and growth rate were reported at concentrations above 5.7 μg L^{-1} [235]. This therapeutic class can also induce immunotoxicity in the freshwater mussel at low concentrations, between 2 and 1100 ng L^{-1} [112].

I4.3. Lipid regulators

In this group, the pattern observed with both previous therapeutic groups was not so clear, with average concentrations similar in all trophic levels for acute toxicity. Observing these data, SIM was clearly the pharmaceutical which promoted toxicity at lower concentrations for invertebrates (160 ng L^{-1}), algae (23 μg L^{-1}) and fish (765 μg L^{-1}) [242,262]. However, data on chronic toxicity, only available for GEM on all trophic levels, showed that the highest toxicity regarded fish (1.5 μg L^{-1}), followed by invertebrates (78.0 μg L^{-1}) and algae (6.3 mg L^{-1}) [239,289].

I4.4. Antiepileptics

For CAR, once again, the pattern of acute data, was similar to that registered for anxiolytics and antibiotics, with the lowest concentrations promoting toxicity at 31.6, 76.3 and 35 400 μg L^{-1} for algae, invertebrates and fish, respectively [243,265]. Considering the chronic data, similar concentrations were found to produce toxicity in all trophic levels, ranging from 10 to 25 μg L^{-1} [243,245].

I4.5. SSRIs

This therapeutic group has the peculiarity that the phylogenetically ancient and highly conserved neurotransmitter and neurohormone serotonin has been found in invertebrates and vertebrates, although its specific physiological role and mode of action is unknown for many species [48]. Many biological functions within invertebrates, such as reproduction, metabolism, moulting and behaviour, are under the control of serotonin [345]. Therefore, the pharmaceuticals in this therapeutic group could have tremendous effects on these and other organisms [77]. These facts are in agreement with those found in acute toxicity data found, since for all trophic levels this group had globally the lowest concentrations which promoted toxic effects, being some of these on reproduction, survival and behaviour [346].

When observing these data, the most sensitive trophic level was the invertebrates (0.1 μg L^{-1}), followed by algae (12.1 μg L^{-1}) and fish (72.0 μg L^{-1}) [249,263,296]. The pharmaceuticals with higher toxicity were FLU (100 ng L^{-1}) and its metabolite Nor-FLU (9 μg L^{-1}) and SER (4.6 μg L^{-1}). On the other side, CIT was the one with lower toxicity [249,263]. In algae, the pharmaceutical with highest toxicity was SER, however, in invertebrates, FLU surpassed SER toxicity.

The only metabolite referred in the literature concerning toxicity studies was Nor-FLU, which had data in all trophic levels. When comparing with FLU (algae and invertebrates), it is clear that the average concentrations inducing toxicity were always lower. Regarding fish, only chronic data was available for Nor-FLU, and no data on FLU was provided. Nevertheless, in this trophic level, the chronic data on Nor-FLU showed higher toxicity than SER, which in turn, had higher toxicity on acute toxicity than FLU. This can suggest that if data on chronic toxicity in fish was obtained for FLU, it should be less toxic than Nor-FLU [347].

Studies performed on SER and FLU demonstrated the influence of pH on toxicity, since the uncharged drug can pass easier through the membrane and act inside the cells, showing a tenfold increased toxicity when shifting the pH closest to their pKa, increasing the nonionized form, from 6.5 to 8.5 and from 7.8 to 9, respectively [38,293,296].

I4.6. Anti-inflammatories

Most anti-inflammatories induce the nonspecific inhibition of prostaglandins. This, in turn, means that there is the potential for effects on any of the normal physiological functions mediated by prostaglandins. In fish, for instance, prostaglandins influence mechanisms of behaviour and reproduction and, therefore, they can act as endocrine disruptors or modulators, because they can exert their effects by mimicking or antagonizing the effects of hormones, alter their pattern of synthesis and metabolism and modify hormone receptor levels, leading to possible adverse effects [1,138,154,348]. However, different and unexpected toxicity effects were also observed. One of the first was reported in Pakistan, where a catastrophic decline in Oriental White-backed Vulture population (95%) was originated from the exposure to DIC contaminated live-stock carcasses, which promoted fatal renal disease [12,302].

Overall, excepting anxiolytics, anti-inflammatories were less toxic than the other therapeutic groups. Regarding the lowest concentrations that produced acute toxicity in the three trophic levels, invertebrates had the lowest value (10 ng L^{-1}), followed by algae (10 μg L^{-1}) and fish (1131 μg L^{-1}), however, when using average values, the differences become less clear

[252,263,300]. As for chronic data, higher toxicity was observed in fish (500 ng L^{-1}) and invertebrates (200 µg L^{-1}), when compared with algae (10 mg L^{-1}), which is in line with the already referred anti-inflammatories mode of action [276,299].

Data for each anti-inflammatories showed no clear pattern, nonetheless, NAP and PARA seemed to have lower toxicity than DIC and IBU. When performing a comparison between DIC and its metabolite (4-OH-DIC), one could observe that they have similar toxicities. Conversely, PARA transformation product (4-PARA) presented higher toxicity than the parent molecule in all three trophic levels.

I4.7. Hormones

Although hormones like E1, E2 and EE2 are mainly used for contraception purposes, the physiological effects are not restricted to effects on reproductive and sexual development, and can target mitochondrial function, energy metabolism and cell cycle control [154].

For acute toxicity, there is only data on algae and invertebrates, and algae presented higher toxicity since the lowest concentration promoting toxic effects was at 10 µg L^{-1}, lower than the 1500 µg L^{-1} observed in invertebrates [258,284]. Nonetheless, the toxicity promoted by this therapeutic group is mainly expected to be detected through chronic toxicity, however, these data could only be obtained for invertebrates and fish. Considering chronic data, in these two trophic levels, hormones presented higher toxicity than the other therapeutic groups, since the lowest concentrations reported were of 100 ng L^{-1} and 0.1 ng L^{-1}, for invertebrates and fish, respectively [227,287,288]. It should also be noted that, the highest concentration found that promoted toxicity for fish was also very low (494 ng L^{-1}) [306].

Individually, there were no differences observed between E1 and E2 toxicity, while EE2 seems the most toxic compound regarding chronic toxicity in invertebrates and especially in fish, where the 36 results available presented concentrations below 44 ng L^{-1} [338]. Namely, when two different fish species were exposed to EE2 at 3 and 4 ng L^{-1} they suffered sex gender reversal, from male to female, which can strongly unbalance the aquatic ecosystem [12,327,333].

I5. Environmental risk assessment

The presence of human pharmaceuticals in the environment has raised concerns worldwide. As already referred, they enter the environment through WWTPs and have been found in different aquatic environments, which has led to concerns about their potential to affect non-target species [38,123,349–351].

Based on this knowledge, the EMA issued its Guideline on ERA of medicinal products for human use in 2006, predicting the possible impact that new marketing authorizations for medicinal products may have on the environment following their release [349,352,353].

Therefore, it is critical to evaluate the concentrations of pharmaceuticals in the aquatic environment to assess and manage the possible risk that these compounds pose to aquatic organisms [190]. Pharmaceutical exposure assessments may be conducted by means of either laborious and exhaustive monitoring programs, which result in measured environmental concentrations (MECs), or by means of prediction models based on different parameters that can be used to calculate PECs. Both approaches have advantages and disadvantages [39,354], nonetheless, the number and variability of molecules that may enter the environment, together with the high costs of analysis, led to further development of theoretical models to estimate the PECs [351]. Additionally, only a predictive model could be used to assess newly marketed pharmaceuticals because MECs can only be used to manage the risk related to substances that have already hit the market. However, a comparison between MECs and PECs that considers the calculation methods and particularly the parameters included in the calculation (consumption data, pharmacokinetic parameters and elimination rate) is required to assess the validity of the predicted approaches for the PECs [351].

The ERA Guideline [353] consists of two phases. In Phase I, crude PECs for surface water are calculated and the log K_{ow} is measured. If the PEC is above 0.01 μg L^{-1}, a Phase II assessment is performed; if log K_{ow} > 4.5, persistence, bioaccumulation potential and toxicity must be evaluated (Figure 13). Pharmaceuticals that are known to have toxic activity at concentrations below 0.01 μg L^{-1}, like some endocrine disruptors, should also enter Phase II, following a tailored risk assessment strategy that addresses its specific mechanism of action [349,350]. Phase II is divided into two tiers (A and B). Tier A involves a basic set of aquatic toxicity and fate tests to determine the PNECs for three trophic levels (algae, daphnids and fish) [350]. Tier B consists of an extended assessment using refined values for PEC and PNEC calculations. At this stage, both a fate analysis and effect studies can be performed [350]. The pharmaceutical is then assessed by generating a risk quotient (RQ) evaluating the ratio between the PEC and

the PNEC; when the ratio is below 1, no risk of the pharmaceutical to the aquatic environment is expected [352,355].

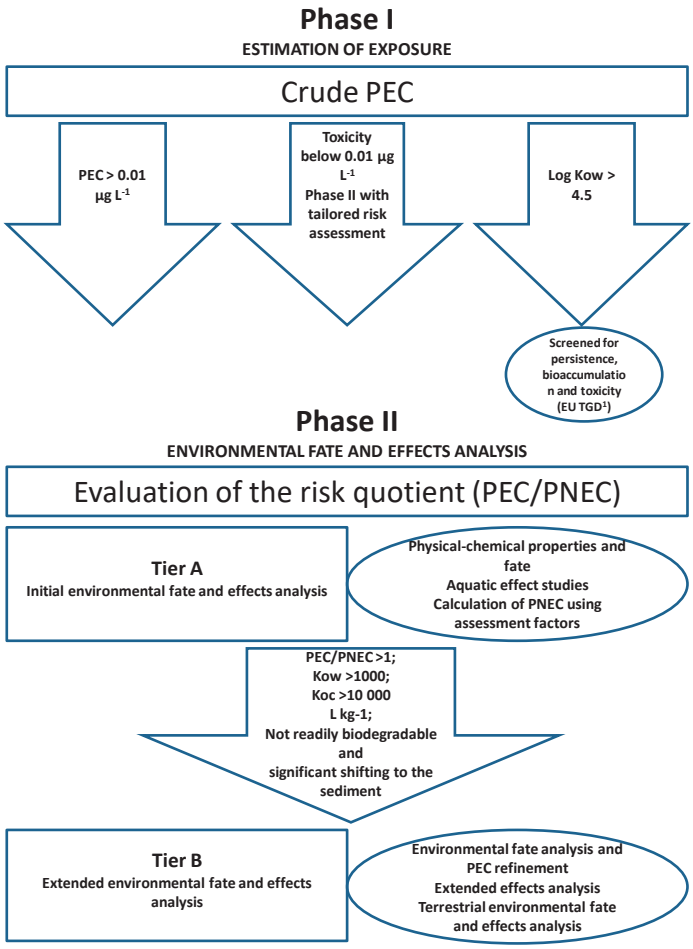


Figure 13. EMA guideline on risk assessment flow chart.

[1] European Chemicals Bureau (2003) Technical Guidance Document in support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances, Commission Regulation (EC) No 1488/94 on Risk Assessment for existing substances and Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market.

The EMA Guideline states that ERA does not constitute a valid criterion upon which to base the refusal of a market authorization of medicinal products for human use in the EU, although for veterinary medicines, this evaluation is included in the risk-benefit analysis. Furthermore, there is no publicly available record of ERAs [356]. Additionally, ERAs should also be performed for products that made it to the market before 2006 because there is no reason to believe that the risks posed by a substance, or the need for a risk assessment, would depend on

the date of market approval [349]. Although it is suggested that metabolites with excretion rates superior to 10% should also be assessed, it is not necessary to perform toxicity tests, which would not clarify whether environmentally relevant concentrations can affect both aquatic and terrestrial environments [357]. Nonetheless, of the approximately 4000 pharmaceuticals on the market today, only roughly 10% have sufficient data to perform a complete ERA, and 10% also have potential environmental risks [349,355,356].

I5.1. Predicted no-effect concentration

Predicted no-effect concentrations (PNECs) values are calculated by applying an UF of 10 to the long-term NOEC values or of 50 and 1000, to the short-term LOEC and L(E)C50 values, respectively, available in the literature. The UF is an expression of the degree of uncertainty in the extrapolation from the test data on a limited number of species to the actual environment [353]. When no experimental data are available, L(E)C50 values can be estimated through quantitative structure–activity relationship models, that predict acute toxicity data, based on the molecular structure, being one of this models provided by ECOSAR 1.11.

I5.2. Predicted environmental concentration

To evaluate the crude PECs in surface water, using the EMA Guideline, the maximum daily dose (DOSEai) (mg day^{-1}) is multiplied with a default penetration factor (Fpen) and divided by the amount of wastewater produced per inhabitant per day (WASTEWinhab) (L inh^{-1} d^{-1}) and considering a dilution factor of 10, which translates the dilution of the WWE in surface water (Equation 1) [57,350,353]. This estimation of exposure uses certain default values: a Fpen of 0.01; the DOSEai, obtained from the Summaries of Product Characteristics; and the WASTEWinhab of 200 L inh^{-1} d^{-1}. Not factoring in any human metabolism or removal by the WWTPs [350].

Equation 1. Predicted environmental concentration in surface water using EMA default formula.

$$PEC(Surface\ Water) = \frac{DOSEai * Fpen}{WASTEWinhab * 10}$$

However, the Guideline, and the PEC calculation, in particular, have been debated by scholars, some of whom argue that other parameters should also be incorporated, such as consumption data and excretion rates [349–352].

I5.3. Risk assessment

The risk assessment is obtained through the RQ, dividing the PEC or MEC (when available) by the PNEC for each pharmaceutical, observing the three different trophic levels. If RQ is equal or above 1 there is a potential environmental risk situation, whereas when values are lower than 1, no risk is expected. However, a certain risk could be expected for the substances with a RQ between 0.1 and 1 [352,355]. This evaluation can be also an important tool to suggest the inclusion or removal of pharmaceuticals in the watch list of the Directive 2013/39/EU.

As discussed, some concentrations compiled in surface water are higher than their levels that induce toxicity, not applying any uncertainty factor (UF) for the PNEC calculation. Additionally, some studies have indicated that concentrations of several pharmaceuticals belonging to different therapeutic groups can promote toxic effects on negatively impacted aquatic biota, presenting RQ higher than 1 [24,112,151,155,159,198].

As referred, aquatic biota within the receiving environment are unintentionally exposed throughout a lifetime to a complex mixture of residual pharmaceuticals at very low concentrations and these mixtures can exhibit greater effect than individual compounds [34,153]. Therefore, it is a challenge to address the concerns related to the chronic effect, low-level exposure to pharmaceuticals, including exposure of sensitive subpopulations to pharmaceutical mixtures [38,153]. Furthermore, it has been found easily measurable and potentially harmful effects on zebrafish (and probably on other fish), in environmental concentrations observed in the Douro estuary [34,358].

Therefore, an improved understanding of how mixture assessment may be performed could generate benefits in water resource management, by providing the means for cross-compliance measures in environmental regulation, providing risk assessment for pharmaceuticals mixtures [27].

Another way to evaluate the possible risk that a pharmaceutical can pose to the environment is using a persistence bioaccumulation and toxicity (PBT) index. In this method, a numerical value of 3 is assigned if the pharmaceutical possess the following characteristics: persistence, bioaccumulation and toxicity. The sum of these values constitutes the PBT index for the substance, therefore, it can be equal to 0, 3, 6 or 9, and the higher the value the greater the potential of the substance to endanger the environment. The persistence is evaluated based on OECD's test guidelines (test 301). The potential bioaccumulation of a substance is assessed based on its log K_{ow}. Values equal or greater than 3 indicate that the substance may bioaccumulate. Finally, toxicity is evaluated based on a comprehensive literature review for the

different trophic levels of the aquatic ecosystem. If its NOEC (chronic toxicity) is lower than 0.01 mg L^{-1}, if no chronic data is available, or if L(E)C50 (acute toxicity) is lower than 0.1 mg L^{-1}, the substance is considered to be potentially toxic [31].

Human health risks posed by pharmaceuticals in drinking water have been assessed using the admissible daily intake (ADI) as shown in Equation 2. This can be estimated from the lowest daily therapeutic dose (LTD) to obtain the desired pharmacological effect to obtain the point of departure (POD). Using this approach, the potentially exposed population is presumed to include healthy adults as well as susceptible sub-populations (e.g., children, the elderly and infirm). Appropriate UFs are selected based upon extrapolation uncertainties that include: LOEC to NOEC (UF1); duration of exposure (UF2); interspecies variability (UF3); intra-individual susceptibility (UF4); and quality of data (UF5). The UFs approach allows integration of protection for sensitive individuals and sub-populations. It also factors an appropriate protection from known adverse effects as well as therapeutic effects of medicines [86,359]. The values selected for the UFs are 10, 3 or 1 [359].

Equation 2. Calculation of admissible daily intake.

$$ADI\ (\mu g/kg\ bw/day) = \frac{1000 \times\ POD\ (mg/kg\ bw/day)}{UF1 \times UF2 \times UF3 \times UF4 \times UF5}$$

Observing the human health risk assessments performed in the United Kingdom, Australia and the United States of America, the World Health Organization concluded that the occurrence of pharmaceuticals in drinking water is very low and unlikely to present appreciable adverse risks to human health [38,204]. This was corroborated by another study on 19 pharmaceuticals where no risk for humans was observed [360]. Nonetheless, there is a lack of different toxicity endpoints and LTD is used to assess ADI values, which probably underestimates human toxicity, especially for mechanism non-related with the established mode of action, one of the uncertainties related to human health risk assessment [204].

Since the use of pharmaceuticals will tend to increase in the future, some mitigation measures, additionally to the improvement of WWTPs, are needed. These measures should start with the awareness of this problem; for example, in Sweden an environmental classification system for drugs has been established in collaboration between producers, authorities and the public health care. This system assesses the environmental risk hazard of the pharmaceuticals and is publicly available, therefore, the market could demand for medicines with less environmental impact, stimulating producers to design future medicines which will be more environmentally friendly

[38]. This includes the concept of green pharmacy, were the design of pharmaceutical products focus also on their high metabolization and environmental degradation, reducing the environmental burden and improving environmental safety and health impacts [12].

The risk assessment is an important tool, since environmental monitoring is facing a complex panorama in which the available analytical possibilities must be directed towards target compounds since not all measurable compounds are worth to be measured [161].

Part B – Experimental part

Chapter II – A one-year follow-up analysis of antidepressants in Portuguese wastewaters: occurrence and fate, seasonal influence and risk assessment

The occurrence and environmental risk assessment (ERA) of pharmaceuticals were first evaluated in wastewater treatment plants (WWTPs), influents (WWIs) and effluents (WWEs), since they are the major point source contamination into the aquatic environment. However, as two distinct methodologies were used, one for the selective serotonin re-uptake inhibitors (SSRIs) antidepressants and another for the remaining pharmaceuticals, and that different sampling periods were assessed, their occurrence and ERA were evaluated separately. Therefore, this publication only addressed the SSRIs citalopram (CIT), fluoxetine (FLU), paroxetine (PAR) and sertraline (SER) in this aquatic compartment.

The work presented and discussed in this chapter resulted in the following publication:
SILVA L.J.G., PEREIRA A.M.P.T., MEISEL L.M., LINO C.M., PENA A.. A one-year follow-up analysis of antidepressants in Portuguese wastewaters: occurrence and fate, seasonal influence, and risk assessment. Science of the Total Environment, 490, 279-287, 2014 (DOI: 10.1016/j.scitotenv.2014.04.131).

II1. Abstract

The occurrence, fate, seasonal influence and environmental risk assessment of four selective serotonin re-uptake inhibitors (SSRIs) antidepressants, citalopram, fluoxetine, paroxetine and sertraline, were studied in 15 different wastewater treatment plants (WWTPs) across Portugal. Influent and effluent samples from four sampling campaigns, in 2013, were extracted through Oasis HLB cartridges, and quantified through liquid chromatography with tandem mass spectrometry (LC-MS*n*). Results showed that citalopram was the SSRI most frequently found, both in influents and in effluents, with mean mass loads ranging between 14.56 and 9.51 mg/day/1000 inhabitants, respectively. Fluoxetine and sertraline were only detected in influent samples, in lower mean mass loads (14.60 and 1.36 mg/day/1000 inhab., respectively), whereas paroxetine was found in influent and effluent samples (12.61 and 18.90 mg/day/1000 inhab., respectively). WWTPs were not capable of completely removing these pharmaceuticals, nonetheless, the mean removal efficiency was 82.24%. Removal efficiency was lower in winter (74.21%), summer (72.02%), and autumn (81.19%), when compared to spring (100%).

Our results translate the variations in SSRIs prescription and use among the five Portuguese regions in study. Influent contaminated samples were found in WWTPs from Lisbon, Alentejo, Center and North (28.25, 19.01, 16.55 and 6.98 mg/day/1000 inhab., respectively). In the Algarve region no contaminated samples were found. A seasonal pattern in the presence of SSRIs in influent wastewaters was observed. The SSRIs mass loads in influent wastewaters was higher in autumn, followed by spring, winter and summer.

Finally, the potential ecotoxicological risk posed by SSRIs to different trophic levels of aquatic organisms exposed to the effluent wastewaters studied was evaluated by means of risk quotients (RQs). Citalopram and paroxetine, the only SSRIs found in these samples, presented RQ lower than 1. According to the results, algae appeared to be the most sensitive followed by fish and daphnids.

Keywords: Environmental contaminants; selective serotonin re-uptake inhibitors; municipal wastewaters; occurrence and fate; seasonal influence; environmental risk.

II2. Introduction

The presence of emerging contaminants, such as pharmaceuticals, in the environment is a growing problem that must be tackled to meet the Water Framework Directive (WFD) of the European Union (EU) [361]. A better knowledge of their environmental occurrence and fate will allow a proper risk assessment [361]. Nowadays, the higher prevalence of psychiatric disorders led to a worldwide increased number of prescriptions for psychiatric pharmaceuticals, namely antidepressants [3]. According to the latest Eurobarometer of 2010 regarding mental health, 7% of the EU citizens took antidepressants during 2009. The same report claims that the use of antidepressants is highest in Portugal, where the prevalence of use doubles that of the EU average [362].

Selective serotonin re-uptake inhibitors (SSRIs) antidepressants are amongst the most prescribed pharmaceuticals throughout the world. Both their increased consumption and their required chronic administration suggest a higher environmental exposure, dictating an environmental risk evaluation. After intake, these highly active compounds undergo metabolic transformations, with subsequent excretion of significant fractions of the unmetabolized or of active metabolites to raw sewage and wastewater treatment plants (WWTPs) [363].

The physicochemical characteristics of SSRIs (Table 7, Supporting Information) outline their environmental behaviour. They are basic drugs, with pKa ranging between 9.05 and 10.5, designed to produce a specific pharmacological response and, in order to reach the specific site of action within the organism, presenting a certain chemical stability. This stability may be later manifested in their insufficient removal during wastewater treatment and by their limited environmental degradation, sometimes resulting in minor structural alteration(s) instead of complete mineralization [3]. Scientific studies have already demonstrated their incomplete removal by WWTPs, being these facilities considered as the major environmental source since their effluents are discharged to the surrounding water bodies [364].

Consequently, their presence in different environmental matrices is ubiquitous. As far as we know, the presence of SSRIs in the environment, specifically fluoxetine, was first reported by Kolpin et al. [205] in US surface waters, and by Metcalfe et al. [365] in Canada WWTP effluents. Later on, in 2005, a study reported the presence of two SSRIs and their metabolites (fluoxetine, sertraline, norfluoxetine, and desmethylsertraline) in different fish tissues residing in a municipal effluent-dominated stream [366]. Since then, several publications, from different countries, referred the presence of these residues in a wide range of water samples, including wastewaters, in concentrations ranging from 0.15 to 32228 ng L^{-1}, surface and groundwaters,

ranging between 0.5 and 8000 ng L^{-1}, and drinking waters, from 0.5 to 1400 ng L^{-1} [3]. Also, in sediments and soils, up to 1033 ng g^{-1} [364,367], and in biota matrices, in concentrations ranging from 0.01 to 73 ng g^{-1} [364,368–370].

These molecules often act by mimicking the effects of the neurotransmitter serotonin, that regulates a wide range of physiological systems in fish, molluscs, and protozoans, and, even at trace levels, have remarkable effects on these and other aquatic organisms [3]. Alteration of the biological activity of aquatic organisms, reproduction reduction, abnormalities in embryo development, delay in physiological development and sexual maturation were described. Decreased aggressiveness and inhibition of feeding responses were also reported [1,133,371]. Recently, Scultz et al. [370] demonstrated that exposure of male fathead minnows (*Pimephales promelas*), for 21 days, to sertraline (5.2 ng L^{-1}) resulted in mortality. Anatomical alterations were noted within the tests of fish exposed to sertraline and fluoxetine. Additionally, fluoxetine at 28 ng L^{-1} induced vitellogenin in male fish, a common endpoint for estrogenic endocrine disruption.

Heavy contamination pressures from extensive urban activities characterize the Portuguese coast and main rivers that might translate into high aquatic contamination levels and consequent environmental exposure. Although, the concentration of pharmaceuticals, such as SSRIs, in influent and effluent of WWTPs is routinely monitored in many countries, sources of SSRIs contamination are geographically diffuse and may be influenced by geographical consumption patterns. Moreover, important fluctuations in concentrations due to seasonal variations might occur. The key driving force of this study was to evaluate, for the first time, the environmental contamination of SSRIs, fluoxetine, paroxetine, sertraline and citalopram, in WWTP influents and effluents from different Portuguese regions, in order to evaluate geographical contamination patterns. Moreover, we aimed to assess seasonal influence and WWTPs removal efficiency. Finally, the potential ecotoxicological risk posed by SSRIs to aquatic organisms, belonging to different trophic levels, when exposed to the studied WWTPs effluents was assessed.

II3. Materials and methods

II3.1. Sampling site and collection

Influents and effluents of 15 different WWTPs, located in 5 Portuguese regions, North, Center, Lisbon and Tagus Valley, Alentejo and Algarve (Figure 18), were collected. These WWTPs are designed for 6850 to 756000 population equivalents, with average loads ranging between 349 and 140000 m^3 per day, having their discharge points in the main Portuguese rivers and Atlantic Ocean. They are designed to treat domestic, hospital and industrial wastewaters, operating with secondary or tertiary treatments, as described in Table 4.

Sampling campaigns, carried out in 2013, were performed during a one year follow-up study, embracing four sampling periods; between 25 February/19 March – winter, 14 May/04 June – spring, 11 July/14 August – summer, and 24 October/7 November – autumn. The characterization of influent and effluent parameters of each WWTP, for the different sampling periods, is shown in Table 8, Supporting Information. For each plant, influent and effluent samples were collected in high-density polyethylene containers previously rinsed with bi-distilled water, as time proportional 24-h composite influent and effluent samples. Samples were kept refrigerated (± 4 ºC) during the transport to the laboratory. Upon reception, samples were frozen and stored at −20 ºC until analysis.

Table 4. Codes of the sampling points and characteristics of the wastewater treatment plants (WWTPs).

WWTP Code	Region	Population equivalent	Type of wastewater treated	Discharging points	Average loads (m^3/day)	Type of treatment	Process
WWTP 1	North	41955	Domestic, industrial (residual)	Fervença River	5685	Secondary	Activated Sludge with conventional aeration
WWTP 2	North	10000	Domestic, industrial (mainly)	Tua River	349	Tertiary with UV	Activated Sludge with extended aeration
WWTP 3	North	57748	Domestic, industrial (residual)	Tâmega River	8069	Tertiary with UV	Activated Sludge with extended aeration
WWTP 4	North	45257	Domestic, hospital and industrial	Atlantic Ocean	8580	Tertiary with UV	Activated Sludge with medium load aeration
WWTP 5	North	255557	Domestic and industrial	Ave River	15000	Tertiary with UV	Activated Sludge with conventional and extended aeration
WWTP 6	North	300000	Domestic	Atlantic Ocean	66718	Tertiary with UV	Activated Sludge with extended aeration
WWTP 7	Center	213000	Domestic and industrial	Mondego River	36000	Secondary	Trickling Filters
WWTP 8	Center	6850	Domestic, hospital and industrial	Mondego River	600	Secondary	Activated Sludge
WWTP 9	Lisbon and Tagus Valley	700000	Domestic and industrial	Trancão River	60000	Secondary with biofiltration	Activated Sludge
WWTP 10	Lisbon and Tagus Valley	215000	Domestic and industrial	Tagus River	50000	Tertiary with UV	Activated Sludge
WWTP 11	Lisbon and Tagus Valley	756000	Domestic	Tagus River	140000	Tertiary with UV	Biofiltration
WWTP 12	Alentejo	60000	Domestic, hospital and industrial	Xarrama River	13720	Tertiary with UV	Activated Sludge with medium load aeration
WWTP 13	Alentejo	8700	Domestic	Álamo Brook	1239	Tertiary with UV	Activated Sludge with extended aeration
WWTP 14	Algarve	49547	Domestic	Atlantic Ocean	9239	Tertiary with UV	Activated Sludge with extended aeration
WWTP 15	Algarve	30766	Domestic	Guadiana River	6141	Tertiary with UV	Lagoons with extended aeration

II3.2. Standards and chemicals

Reference standards of fluoxetine hydrochloride, sertraline hydrochloride, paroxetine hydrochloride hemihydrate, citalopram hydrobromide and the labelled surrogate fluoxetine-d_5 hydrochloride, all with ≥ 98 % purity, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock and intermediate solutions were prepared in methanol at 5 mg mL^{-1} and 250 µg mL^{-1}, respectively, and were stored at −20 °C, for a maximum of 6 months. Mixed standard working solutions, renewed before each analytical run, were prepared at 7.5 and 50 ng mL^{-1}, of each SSRI, and used for linearity, accuracy, and repeatability assays. The labelled surrogate fluoxetine-d_5 hydrochloride was typically prepared to obtain a final concentration of 50 ng mL^{-1}.

HPLC-grade acetonitrile and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was prepared from a Millipore Milli Q system (Bedford, MA, USA). Ammonium acetate and formic acid (98%) were obtained from Merck (Darmstadt, Germany); glacial acetic acid was from Panreac (Barcelona, Spain).

II3.3. Experimental procedure

Samples were acidified with 0.1% formic acid (to a final pH that ranged between 3.0 and 3.2) and vacuum filtered through glass microfiber filters (1.0 µm, 934-AH, from Whatman Schleicher and Schuell, USA), followed by 0.45 and 0.2 µm polyamide membrane filters (from Whatman, Dassel, Germany). As the suspended solids were removed during sample preparation, the measured concentrations of SSRIs correspond to their dissolved fraction.

Based on previously reported methodologies [363], 100 mL of influent and effluent samples were spiked at 500 ng L^{-1} with the labelled surrogate fluoxetine-d_5, and loaded into the solid phase extraction (SPE) cartridges, Oasis HLB (200 mg, 6 mL, from Waters, Milford, MA, USA), previously conditioned with 5 mL water and 5 mL methanol. The cartridges were then washed with 5 mL 20% methanol in 2% ammonium acetate, and eluted with 8 mL 2% acetic acid in methanol. Finally, the eluate was evaporated to dryness under a gentle stream of nitrogen, at 40 ºC, and the dried extracts were stored at −20 ºC until analysis, that took place in 48 h maximum.

For liquid chromatography coupled to tandem mass spectrometry (LC-MS*n*) analysis, the dried eluate was taken in 1 mL methanol and microfiltered. A 20 µL (partial loop) injection volume was used with a flow rate at 200 µL min^{-1} and a gradient of (A) water with 1% formic acid and

(B) acetonitrile, as presented in Table 9, Supporting Information. A chromatographic column ZORBAX Eclipse XDB-Phenyl (150 x 3.0 mm; 3.5 µm), maintained at 45ºC, and guard-column of the same packing material were used. A hybrid Quadrupole Ion Trap Mass Spectrometer (LCQ Advantage MAX, Thermo Finnigan, San Jose, California, USA) was operated in the positive electrospray ionization (ESI) mode using selected reaction monitoring (SRM) acquisition. Source and capillary temperatures were set at 0 and 220 ºC and voltages at 4.5 and 34 V, respectively. Nitrogen was used as nebulizing gas, with a sheath gas flow of 40 (arbitrary unit) and the auxiliary sweep gas flow of 10 (arbitrary unit). The collision gas was helium with normalized collision energy of 35%. A precursor ion (MS1), a MS2 product ion and, at least, one MS3 product ion were obtained, as following, for each SSRI: citalopram (m/z 325→ m/z 266→ m/z 234), paroxetine (m/z 330→ m/z 192→ m/z 70), fluoxetine (m/z 310→ m/z 148→ m/z 117), and sertraline (m/z 306→ m/z 275→ m/z 159, 129, 197).

II3.4. Mass loading estimations

Mass loadings of SSRIs were calculated for each sampling period by multiplying individual concentrations of each SSRI found by the mean daily flow rate of wastewater provided by each WWTP. Discharges of pharmaceuticals can fluctuate daily, monthly or seasonally. Nonetheless, antidepressants, namely SSRIs, are used chronically, and it would be very demanding to conduct a more comprehensive monitoring using a more periodical sampling. The WWTP loads were normalized by the population equivalent (Table 8, Supporting Information). Removal efficiency of SSRIs was evaluated by means of Equation 3.

Equation 3. Removal efficiency.

$$\text{Removal efficiency (\%)} = \frac{m_{inf} - m_{eff}}{m_{inf}} \text{ x } 100$$

Where m_{inf} is the load of the pharmaceutical in WWTP influent and m_{eff} is the load of the pharmaceutical in WWTP effluent.

II3.5. Environmental risk assessment

The evaluation of the potential ecotoxicological risk posed for the aquatic compartment has been based on the guideline on the environmental risk assessment (ERA) of medicinal products for human use [353]. Following this guideline, the risk evaluation is performed calculating the risk quotient (RQ), using three different trophic levels representatives of the aquatic ecosystem (algae, daphnids and fish), between measured environmental concentration (MEC) and predicted no-effect concentration (PNEC), where the maximum individual concentrations of pharmaceuticals found in the different wastewaters were used as MEC [23,372]. PNEC values were calculated by dividing the lowest short-term L(E)C50 or long-term NOEC (no-observed-effect-concentration) value, available in the literature, by an assessment factor (AF) of 1000 or 10, respectively. The AF is an expression of the degree of uncertainty in the extrapolation from the test data on a limited number of species to the actual environment [353], therefore AF values of 10 and 1000 were used for long-term and short-term data, respectively. When no experimental values were available, L(E)C50 values estimated with ECOSAR 1.11 were used. If RQ is equal or above 1 there is a potential environmental risk situation, whereas when values are lower than 1, no risk is expected.

II4. Results and discussion

II4.1. Method validation

Validation was performed to assure the fitness for purpose of the analytical method for the determination of the selected SSRIs in wastewaters. Validation procedures were carried out in influent and effluent samples, encompassing different performance criteria such as sensitivity, linear range, matrix effects, accuracy, and precision. Results are summarized in Table 10, Supporting Information.

Linearity was studied using standard solutions and matrix-matched calibrations by analysing in triplicate eight concentration levels, between 7.5 and 50 ng mL^{-1}, that correspond, according to the analytical methodology, to the range of 100 to 500 ng L^{-1}, and 75 to 500 ng L^{-1}, studied in influent and effluent wastewater, respectively. Linearity, achieved for every compound, in the working standard solutions, was good, as shown by the fact that the correlation coefficients (r^2) were 0.9985, 0.9987, 0.9988, and 0.9983 for citalopram, paroxetine, fluoxetine and sertraline,

respectively. In influent and effluent matrix-matched solutions adequate r^2 values greater than 0.996 were obtained.

Matrix effects (ME) equalled the percentage of the matrix-matched calibration slope (B) divided by the slope of the standard calibration in solvent (A). Thus, the ratio (B/A x 100) was defined as the absolute matrix effect (ME %). The obtained value was interpreted as follows: a value of 100% denoted an absence of matrix effects, above 100% a signal enhancement and below 100% a signal suppression. Matrix effects were investigated, both in influent and effluent samples, and ranged between 84.6 and 116.6%, and so were considered negligible.

The method detection limits (MDL) and method quantification limits (MQL) were calculated through the matrix-matched calibration curve as $|3.3S_{y/x}|/b$ and $|10S_{y/x}|/b$, respectively, where b is the slope and $S_{y/x}$ the residual standard deviation of the linear function. Influent MQL and MDL values ranged from 63.2 to 92.3 ng L^{-1}, and from 20.8 to 30.4 ng L^{-1}, respectively. Regarding effluent samples, MQL and MDL values ranged from 35.3 to 70.9 ng L^{-1}, and from 11.7 to 23.4 ng L^{-1}, respectively.

For accuracy and repeatability assays, recoveries were determined in triplicate, at three different spiking levels, in three different days, and each extract was analysed three times. SSRIs accuracy in influent wastewater, evaluated through spike assays at 100, 250 and 500 ng L^{-1}, varied between 72.5% and 125.9%, with an intra-day and inter-day repeatability ranging between 0.2 – 5.0% and 0.1 – 5.9%, respectively. For effluent wastewater, spike assays were done at 75, 250 and 500 ng L^{-1}, and accuracy varied between 86.3% and 122.2%, with intra-day and inter-day repeatability (RSD %) ranging from 0.2 to 5.0%, and from 0.1 to 5.9 %, respectively.

II4.2. Occurrence and removal efficiency

Table 5 and Figure 14 outline a summary of the occurrence data of the selected SSRIs in influents and effluents of the studied WWTPs, the range and mean detected concentrations, detection frequency, together with the estimated error loads of each compound and the removal efficiencies observed. The results showed that citalopram was the SSRI most frequently found. Regarding influent samples, 23.33% were contaminated in levels ranging between 99.20 and 213.60 ng L^{-1}. As expected, citalopram concentrations were lower in the 8.33% of contaminated effluent samples, with levels ranging between 82.80 and 95.60 ng L^{-1}. Citalopram mean detected concentrations in influent and effluent samples were 147.54 and 90.02 ng L^{-1}, respectively, which corresponds to mean mass loads of 14.56 and 9.51 mg/day/1000

inhabitants. Fluoxetine and sertraline were only detected in influent samples, with a frequency of 5% and 1.67%, respectively, in mean detected levels of 127.97 and 100.4 ng L^{-1} (14.6 and 1.36 mg/day/1000 inhab., respectively). Paroxetine was found in influent and effluent samples, with a frequency of 5 and 1.67%, in mean detected concentrations of 169.97 and 81.1 ng L^{-1}, respectively (12.61 and 18.90 mg/day/1000 inhab., respectively).

These results, and the fact that citalopram was found in higher frequency and concentrations when compared to the other SSRIs, are largely explained by the following factors: consumption, excretion, sorption to solid matter, transformation, and removal. The latest Portuguese data on consumption of antidepressants are from 2011 and were reported by Infarmed, the National Authority of Medicines and Health Products. Fluoxetine, sertraline, escitalopram, and paroxetine were in the top 100 active substances list of packages sold in the National Health Service (NHS), with 77425, 743332, 540830 and 410133 packages, respectively. Citalopram, with 125620 packages sold [373], being a racemic mixture of (R)-Citalopram and (S)-Citalopram, enantiomers with different potency, is also marketed as the single (S)-enantiomer formulation, escitalopram [3]. Since the LC methodologies used are unable to separate enantiomers, the concentrations found correspond to the sum of both pharmaceuticals. Therefore, in 2011, 666450 packages of citalopram and escitalopram were sold [373], that places this active substance in the third place of the list of SSRIs most consumed in Portugal.

Table 5. Detected concentrations (ng L^{-1}), frequencies (%), mass loads (mg/day/1000 inhab) and removal efficiencies (%) of SSRIs in WWTP influents and effluents.

Sampling		**Concentration**		**Mass Loads**		
WWTP	**Period**	**WWI**	**WWE**	**WWI**	**WWE**	**Removal**
Citalopram						
WWTP2	Winter	137.40	87.10	3.89	2.47	36.50
	Spring	101.20	n.d.	1.36	n.d.	100.00
	Summer	172.00	n.d.	3.87	n.d.	100.00
	Autumn	213.60	n.d.	3.74	n.d.	100.00
WWTP3	Winter	99.20	n.d.	21.0	n.d.	100.00
	Autumn	158.30	n.d.	18.1	n.d.	100.00
WWTP5	Winter	125.70	82.80	5.50	3.60	34.55
WWTP7	Spring	167.20	n.d.	25.30	n.d.	100.00
WWTP8	Winter	110.50	n.d.	7.80	n.d.	100.00
WWTP10	Autumn	162.20	89.70	37.70	20.90	44.56
WWTP11	Winter	100.50	n.d.	18.60	n.d.	100.00
WWTP13	Spring	179.70	n.d.	20.24	n.d.	100.00
	Summer	167.70	94.90	15.90	8.90	44.03
	Autumn	170.30	95.60	20.90	11.70	44.02
Frequency	–	23.33	8.33	–	–	–
Range	–	99.20 – 213.60	82.80 – 95.60	1.36 – 37.70	2.47 – 20.90	34.55 – 100.00
Mean±SD	–	147.54±35.40	90.02±5.38	14.56±10.53	9.51±7.41	78.83±29.59
Fluoxetine						
WWTP2	Autumn	120.70	n.d.	2.11	n.d.	100.00
WWTP6	Autumn	157.40	n.d.	17.10	n.d.	100.00
WWTP10	Autumn	105.80	n.d.	24.60	n.d.	100.00
Frequency		5.00	0.00	–	–	–
Range	–	105.80 – 157.40	–	2.11 – 24.60	–	–
Mean±SD	–	127.97±26.56	–	14.60±11.45	–	100.00±0.00
Paroxetine						
WWTP2	Spring	186.40	n.d.	2.50	n.d.	100.00
WWTP2	Autumn	185.60	n.d.	3.25	n.d.	100.00
WWTP10	Autumn	137.90	81.10	32.10	18.90	41.12
Frequency		5.00	1.67	–	–	–
Range	–	137.90 – 186.40	–	2.5 – 32.10	–	41.12 - 100
Mean±SD	–	169.97±27.77	81.10±0.00	12.61±16.88	18.90±0	80.37±33.99
Sertraline						
WWTP2	Spring	100.40	n.d.	1.36	n.d.	100.00
Frequency		1.67	0.00	–	–	–
All SSRIs						
Frequency		25	8.33	–	–	–
Range	–	99.20 – 213.60	81.10 – 95.60	1.36 – 37.70	2.47 – 20.90	34.55 – 100.00
Mean±SD	–	147.97±33.95	88.53±6.03	14.28±10.90	11.08±7.65	82.24±27.92

n.d. – not detected

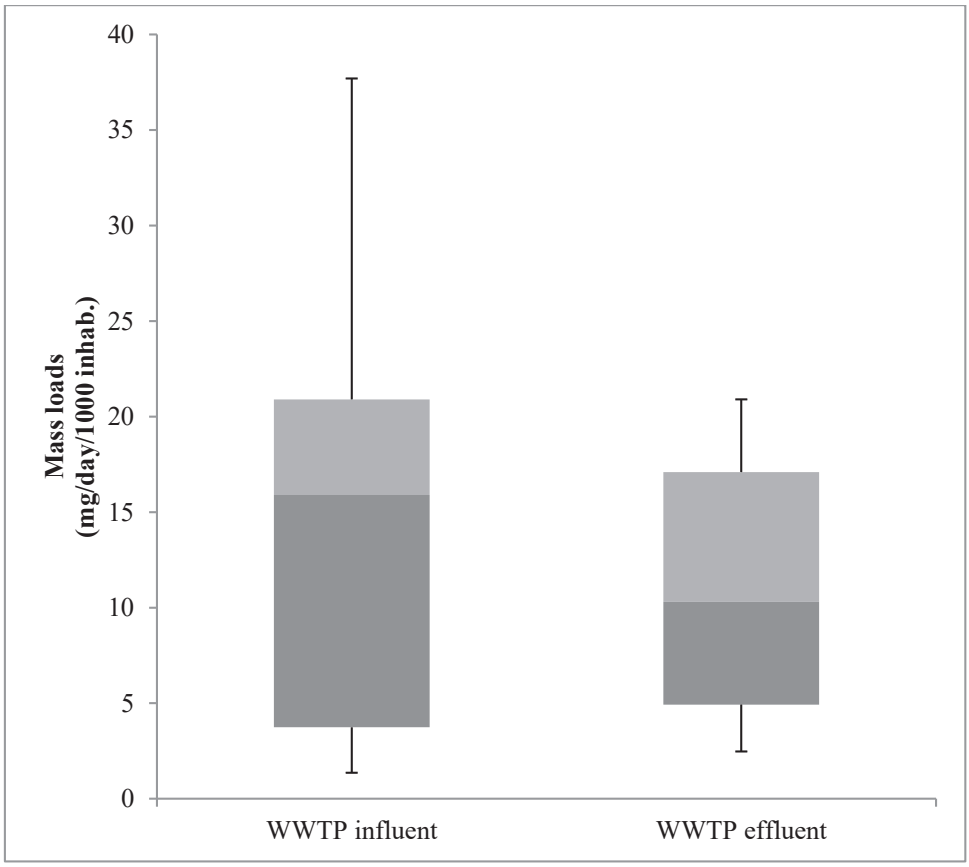


Figure 14. Boxplots indicating mass load values, expressed in mg/day/1000 inhabitants, of the total SSRIs in WWTP influents and effluents.

Although citalopram becomes in the third place of the Portuguese consumption list, it accounts, according to the scientific literature, with the larger percentage of excretion as unchanged compound, ranging between 12 and 20%. Fluoxetine, with less than 10% excreted unchanged, is mainly excreted as norfluoxetine, and 2% and 1% of paroxetine is excreted as parent compound in urine and faeces, respectively. Although information on sertraline metabolism is rather limited, only 0.2% of its oral dose is excreted unchanged [3].

Some studies have examined a suite of antidepressants in wastewater matrices being less ambitious regarding the geographical distribution of the WWTPs evaluated [77,128,129,374–377]. Several authors included few SSRIs in their multiclass monitoring [23,378]. Our results are in good agreement with those found in the scientific literature reviewed since citalopram is typically found at higher frequencies and concentrations when compared to the other SSRIs [3]. For instance, recently, in Canada, citalopram was found in wastewater influents and effluents at mean levels of 236 and 173 ng L^{-1}, whereas fluoxetine, paroxetine and sertraline were found in these same matrices ranging between 8 and 20, and 5.6 and 15 ng L^{-1}, respectively [367]. This disparity is also observed in other studies from Canada [77,375,379], and in studies from

Norway, where citalopram was found in influents and effluents ranging between 13 and 612 ng L^{-1} and 9.2 and 318 ng L^{-1}, respectively, whereas fluoxetine ranged between 0.4 and 2.4 ng L^{-1} and <0.12 and 1.3 ng L^{-1}, respectively [128]. In Spain, in 2012, citalopram was found ranging between 319 and 163 ng L^{-1} in influent wastewaters, and 288 and 21 ng L^{-1} in effluent wastewaters, while fluoxetine was detected at lower concentrations, 23 and 28 ng L^{-1}, respectively [380]. On the contrary to our results paroxetine was found at higher concentrations in Spanish influents, at 1649 ng L^{-1}, but at levels similar to ours, 89 ng L^{-1} in effluents [380]. High citalopram levels were also reported in effluent samples from Austria, between 44 and 322 ng L^{-1} [381], and from India, in mean levels of 430 ng L^{-1} [9]. Recently, in 2012, an EU wide monitoring survey on WWTP effluents was published and, accordingly to their results, citalopram was detected in a mean concentration of 34 ng L^{-1}, a value higher to that of our study, 7.63 ng L^{-1}. Fluoxetine and sertraline were determined in lower mean concentration of 2 ng L^{-1}, whereas paroxetine was not detected [21].

In Portugal four studies are available including the SSRIs escitalopram, fluoxetine and paroxetine [22,157], fluoxetine and paroxetine [20] and citalopram, fluoxetine, paroxetine and sertraline [23]. In the former, remarkably high values, ranging between 14 ng L^{-1}, for paroxetine, and 39732 ng L^{-1}, for escitalopram, were observed [157]. Our results differ from those presented in the study of Santos et al. [23], where mean levels of citalopram of 23.3 and 34.0 ng L^{-1}, in influent and effluent samples, respectively, were reported, whereas fluoxetine, paroxetine and sertraline were not detected. Sousa et al. [20], also observed paroxetine at higher concentrations (45 – 240 ng L^{-1}) than fluoxetine (< 5 ng L^{-1}). According to the recently, above mentioned, EU monitoring, in Portuguese effluents, citalopram was the SSRI found in higher concentrations (16.9 - 47.8 ng L^{-1}), corroborating our study. Fluoxetine was found in lower levels (16.6 – 21.5 ng L^{-1}), while paroxetine and sertraline were not detected [21].

In the present study, the fate of the selected SSRIs was determined in 15 Portuguese WWTPs employing different treatment processes (e.g. secondary and tertiary treatments). The WWTPs were operating normally during all sampling events, and generally achieved good removals on what concerns biochemical oxygen demand (BOD), chemical oxygen demand (COD) and total suspended solids (TSS) (Table 8, Supporting Information). One should note that the influent and effluent composite samples were collected concurrently, with no allowance for the hydraulic retention times (HRTs) of the treatment systems. Removal efficiencies of SSRIs were evaluated by comparing the load of each compound in WWTP influent and effluent. Table 5 shows the mass loads found for each SSRI in the different sampling campaigns, ranges and mean values, as well as their removal rates. The results obtained show that some WWTPs were

not able to completely remove these pharmaceuticals; nonetheless, the overall removal efficiency for SSRIs was 82.24%. The removal efficiency of citalopram ranged between 34.55 and 100.00%, with a mean value of 78.83%. Fluoxetine, paroxetine and sertraline, found in lower frequencies, had mean removal efficiencies oscillating between 80.37 and 100.00%.

The occurrence of emerging contaminants in environmental waters is directly related to their removal in WWTPs [382]. Since SSRIs are designed to produce a specific pharmacological response, and, in order to reach the specific site of action within the organism, they require a certain chemical stability. This stability may be later manifested in their incomplete removal during water treatment [3]. As seen in Table 5, systems that use an activated sludge process are still widely employed for wastewater treatment, mostly because they produce an acceptable quality effluent at reasonable operating and maintenance costs. However, this type of treatment has limited capability of removing pharmaceuticals from wastewater [383–385]. Even though, the removal rates obtained were better than those reported by Gros et al. [386], who stated that SSRIs show either poor or no elimination, and also better than those reported by Lajeunesse et al. [367], who observed removal rates of 27% for citalopram, and 38% for sertraline.

Although, SSRIs concentrations in sludge or suspended solids were not considered nor measured, one should note that good removal rates obtained in aqueous phase do not imply degradation to the same extent. SSRIs are persistent compounds [387], presenting high sorption coefficients with soils and sediments, with a range of log k_{oc} (organic carbon normalized sorption coefficient) values ranging from 4.17 to 5.63, for sertraline (lowest degree of sorption) and citalopram (highest degree of sorption), respectively. In the absence of other transformation processes, the environmental concentration of each of these chemicals would increase in the solid matter and their concentration in the overlying water reduced [3]. Moreover, the conversion of a given pharmaceutical to transformation products other than the analysed might lead to lower pharmaceutical levels in effluent samples, and to an apparent removal [96,113].

II4.3. Geographical and seasonal influence

Despite the fact that some research for understanding the fate of pharmaceuticals, namely SSRIs, in Portuguese WWTPs has been performed, specific geographical surveys still need to be considered since the occurrence pattern of pharmaceuticals in WWTPs is normally related to local consumption or sales figures [20].

Based on Portuguese data, different psychodrugs consumption patterns, including antidepressants, are observed for the 5 regions in study. In 2008, the regions which registered

a higher rate were Alentejo and Centro (172.9 and 165.1 defined daily dose—DDD/1000 inhab./day, respectively), followed by North (157.4 DDD/1000 inhab./day) and Lisbon and Tagus Valley (142.6 DDD/1000 inhab./day). The lowest values were registered in Algarve (106.9 DDD/1000 inhab./day) [388].

As seen in Figure 15, our results translate these variations in prescription and use patterns between the five Portuguese regions in study. Influent contaminated samples were found in WWTPs from Lisbon, Alentejo, Center and North, in levels that decreased by this order: 28.25, 19.01, 16.55 and 6.98 mg/day/1000 inhab., respectively. In the Algarve region none contaminated samples were found.

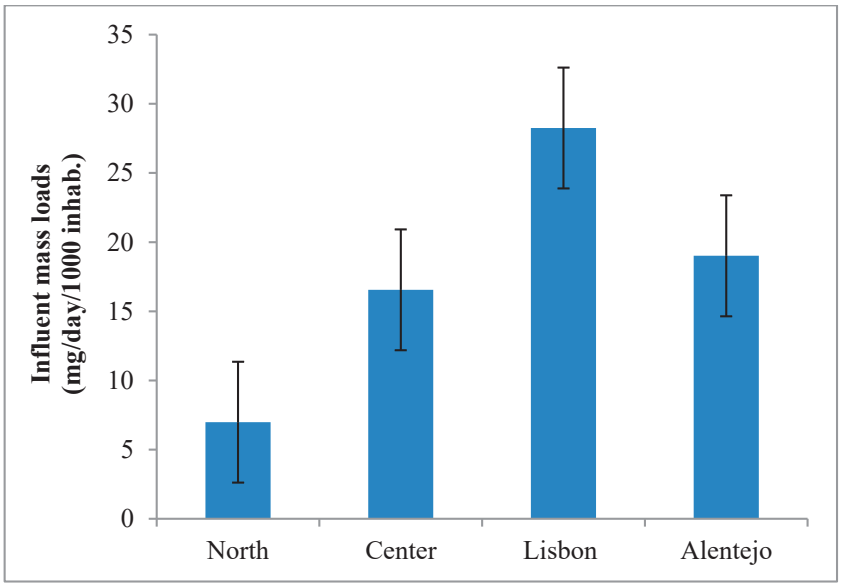


Figure 15. Geographical variations on the occurrence of the selected SSRIs in influent wastewaters.
Error bars represent the standard error of the mean of each geographic region.

Seasonal affective disorder is a combination of biologic and mood disturbances with a seasonal pattern, typically occurring in the autumn and winter with remission in the spring or summer. Pharmacotherapy with antidepressants is usually an option for an appropriate treatment [389]. Our results (Figure 16) indicate a seasonal pattern in the presence of SSRIs in the influent wastewater studied. The mass loads of each compound in influent wastewater decreased in the following order: autumn (ranging between 14.6 and 20.11 mg/day/1000 inhab. for fluoxetine and citalopram, respectively), spring (ranging between 1.35 and 15.63 mg/day/1000 inhab. for sertraline and citalopram, respectively), winter (only citalopram was found in mass loads of 11.3 mg/day/1000 inhab.), and summer (only citalopram was found in mass loads of 1.32

mg/day/1000 inhab.), which translates the consumption of antidepressants, including SSRIs, during these periods.

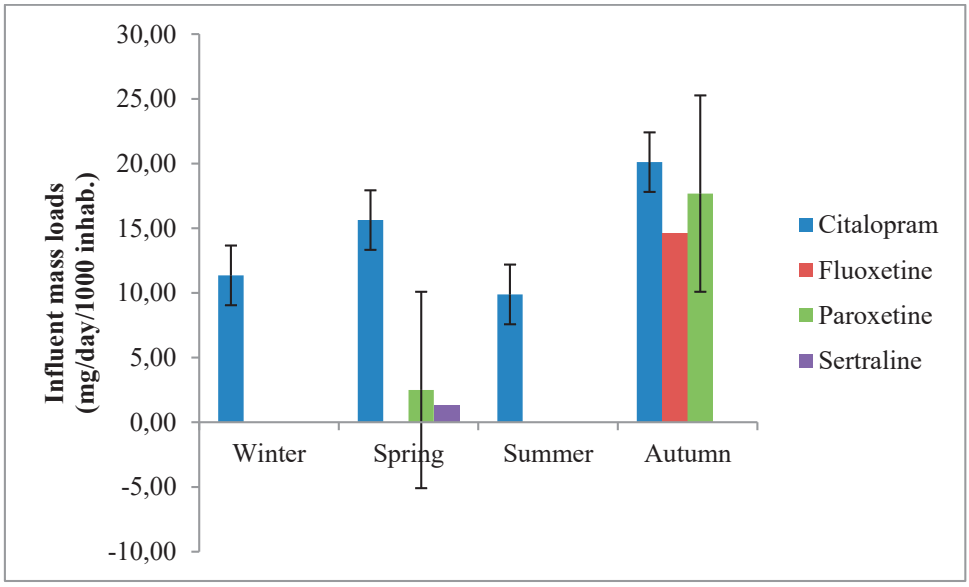


Figure 16. Seasonal variations on the occurrence of the selected SSRIs in influent wastewaters.
Error bars represent the standard error of the mean of each season.

Many factors, including HRT, organic load, microbial community, raw sewage temperature and pH were shown to have pronounced effects on the efficiency of activated sludge treatments [367]. As such, seasonal variations may also affect the efficiency of WWTPs, leading to increased concentrations of pharmaceuticals in the effluent water since in winter the microbial activity and biological reactions are reduced due to low temperatures and reduced HRTs [89,367,390,391]. With the heavy raining conditions that were registered during the winter 2013 sampling campaign, especially in March, that registered a precipitation rate higher than 220 mm, about 2.5 to 5 times higher than the average (http://www.ipma.pt/pt/oclima/observatorio.secas/pdsi/monitorizacao/evolucao/), reduced HRTs were to be expected. According to our results, the overall mean removal efficiency (Figure 17) was lower in summer (72.02), followed by winter (74.21%), autumn (81.19%) and spring (100%).

In summer, the mean percentage of removal observed was similar to the winter period due to the low removal of WWTP 13 during this period.

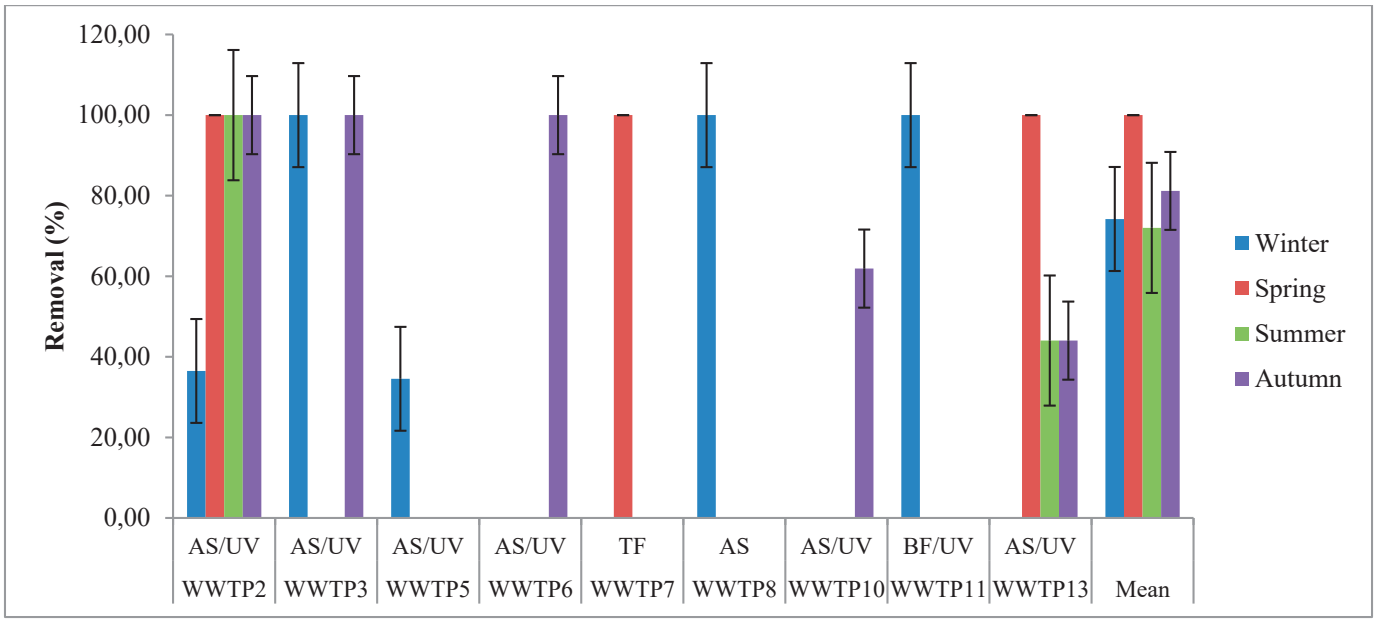


Figure 17. Seasonal variations on the removal of all SSRIs.
(AS/UV—activated sludge with UV disinfection; TF—trickling filters; BF/UV—biofiltration with UV disinfection). Error bars represent the standard error of the mean of each season.

II4.4. Environmental risk assessment

The above-mentioned data about occurrence and fate of SSRIs are crucial in order to improve ERA in a way to evaluate health, ecological and economic consequences. Since SSRIs concentration in water is low, ecotoxicological long-term data are preferred to short-term data. However, due to the lack of long-term toxicological studies, a widespread approach is the use of data from short-term studies (EC50 or LC50) to calculate PNECs [23,372]. It should be taken into account that the choice of data can obviously affect the outcome. The highest concentrations of SSRIs in the effluent wastewater samples (to set in the worst-case scenario), PNEC values (together assessment factors used) and risk quotients deemed for each analyte are shown in Table 6.

Table 6. Maximum environmental concentrations (MEC) in effluent wastewaters, PNEC and RQ for algae, daphnids and fish for the studied SSRIs.

SSRI	MEC (ng L^{-1})	PNEC (ng L^{-1}) algae	RQ algae	PNEC (ng L^{-1}) daphnids	RQ daphnids	PNEC (ng L^{-1}) fish	RQ fish
Citalopram	213.60	360.00 [a, b]	0.59	80000 [c, d] [266]	0.0027	4467 [b,e]	0.048
Paroxetine	81.10	260.00 [a, b]	0.31	22000 [c, d] [266]	0,0037	3293 [b,e]	0.025

[a] EC50 was estimated with ECOSAR
[b] AF=1000
[c] long-term data
[d] AF=10
[e] LC50 was estimated with ECOSAR

According to these results both citalopram and paroxetine, the only SSRIs found in effluent wastewaters, have RQ lower than 1, therefore, no risk is expected. Nonetheless, a certain risk could be expected for these substances with a RQ calculated for algae between 0.1 and 1, more precisely, 0.59 and 0.31, for citalopram and paroxetine, respectively. According to the results, algae appeared to be the most sensitive species followed by fish and daphnids. As far as we know, scarce information is available on the individual ecotoxicity of citalopram and paroxetine [266,272,292]. However, it should be noted that, given the mixture of these compounds with the same pharmacological mechanisms, additive or even synergistic effects could be expected, being the real hazard greater than the calculated. For instance, Henry and Black [392] reported that concentrations estimated to induce 50% *Ceriodaphnia dubia* mortality in 48 h for paroxetine, and citalopram ranged from 2.23 to 3.57, and from 10.47 to 14.53 μM, respectively, whereas for the mixture of these compounds (relative concentration factors of 1 and 5.27, respectively) the concentration was 8.76 μM, for the sum of both compounds.

Probably the dilution of wastewaters in receiving surface waters may be enough to mitigate the estimated ecotoxicological risk. Indeed, the mitigation of the risk posed by the occurrence of pharmaceuticals in the treated effluent is due not only to dilution of the receiving water body, but also to auto-depurative processes occurring within the water phase in the bulk of the receiving water body, as well as photocatalytic processes once pharmaceuticals reach the environment and remain in the free water systems (rivers, lakes, sea, etc.) [23].

This risk evaluation has its limitations given the lack of toxicological studies, namely long-term studies and long-term studies across the lifespan of the organisms (especially with fishes). Nonetheless, it is a contribution to assess the ecotoxicological risk posed by these pharmaceuticals to aquatic organisms that were already described to undergo remarkable effects including estrogenic endocrine disruption [370].

II5. Conclusions

Based upon our results, the presence of citalopram in the aquatic environment of some Portuguese regions is evident. Citalopram was the SSRI most frequently found, with higher mean mass loads, in influent and effluent samples. Fluoxetine, sertraline, and paroxetine were detected in lower mean mass loads. Paroxetine was found in influent and effluent samples, whereas fluoxetine and sertraline were only detected in influents. WWTPs were not able to completely remove these pharmaceuticals; nonetheless, the overall removal efficiency was

82.24%. Removal efficiency was lower in winter (74.21%), summer (72.02%), and autumn (81.19%), when compared to spring (100%).

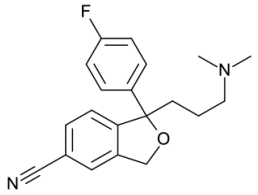
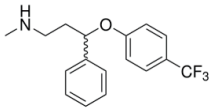
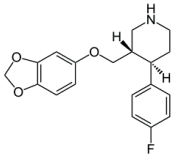
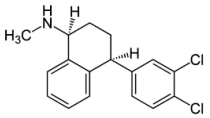
Our results demonstrate the variations in SSRIs prescription and use between the five Portuguese regions in study. Influent contaminated samples were found in WWTPs from Lisbon, Alentejo, Center and North (28.25, 19.01, 16.55 and 6.98 mg/day/1000 inhab., respectively). In the Algarve region no contaminated samples were found. As expected, a seasonal pattern in the presence of SSRIs in influent wastewater was observed. The SSRIs concentrations in influent wastewater were higher in autumn, followed by spring, winter, and summer.

Finally, after evaluating the potential ecotoxicological risk posed by SSRIs to different trophic levels of aquatic organisms, exposed to the effluents studied, we conclude that citalopram and paroxetine, the only SSRIs found in effluent wastewaters, have RQ lower than 1. Algae appeared to be the most sensitive species followed by fish and daphnids.

In order to evaluate health, ecological and economic consequences, these are important data to estimate the European contamination pattern and address SSRIs ERA. Sustainable strategies for minimizing SSRIs impact on the environment and prioritizing measures should be established.

II6. Supporting information

Table 7. CAS number and physicochemical characteristics of the selected SSRIs (adapted from Kwon et al. [393]).

Name	CAS number	MW	pka	log k_{ow}[a]	log k_{oc}[a,b]	Molecular structure (formula)
Citalopram	59729-33-8	324.16	9.59	1.39	5.63	 ($C_{20}H_{21}FN_2O$)
Fluoxetine	54910-89-3	309.13	10.05	1.22	4.65	 ($C_{17}H_{18}F_3NO$)
Paroxetine	61869-08-7	329.14	10.32	1.37	4.47	 ($C_{19}H_{20}FNO_3$)
Sertraline	79617-96-2	305.07	9.47	1.37	4.17	 ($C_{17}H_{17}Cl_2N$)

[a] Measured on salt form (HCl) of each SSRI.

[b] Average calculated from experiments with five different soils and sediments at pH 5.0-7.8.

Table 8. Characterization of WWTP parameters for the different sampling periods.

WWTP	Sampling date	Flow rate (m^3/ day)	Flow rate (L/day/1000 inhab)	WWTP influent				WWTP effluent			
				pH	BOD5 (mg O_2 L^{-1})	COD (mg O_2 L^{-1})	TSS (mg L^{-1})	pH	BOD5 (mg O_2 L^{-1})	COD (mg O_2 L^{-1})	TSS (mg L^{-1})
1	17-03-2013	9664	230342	7.6	300	515	200	7.7	8	15	12
	12-05-2013	3819	91026	8.3	480	768	453	7.3	10	47	18
	23-07-2013	4728	112692	8.0	700	1275	644	7.3	36	94	22
	29-10-2013	5631	134215	8.2	440	722	331	7.5	8	20	11
2	19-03-2013	283	28300	7.9	850	1765	1020	7.2	11	28	9
	15-05-2013	134	13400	8.0	2600	3750	1460	8.2	6	23	8
	23-07-2013	225	22500	7.8	1700	4230	3777	7.3	12	68	9
	30-10-2013	175	17500	7.8	1800	2825	556	7.2	8	72	17
3	18-03-2013	12228	211748	7.6	82	169	66	7.8	5	9	10
	14-05-2013	7236	125303	7.2	50	100	26	6.9	8	40	6
	23-07-2013	5673	98237	8.2	– [a]	– [a]	– [a]	8.0	– [a]	– [a]	– [a]
	30-10-2013	6591	114134	7.5	195	286	150	7.5	7	11	12
4	28-02-2013	6036	133372	– [a]	240	551	157	– [a]	50	134	70
	29-05-2013	6356	140442	– [a]	400	753	310	– [a]	45	114	51
	23-07-2013	5151	113817	– [a]	280	843	294	– [a]	45	112	72
	30-10-2013	5031	111165	– [a]	250	375	110	– [a]	17	56	16
5	27-02-2013	11199	43822	7.3	150	610	340	6.8	<4	32	<10
	28-05-2013	11216	43888	7.3	260	690	310	7.2	<4	47	<10
	22-07-2013	14884	58241	7.5	480	896	512	7.1	9	30	10
	30-10-2013	12136	47488	7.7	225	334	200	7.4	6	67	11
6	04-03-2013	31850	106167	7.3	230	340	180	6.8	3	33	<10
	23-05-2013	31770	105900	7.5	260	580	250	7.4	6	47	18
	11-07-2013	29690	98967	7.2	680	720	470	7.5	6	42	13
	07-11-2013	32530	108433	7.4	250	810	590	7.5	8	76	<10
7	25-02-2013	36734	172460	7.3	220	440	175	7.6	27	108	33
	22-05-2013	32258	151446	– [a]	640	1000	250	7.5	50	110	24
	14-08-2013	18180	85352	– [a]	370	510	200	7.5	50	110	42
	24-10-2013	30299	142249	– [a]	340	490	210	7.4	29	80	20
8	12-03-2013	489	71387	7.0	332	673	440	7.1	47	146	23
	07-05-2013	495	72263	7.4	208	512	150	7.4	13	88	10
	23-07-2013	634	92555	7.4	306	729	360	7.4	16	74	26
	01-10-2013	996	145401	7.4	22	94	33	7.4	10	68	10
9	18-03-2013	60000 [b]	85714	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]
	03-06-2013	60000 [b]	85714	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]
	22-07-2013	60000 [b]	85714	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]
	04-11-2013	60000 [b]	85714	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]

Table 8. Characterization of WWTP parameters for the different sampling periods. *(continued)*

WWTP	Sampling date	Flow rate (m^3/ day)	Flow rate (L/day/1000 inhab)	WWTP influent				WWTP effluent			
				pH	BOD5 (mg O_2 L^{-1})	COD (mg O_2 L^{-1})	TSS (mg L^{-1})	pH	BOD5 (mg O_2 L^{-1})	COD (mg O_2 L^{-1})	TSS (mg L^{-1})
10	18-03-2013	50000[b]	232558	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]
	03-06-2013	50000[b]	232558	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]
	22-07-2013	50000[b]	232558	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]
	04-11-2013	50000[b]	232558	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]
11	19-03-2013	140000[b]	185185	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]
	04-06-2013	140000[b]	185185	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]
	23-07-2013	140000[b]	185185	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]
	05-11-2013	140000[b]	185185	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]
12	13-03-2013	12444	207400	7.6	267	550	203	7.8	10	47	23
	21-05-2013	12305	205083	–[a]	220	510	158	7.5	8	41	54
	23-07-2013	6001	100017	–[a]	389	510	231	7.6	7	52	16
	24-10-2013	6113	101883	–[a]	132	430	158	6.7	6	44	14
13	13-03-2013	1889	217126	8.0	364	600	330	7.7	13	55	17
	21-05-2013	980	112644	–[a]	177	580	116	7.6	<3	17	<10
	23-07-2013	822	94483	–[a]	848	1380	597	7.1	18	<3	<10
	24-10-2013	1067	122644	–[a]	70	610	73	6.7	<3	28	<10
14	05-03-2013	8795	177508	8.0	150	430	120	7.8	<10	29	5
	21-05-2013	7887	159182	7.4	400	800	300	7.8	<10	34	5
	23-07-2013	12554	253376	–[a]	–[a]	900	–[a]	–[a]	–[a]	39	–[a]
	05-11-2013	5047	101863	7.5	379	1100	460	7.7	3	30	6
15	01-03-2013	4769	155009	7.5	200	300	90	7.9	<10	26	3
	24-05-2013	4910	159592	7.7	260	470	220	8.2	<10	25	4
	19-07-2013	8146	264773	7.5	400	700	600	7.7	11	38	8
	01-11-2013	4755	154554	7.6	146	800	220	8.0	<10	22	5

BOD – Biochemical oxygen demand; COD – Chemical oxygen demand; TSS – Total suspended solids; [a] Data not available; [b] Average loads

Table 9. Gradient elution scheme.

Time (min)	% A	% B
0	90	10
3	90	10
3.1	55	45
5	55	45
8	15	85
9	15	85
9.1	5	95
14	5	95
14.1	90	10
20	90	10

Table 10. Performance data obtained for SSRIs in spiked influent and effluent samples.

SSRI	Matrix matched linearity (r^2)	MDL (ng L^{-1})	MQL (ng L^{-1})	ME (%)	Recovery (%)			RSD within-day (%)			RSD between-day (%)		
					100 ng L^{-1}	250 ng L^{-1}	500 ng L^{-1}	100 ng L^{-1}	250 ng L^{-1}	500 ng L^{-1}	100 ng L^{-1}	250 ng L^{-1}	500 ng L^{-1}
Influent samples													
Citalopram	0.9984	20.8	63.2	116.6	99.2	100.8	101.6	0.6	0.2	0.3	0.4	0.7	1.0
Fluoxetine	0.9975	25.6	77.5	97.3	125.9	93.7	100.6	5.0	2.0	0.5	5.9	0.9	1.1
Paroxetine	0.9965	30.4	92.3	104.1	95.8	94.0	100.9	2.0	0.6	0.6	0.9	0.1	0.5
Sertraline	0.9977	25.1	75.9	84.6	72.5	101.4	99.2	0.3	0.5	0.5	0.8	0.7	0.4
Effluent samples					75 ng L^{-1}	250 ng L^{-1}	500 ng L^{-1}	75 ng L^{-1}	250 ng L^{-1}	500 ng L^{-1}	75 ng L^{-1}	250 ng L^{-1}	500 ng L^{-1}
Citalopram	0.9982	22.5	68.0	109.2	86.3	100.9	102.4	1.0	1.1	0.5	0.2	0.4	2.2
Fluoxetine	0.9977	23.4	70.9	100.5	122.2	92.9	103.2	2.4	0.9	0.6	2.2	0.7	0.9
Paroxetine	0.9994	11.7	35.3	101.0	106.4	96.0	95.3	1.2	1.0	4.3	1.0	0.4	2.9
Sertraline	0.9992	13.1	39.7	107.5	98.0	98.7	101.4	1.2	1.1	0.6	0.5	0.4	0.8

Figure 18**.** Map of the studied area and sample site locations.

Chapter III – Environmental impact of pharmaceuticals from Portuguese wastewaters: geographical and seasonal occurrence, removal and risk assessment

In this publication, as already referred, a different analytical methodology was used which embraced pharmaceuticals belonging to therapeutic groups other than selective serotonin re-uptake inhibitors (SSRIs), including alprazolam (ALP), lorazepam (LOR) and zolpidem (ZOL) (anxiolytics and hypnotics), azithromycin (AZI) and ciprofloxacin (CIP) (antibiotics), simvastatin (SIM), bezafibrate (BEZ) and gemfibrozil (GEM) (lipid regulators), and ibuprofen (IBU), diclofenac (DIC) and paracetamol (PARA) (anti-inflammatories and/or analgesics).

After the two first sampling campaigns (spring and summer) were evaluated, and since there was already a large amount of data, the option was to publish these first sampling campaigns separately. Therefore, these works embraced methodology validation, geographical and seasonal occurrence, removal efficiencies in wastewater treatment plants (WWTPs) and environmental risk assessment (ERA).

The work presented and discussed in this chapter resulted in the following publication:
PEREIRA A.M.P.T., SILVA L.J.G., MEISEL L.M., LINO C.M., PENA A.. Environmental impact of pharmaceuticals from Portuguese wastewaters: geographical and seasonal occurrence, removal and risk assessment. Environmental Research, 136, 108–119, 2015 (DOI: 10.1016/j.envres.2014.09.041).

III1. Abstract

The occurrence, fate, geographical and seasonal influence and environmental risk assessment of eleven of the most consumed pharmaceuticals in Portugal were studied in wastewater treatment plants (WWTPs) influents (WWIs) and effluents (WWEs). WWI and WWE samples, from two sampling campaigns (spring and summer), in 2013, were evaluated in 15 different WWTPs across the country, by solid phase extraction (SPE) and liquid chromatography coupled with tandem mass detection (LC-MS*n*).

Lipid regulators were the most frequently found in WWIs and WWEs (184.1 and 22.3 mg/day/1000 inhab., respectively), followed by anti-inflammatories (1339.4 and 15.0 mg/day/1000 inhab., respectively), and antibiotics (330.7 and 68.6 mg/day/1000 inhab., respectively). Anxiolytics were the least detected, with 3.3 and 3.4 mg/day/1000 inhab. in WWIs and WWEs, respectively.

The mass loads, both in WWIs and WWEs, were higher in summer than those found during the spring season, being remarkable the high values registered in a region where population triplicates in this time of the year. The mean removal efficiency achieved was of 94.5%, nonetheless, among the different therapeutic groups, as well as within each group, important variations in removal were observed, going from not eliminated to 100%. In the summer, higher efficiencies were observed regarding lipid regulators and antibiotics.

Furthermore, an important outcome was the evaluation, by means of risk quotients (RQs), of the potential ecotoxicological risk posed by the selected pharmaceuticals to different aquatic organisms, exposed to the effluents studied. Ciprofloxacin, bezafibrate, gemfibrozil, simvastatin and diclofenac showed RQs higher than one, being expected that these pharmaceuticals might pose a threat to the three trophic levels (algae, daphnids and fish) evaluated. These results highlight the importance of these monitoring studies, as required by the Directive 2013/39/EU, in order to minimize their aquatic environmental contamination and support future prioritization measures.

Keywords

Environmental contaminants; pharmaceuticals; municipal wastewaters; occurrence and fate; seasonal variation; environmental risk assessment.

III2. Introduction

Human pharmaceuticals represent a group of widely used chemicals that contaminate the environment. Albeit in trace amounts, they are of concern since they are designed to perform a biological effect. Moreover, given their continuous introduction into the environment, their environmental impact, both as stressors and as agents of change, is of great importance [1].

The environmental impact of medicinal products has been recognized worldwide. Although no legal limits have been established in water, relevant legislation and regulatory guidance have been issued by the European Union (EU) [39]. The Water Framework Directive (WFD) (Directive 2000/60/CE) establishes the priority substances in the policies of the water domain of the EU [221,361], whereas, the Directive 2001/83/EC, as amended by the Directive 2004/27/EC, requires an evaluation of the potential environmental risks to be performed for every new marketing authorization. In January 2012, the EU published a report regarding the revision of the Directive 2000/60/CE, and several new substances were proposed, including diclofenac (European Commission 2012). Moreover, Directive 2013/39/EU sets a watch list, that includes three pharmaceuticals, being one of them diclofenac, and requires relevant monitoring data from each member state, in order to minimize their aquatic environmental contamination and support future prioritization measures.

In recent years, has been observed an increased and chronic consumption of several medicines all across the world. In Portugal, the highest prescription and consumption regard, among others, alprazolam, lorazepam and zolpidem (anxiolytics and hypnotics), azithromycin and ciprofloxacin (antibiotics), simvastatin, bezafibrate and gemfibrozil (lipid regulators), and ibuprofen, diclofenac and paracetamol (non-steroidal anti-inflammatories and analgesics) [373] (Table 11). As their use cannot be avoided, a sound risk assessment of their presence in the environment is a key problem. The selected pharmaceuticals were chosen within each group by the ranking of national sales, by package, in 2011 [373] (Table 11).

Table 11. Therapeutic groups, characteristics, CAS number and national sales for the selected pharmaceuticals.

Therapeutic group	Pharmaceutical	Molecular formula	Molecular weight	CAS no.	National sales by package
Anxiolytics and hypnotics	Alprazolam	$C_{17}H_{13}ClN_4$	308.8	28981-97-7	2 384 299
	Lorazepam	$C_{15}H_{10}N_2Cl_2O_2$	321.2	846-49-1	1 947 305
	Zolpidem	$C_{19}H_{21}N_3O$	307.4	82626-48-0	1 089 029
Antibiotics	Azithromycin	$C_{38}H_{72}N_2O_{12}$	749	83905-01-5	944 513
	Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	331.4	85721-33-1	618 465
Lipid regulators	Bezafibrate	$C_{19}H_{20}ClNO_4$	361.8	41859-67-0	41 450
	Gemfibrozil	$C_{15}H_{22}O_3$	250.3	25812-30-0	n.a.
	Simvastatin	$C_{25}H_{38}O_5$	418.6	79902-63-9	3 440 703
Anti-Inflammatories and/or analgesics	Diclofenac	$C_{14}H_{10}Cl_2NNaO_2$	318.1	15307-79-6	1 295 809
	Ibuprofen	$C_{13}H_{18}O_2$	206.3	15687-27-1	2 063 414
	Paracetamol	$C_8H_9NO_2$	151.2	103-90-2	3 239 035

n.a. – Not available

The main source of pharmaceuticals residues in the aquatic environment is from human excretion, consequently, the widespread presence of pharmaceuticals in environmental samples is most likely to occur from wastewater treatment plants (WWTPs), which incompletely remove these compounds. Pharmaceuticals are then released into the environment as parent compounds, metabolites, as well as transformation products formed during water treatments, by biodegradation, photolysis or hydrolysis [5], leading to the contamination of surface waters, seawaters, groundwater and some drinking waters. Nevertheless, there are also other pathways of aquatic contaminations such as sewage overflow, aquaculture and leaching from agricultural fields resulting from the spreading of manure and presence of livestock [6–13].

Heavy contamination pressures from extensive urban activities characterize the Portuguese coast and main rivers that might lead to high aquatic contamination levels and consequent environmental and human exposure. Although the concentrations of pharmaceuticals in influents (WWIs) and effluents (WWEs) of WWTPs are routinely monitored in many countries, there is little knowledge on pharmaceuticals occurrence/fate and their environmental exposure

profile in Portugal [20,21,23,92]. Moreover, their sources of contamination may be influenced by different geographical patterns of pharmaceuticals consumption and important fluctuations due to seasonal variations might also occur.

These are important issues for an integrated management of the possible environmental risk assessment, which is essential for the implementation of minimizing measures. Frequently, a pragmatic approach for identifying hazards or prioritizing critical substances has been made [353], but this concept is not sufficiently precise for an accurate assessment of pharmaceuticals risk. Nevertheless, information on real measured concentrations of pharmaceuticals in the environmental aquatic compartment, allows a good insight into human exposure.

The key driving force of this study was to perform, for the first time, a nationwide environmental contamination mapping of the above mentioned 11 pharmaceuticals, in 15 WWTPs from 5 different Portuguese regions, in order to evaluate geographical/national contamination patterns and to assess vulnerable areas. Moreover, we aimed to assess seasonal influence, in spring and summer seasons, and WWTPs removal efficiency. Furthermore, an important outcome was the evaluation of the potential ecotoxicological risk posed by these pharmaceuticals to different aquatic organisms, when exposed to the studied WWEs, allowing a better understanding of the environmental risk in the Portuguese context.

III3. Materials and methods

III3.1. Sampling site and collection

WWIs and WWEs of 15 different WWTPs, located in 5 Portuguese regions, North, Center, Lisbon and Tagus Valley, Alentejo and Algarve (Figure 18), were collected. These WWTPs are designed for 6850 to 756000 population equivalents, representing 26.1% of the national population (10526700, in 2012). With average flow rates ranging between 349 and 140000 m^3 per day, these facilities have their discharge points in the main Portuguese rivers and Atlantic Ocean. They treat domestic, hospital and industrial wastewaters, operating with secondary or tertiary treatments, as described in Table 4.

Sampling campaigns, carried out in 2013, were performed during two sampling periods: between 14 May/04 June – spring, and 11 July/14 August – summer, one sample by sampling site (WWI and WWE) for each season. The characterization of WWIs and WWEs, for the different sampling periods, is shown in Table 14 (Supporting information). WWI and WWE

samples were collected in high-density polyethylene containers previously rinsed with bi-distilled water, as time proportional 24-h composite samples. Samples, kept refrigerated (4 ºC) during the transport to the laboratory, upon reception, were frozen and stored at -20 ºC until analysis.

III3.2. Standards, chemicals and materials

Pharmaceutical standards, with purity degree ≥ 98%, were purchased from Fluka, Sigma and Riedel-de-Haen (Sigma-Aldrich, Spain), with the exception of alprazolam, lorazepam and zolpidem that were acquired from LGC Standards (Barcelona, Spain). Individual stock solutions were prepared in methanol at 500 μg mL^{-1} and stored at -20 °C in the dark. An intermediate solution was prepared, in mixture, at a concentration of 5 μg mL^{-1}, in methanol. Daily, a working solution at 0.5 μg mL^{-1}, in methanol/water (25:75 v/v), was used.

Internal standards (paracetamol-D4 and fluoxetine-D5) were added to the samples extracts at a final concentration of 500 μg L^{-1}.

J.T. Baker (Deventer, Netherlands) supplied Baker-analyzed methanol for LC-MS and ultrapure Milli-Q water was obtained from a Milli-Q apparatus from Millipore (Molsheim, France). Formic acid (50%) and hydrochloric acid (37%) were obtained from Fluka, Sigma and Riedel-de-Haen (Sigma-Aldrich, Spain). Glass microfiber filters (1.0 μm, 934-AH) and 0.45 and 0.2 μm polyamide membrane filters were aquired from Whatman Schleicher and Schuell (USA) and from Whatman, (Dassel, Germany), respectively. Oasis MAX (500mg, 6mL) cartridges, from Waters Corporation (Milford, Massachusetts, USA), were used for solid phase extraction (SPE).

III3.3. Experimental procedure

The method used for identification and quantification of these pharmaceuticals was based on the methodology reported by Sousa et al. [20]. Briefly, after defrosting and reaching room temperature, samples were acidified with hydrochloric acid (37%) to pH 2 and, to remove suspended material, consecutively filtrated through a glass microfiber filter, 0.45 and 0.2 μm polyamide membrane filters.

For SPE, the Oasis MAX cartridges were pre-conditioned with 6 mL methanol followed by 3 mL Milli-Q water at pH 2. Samples (50 mL of WWI and 100 mL of WWE) were applied to the cartridge, with a flow of 10 mL min^{-1}, that was then washed with 3 mL Milli-Q water. After left

to dry for 15 minutes, elution was performed with 2 x 3 mL methanol. The eluent was evaporated to dryness at 45 ºC under a gentle stream of nitrogen and the residue was redissolved in 200 µL of methanol/Milli-Q water (35:65 v/v).

Instrumentation analysis was performed in a liquid chromatography with tandem mass detection (LC-MS*n*) system equipped with two 210 HPLC pumps, a 500 MS ion trap mass spectrometer and a ProStar 410 autosampler kept at 10 ºC, all from Varian (Walnut Creek, CA, USA). The system, assembled with a Varian analytical column Pursuit UPS C18 (2.1mm i.d.x50 mm, 2.4 mm), kept at 35 ºC, and a guard column of the same characteristics (2.1mm i.d.x10 mm, 3 mm), was fitted with a 10 µL sample loop. Chromatographic separation was achieved using a flow rate of 300 µL min^{-1} and a gradient of methanol and 10 mM formic acid in Milli-Q water as follows: 25% methanol, rising to 75% methanol in 8 min, then to 100% methanol at 10 min and holding until 13 min; at the end of the chromatographic run the column re-equilibrated to the initial conditions in 1 min and stabilized for 8 min.

The electrospray ionization (ESI) source parameters (ionization polarity, drying gas temperature, needle voltage and capillary voltage) and the detector storage and fragmentation conditions (RF loading voltage and collision induced dissociation (CID) voltage, precursor and product ions) are described in Table 15 (Supporting information). The software used for data processing was the Varian MS Workstation version 6.9.1. Identification of positive samples was made by comparison of the MS/MS spectra against authentic standards and also by setting two to three qualifiers and 20% tolerance criteria. Quantification of each compound was based on the main characteristic MS^2 precursor/product ion transition.

III3.4. Mass loading estimations and removal efficiency

Mass loadings of all pharmaceuticals were calculated for each sampling period by multiplying individual concentrations of each pharmaceutical found by the mean daily flow rate of wastewater provided by each WWTP (Table 14, Supporting information). The WWTPs loads were normalized by the population equivalent (Table 14, Supporting information). Removal efficiency of the selected pharmaceuticals was evaluated by means of Equation 3 [23].

III3.5. Ecotoxicological risk assessment

The risk assessment for the aquatic compartment has been based on the guideline on the environmental risk assessment of medicinal products for human use [353]. Following this

guideline, the risk quotients (RQs) associated to the selected pharmaceuticals were calculated by the ratio of measured environmental concentration (MEC) and predicted no-effect concentration (PNEC).

The maximum individual concentrations of pharmaceuticals found in the 30 different WWEs were used as MEC, to set a worst-case scenario approach [23,372]. PNEC values were calculated by applying an uncertainty factor (UF) of 10 to the long-term no-observed-effect-concentration (NOEC) values or of 50 and 1000, to the short-term lowest-observed-effect-concentration (LOEC) and L(E)C50 values, respectively, available in the literature. The UF is an expression of the degree of uncertainty in the extrapolation from the test data on a limited number of species to the actual environment [353]. When no experimental data were available, L(E)C50 values were estimated with ECOSAR 1.11. If RQ is equal or above 1 there is a potential environmental risk situation, whereas when values are lower than 1, no risk is expected.

III4. Results and discussion

III4.1. Method validation

Revalidation was performed, to assure the fitness for purpose of the multi-residue analytical method for the determination of the selected pharmaceuticals in wastewaters (Table 16, Supporting information). Several procedures were carried out in WWI and WWE samples, encompassing sensitivity, linear range, matrix effects accuracy and precision features, according to Sousa et al. [20].

Linearity, achieved for every compound, in triplicate, in the concentration range from 0.01 to 2 μg L^{-1}, was good, as shown by the correlation coefficients (r^2) observed, ranging from 0.9926 to 0.9992.

The method detection limits (MDLs) and the method quantification limits (MQLs) were estimated as the concentration giving a signal-to-noise (S/N) ratio of 3 and 10, respectively, are within the range of other methods developed for the same purpose [22,117,130,163,164,394–399]. MDL values ranged from 0.4 to 60.0 ng L^{-1} in WWEs and from 0.5 to 61.2 in WWIs. MQL ranged from 1.4 to 200.0 ng L^{-1} in WWEs and from 1.7 to 204.1 ng L^{-1} in WWIs.

Recovery tests were performed to determine the accuracy and precision of the method by spiking of WWI and WWE samples. Precision was evaluated through the RSD (%) of the

fortified samples. Recoveries were all above 65.2% and relative standard deviation ranged from 5.9 to 23.0%.

III4.2. Occurrence and geographical variations

III4.2.1. Frequency and occurrence

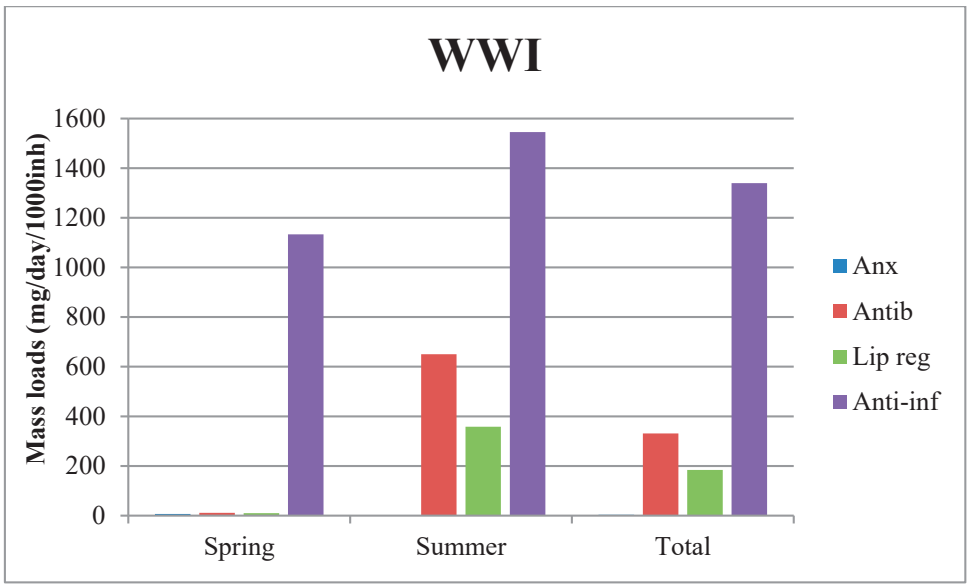
Table 12, Figure 19, and Table 17 (Supporting information) present the occurrence data of the selected pharmaceuticals in the WWI and WWE samples, their frequency, range, and mean concentration, together with the estimated mass loads of each compound and the removal efficiencies observed. Generally, the results showed that, as expected, the frequencies of contamination, concentration levels and mass loads were higher in WWI samples, although some exceptions were observed. From the 11 targeted pharmaceuticals, only two, alprazolam and zolpidem, were not detected, being all samples contaminated with at least one, and up to 8 pharmaceuticals.

Table 12. WWI and WWE mass loads (mg/day/1000 inhab.), concentrations (ng L^{-1}) and removal efficiencies (percentage) of the selected pharmaceuticals.

	WWI						WWE						Removal					
	Spring		Summer		Total		Spring		Summer		Total		Spring		Summer		Total	
Therapeutic group	**Range**	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**	**Mean**
Anxiolytics and hypnotics	0.8-54.5 (38.4-475.8)	6.5 (53.8)	-	-	0.8-54.5 (38.4-475.8)	3.3 (26.9)	0.3-49.2 (22.8-399.8)	6.8 (56.5)	-	-	0.3-49.2 (22.8-399.8)	3.4 (28.2)	NE-70.0	NE	-	-	NE-70.0	NE
Alprazolam	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lorazepam	0.8-54.5 (38.4-475.8)	19.5 (161.5)	-	-	0.8-54.5 (38.4-475.8)	9.8 (80.8)	0.3-49.2 (22.8-399.8)	20.5 (169.4)	-	-	0.3-49.2 (22.8-399.8)	10.3 (84.7)	NE-70.0	NE	-	-	NE-70.0	NE
Zolpidem	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Antibiotics	n.d.-133.2 (n.d.-719.3)	11.3 (82.5)	n.d.-3627.4 (n.d.-17500.0)	650.1 (4333.4)	n.d.-3627.4 (n.d.-17500.0)	330.7 (2208.0)	n.d.-85.5 (n.d.-608.6)	6.0 (40.0)	n.d-836.5 (n.d-9800.0)	131.3 (1191.3)	n.d-836.5 (n.d-9800.0)	68.6 (615.7)	NE-100.0	47.2	NE-100.0	79.8	NE-100.0	79.3
Azithromycin	n.d.-133.2 (n.d.-719.3)	14.4 (84.5)	-	-	n.d.-133.2 (n.d.-719.3)	7.2 (42.3)	-	-	n.d.-11.7 (n.d.-200.0)	0.8 (13.3)	n.d.-11.7 (n.d.-200.0)	0.4 (6.7)	100.0	100.0	NE	NE	NE-100.0	94.6
Ciprofloxacin	n.d.-67.7 (n-d—792.7)	8.1 (80.5)	n.d.-3627.4 (n.d.-17500.0)	1300.2 (8666.7)	n.d.-3627.4 (n.d.-17500.0)	654.2 (4373.6)	n.d.-85.5 (n.d.-608.6)	11.9 (80.0)	n.d-836.5 (n.d-9800.0)	261.7 (2369.3)	n.d-836.5 (n.d-9800.0)	136.8 (1224.7)	NE-100.0	NE	NE-100.0	79.9	NE-100.0	79.1
Lipid regulators	0.7-38.5 (49.4-187.9)	9.9 (77.9)	n.d.-2052.4 (n.d.-8500.0)	358.2 (2802.1)	n.d.-2052.4 (n.d.-8500.0)	184.1 (1440.0)	n.d.-26.7 (n.d.-369.8)	6.0 (63.6)	n.d.-240.0 (n.d.-2400.0)	38.3 (332.2)	n.d.-240.0 (n.d.-2400.0)	22.3 (192.9)	NE-100.0	36.2	NE-100.0	89.3	NE-100.0	87.9
Bezafibrate	0.7-28.3 (35.6-152.8)	11.3 (87.4)	32.0-1348.8 (420.0-6000.0)	331.8 (2651.3)	0.7-1348.8 (35.6-6000.0)	171.6 (1369.4)	n.d.-13.6 (n.d.-73.3)	2.6 (19.7)	0.9-240.0 (40.0-2400.0)	68.9 (584.7)	n.d.-240.0 (n.d.-2400.0)	35.6 (302.2)	24.1-100.0	77.2	25.3-99.3	79.2	24.1-100.0	79.2
Gemfibrozil	0.7-38.5 (49.4-187.9)	10.6 (85.4)	n.d.-1138.5 (n.d.-4300.0)	103.2 (511.7)	n.d.-1138.5 (n.d.-4300.0)	56.9 (298.6)	0.6-14.9 (29.2-133.9)	7.3 (62.5)	n.d.-169.0 (n.d.-1500.0)	45.9 (412.0)	n.d.-169.0 (n.d.-1500.0)	26.6 (237.3)	NE-79.7	31.0	NE-100.0	55.5	NE-100.0	53.2
Simvastatin	0.8-15.7 (45.7-76.5)	7.8 (60.8)	n.d.-2052.4 (n.d.-8500.0)	639.6 (5243.3)	n.d.-2052.4 (n.d.-8500.0)	323.7 (2652.1)	0.7-26.7 (26.3-369.8)	9.1 (78.6)	-	-	0.7-26.7 (26.3-369.8)	4.6 (39.3)	NE-53.4	NE	100.0	100.0	NE-100.0	98.6
Anti-inflammatories and/or analgesics	n.d.-7780.5 (n.d.-48878.0)	1133.5 (8744.8)	n.d.-16900.2 (n.d.-66700.0)	1545.3 (10929.4)	n.d.-16900.2 (n.d.-66700.0)	1339.4 (9837.2)	n.d.-139.8 (n.d.-995.4)	10.0 (78.8)	n.d.-177.4 (n.d.-670.0)	20.0 (164.3)	n.d.-177.4 (n.d.-670.0)	15.0 (121.6)	NE-100.0	99.1	NE-100.0	98.7	NE-100.0	98.9
Diclofenac	n.d.-43.1 (n.d.-232.7)	8.7 (67.4)	n.d.-635.5 (n.d.-2400.0)	46.0 (183.0)	n.d.-635.5 (n.d.-2400.0)	27.4 (125.2)	n.d.-16.4 (n.d.-90.1)	3.1 (24.9)	n.d.-177.4 (n.d.-670.0)	26.7 (154.8)	n.d.-177.4 (n.d.-670.0)	14.9 (89.9)	NE-100.0	64.6	NE-100.0	42.0	NE-100.0	45.6
Ibuprofen	5.3-1100.7 (305.2-6810.0)	404.4 (2982.3)	n.d.-1266.9 (n.d.-8600.0)	505.2 (3920.0)	n.d.-1266.9 (n.d.-8600.0)	454.8 (3451.2)	n.d.-139.8 (n.d.-995.4)	20.7 (157.8)	n.d.-116.9 (n.d.-1370.0)	33.3 (338.0)	n.d.-116.9 (n.d.-1370.0)	27.0 (247.9)	57.4-100.0	94.9	61.4-100.0	93.4	57.4-100.0	94.1
Paracetamol	59.2-7780.5 (1347.7-48878.0)	2987.4 (23184.8)	217.8-16900.2 (4200.0-66700.0)	4084.6 (28685.3)	59.2-16900.2 (1347-66700.0)	3536.0 (25935.1)	n.d.-80.4 (n.d.-530.7)	6.1 (53.7)	-	-	n.d.-80.4 (n.d.-530.7)	3.1 (26.9)	82.5-100.0	99.8	100.0	100.0	82.5-100.0	99.9
All pharmaceuticals	n.d.-7780.5 (n.d.-48878.0)	231.5 (1786.3)	n.d.-16900.2 (n.d.-66700.0)	467.4 (3324.1)	n.d.-16900.2 (n.d.-66700.0)	429.3 (3505.1)	n.d.-139.8 (n.d.-995.4)	5.4 (43.1)	n.d-836.5 (n.d-9800.0)	29.2 (258.1)	n.d-836.5 (n.d-9800.0)	23.6 (205.4)	NE-100.0	97.7	NE-100.0	93.8	NE-100.0	94.5

n.d. - not detected; NE - not eliminated (compounds for which the concentrations found in WWE were higher than the concentrations found in WWI)

(A)



(B)

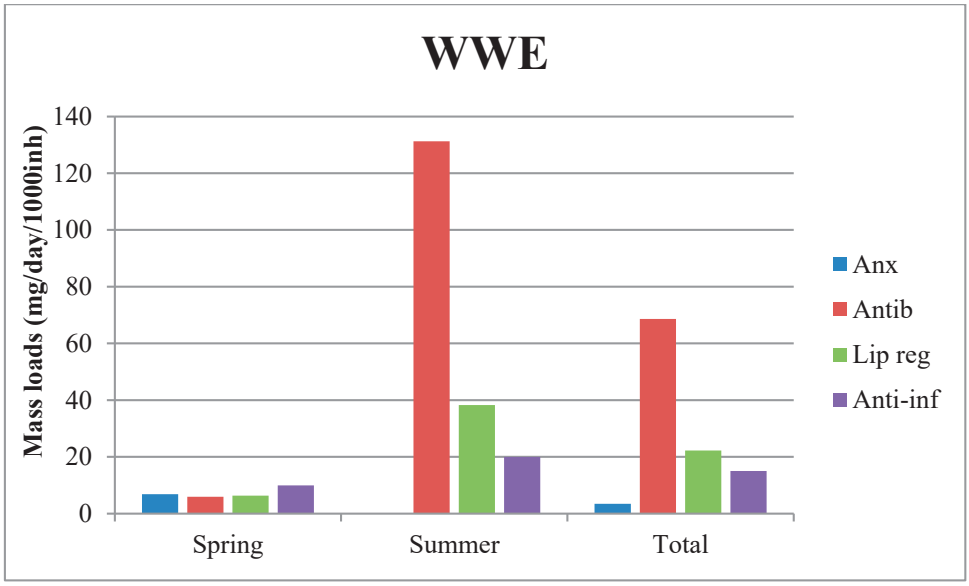


Figure 19. Mass loads (mg/day/1000 inhab.) of the therapeutic groups in WWIs (A) and WWEs (B).
(Anx - anxiolytics and hypnotics; Antib - antibiotics; Lip reg - Lipid regulators; Anti-inf - anti-inflammatories and analgesics).

Anti-inflammatories, found in WWI and WWE samples with a frequency of 84% and 30%, respectively, reached the highest average concentration level in WWI samples, up to 9837.2 ng L^{-1}, corresponding to a mean mass load of 1339.4 mg/day/1000 inhab.. Paracetamol, with the highest average WWI frequency (100%) and average concentration level, 25935.1 ng L^{-1} (3536.0 mg/day/1000 inhab.), accounted for the highest concentration, among all pharmaceuticals, in WWTP 14, with 66700.0 ng L^{-1} (16900.2 mg/day/1000 inhab.). Diclofenac had the lowest WWI frequency (54%) and average concentration, with 125.2 ng L^{-1} (27.4 mg/day/1000 inhab.).

Antibiotics accounted with 32% of positive samples, in WWIs, with ciprofloxacin having the highest frequency, 57%. Their average contamination level reached up to 2208.0 ng L^{-1} (330.7 mg/day/1000 inhab.), with ciprofloxacin accounting with the second highest average concentration, 4373.6 ng L^{-1} (654.2 mg/day/1000 inhab.), among all pharmaceuticals. The highest average concentrations in WWEs were observed for antibiotics, with 615.7 ng L^{-1} (68.6 mg/day/1000 inhab.), being ciprofloxacin the most prevalent compound, with 1224.7 ng L^{-1} (136.8 mg/day/1000 inhab.).

Concerning the lipid regulators, the therapeutic group most widely detected (94% in WWIs, and 68% for WWEs), a mean concentration of 1440.0 ng L^{-1} (184.1 mg/day/1000 inhab.) was found, with simvastatin and bezafibrate having higher averages than gemfibrozil.

Anxiolytics were the group that presented the lowest frequency (17%, both in WWIs and WWEs), with an average concentrations of 26.9 ng L^{-1} (3.3 mg/day/1000 inhab.) and 28.2 ng L^{-1} (3.4 mg/day/1000 inhab.), for WWIs and WWEs, respectively, being lorazepam the only one found.

III4.2.2. Comparison with national consumption and excretion data

The results found in our study are largely explained by consumption and excretion data. The latest Portuguese figures on pharmaceuticals consumption are from 2011 and were reported by INFARMED, the National Authority of Medicines and Health Products. The group of anti-inflammatories, with excretion rates ranging from 5% to 39% [1,8], is the one with higher sales ranking, with a total of 6598258 packages sold, with the decreasing rank order: paracetamol>ibuprofen>diclofenac [373], that equals the ranking of WWI average mass loads found in our study: 3536.0, 454.8 and 27.3 mg/day/1000 inhab., respectively (Table 11 and Table 12).

Anxiolytics are the second group in the ranking of national sales, with 5420633 packages sold [373], however, due to their negligible excretion rates [1,20], they presented low WWI mass loads (Table 11 and Figure 19).

Regarding lipid regulators, bezafibrate has the lower selling rates from all of the selected pharmaceuticals; however, it has high excretion rates (up to 69%) and higher stability than most of the studied compounds, which led to WWI mean mass loads of 171.6 mg/day/1000 inhab., approximately half than simvastatin mass loads (323.7 mg/day/1000 inhab.), the best-selling pharmaceutical (with 3440703 packages), but with only 15% of the consumed dose being released in the environment in his original form [1,8].

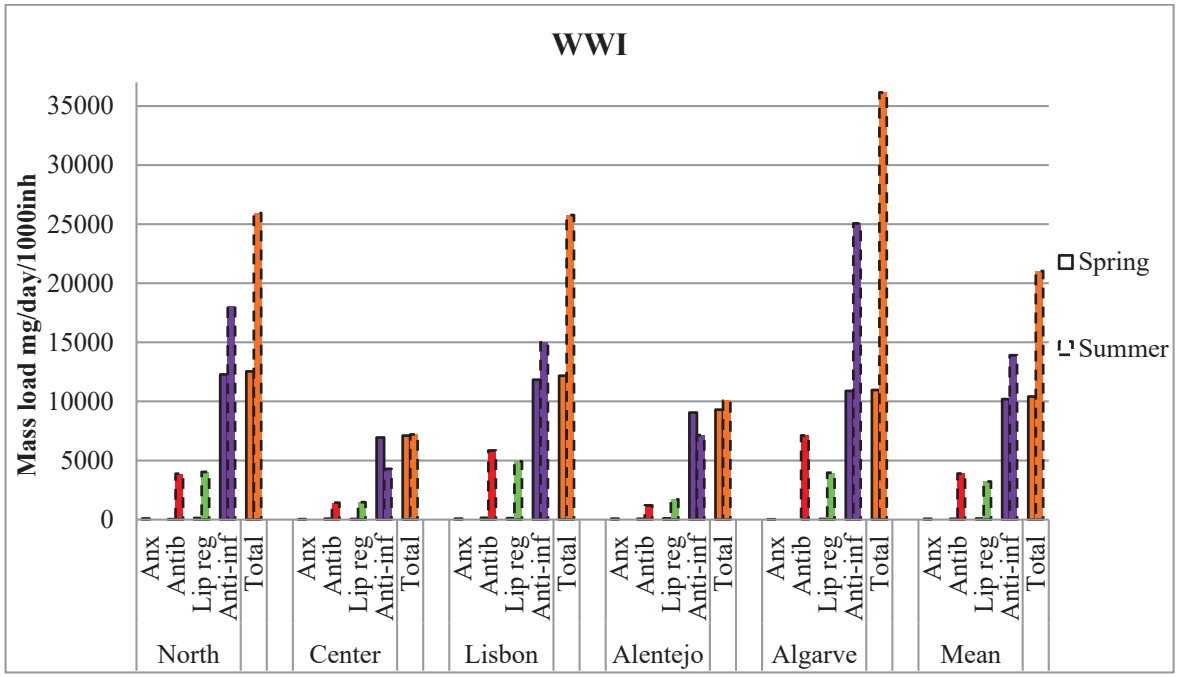
Although lipid regulators present higher selling rates than antibiotics, 3482153 and 1562978 packages, respectively [373], they show lower WWI mass loads (Table 11 and Figure 19). This fact is due to the lower excretion rates of the former, especially of simvastatin when compared with the excretion of up to 84% of ciprofloxacin [1,8].

III4.2.3. Geographical variations

Despite the fact that some efforts were made for a better understanding of the pharmaceuticals fate in Portuguese WWTPs, specific geographical surveys must be considered, since the occurrence pattern of pharmaceuticals in WWTPs is normally related to local consumption or sales figures [20]. On the other hand, it is necessary to determine whether observations made from geographical sampling sets are representative of environmental concentrations nationwide, being essential to perform contamination maps [20,23].

Portugal is a well-known vacation destination, in particular Algarve, where in summer, the number of inhabitants triplicates and the population-equivalent served during this period is much higher than the annual average, increasing the overall flow rates (Table 14, Supporting information), and promoting the highest mass load determined (36152.2 mg/day/1000 inhab.). The results for the remaining regions are similar, with Lisbon (12178.5 and 25777.1 mg/day/1000 inhab., in spring and summer, respectively) and North (12533.0 and 25945.0 mg/day/1000 inhab., in spring and summer, respectively) regions presenting slightly higher contamination values than Alentejo (9298.1 and 10081.1 mg/day/1000 inhab., in spring and summer, respectively) and Center (7109.4 and 7203.5 mg/day/1000 inhab., in spring and summer, respectively) region (Figure 20).

(A)



(B)

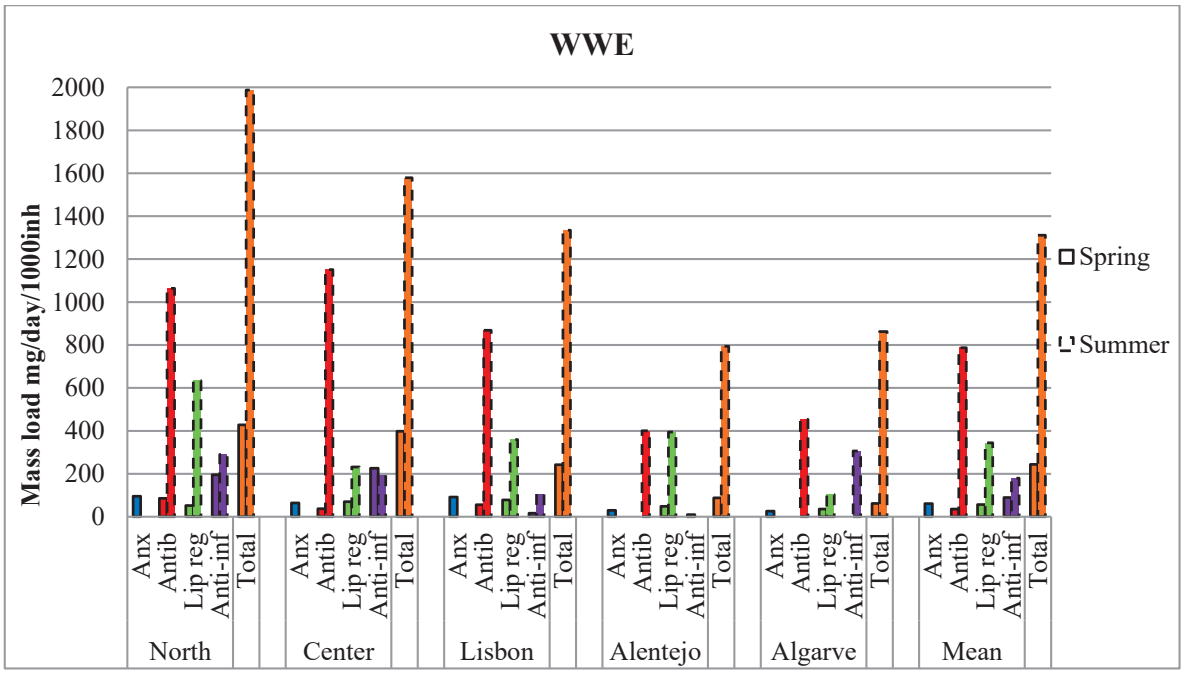


Figure 20. Geographic/seasonal variations on the occurrence of the selected pharmaceuticals in WWIs (A) and WWEs (B).
(Anx - anxiolytics and hypnotics; Antib - antibiotics; Lip reg - Lipid regulators; Anti-inf - anti-inflammatories and analgesics).

For management purposes, information on the distribution of risk due to pharmaceuticals use on a geographical scale and a risk assessment based in a geographic information system can be very useful for an environmental-oriented monitoring [400].

III4.2.4. Comparison with other studies

The range of contamination levels, both in WWI and WWE samples, concur with those found in several other studies reported worldwide. As in our study, others report that anti-inflammatories, the most investigated therapeutic group, present the highest WWI concentration levels. Accordingly, paracetamol shows the highest WWI values (up to 150000 ng L^{-1}), and much lower WWE levels. Moreover, ibuprofen WWI average contamination is also above the reported for diclofenac, and the majority of the results conveyed for WWE samples presented the same tendency [89,90,98,102,113,117,122,185]. This pattern was also observed, by an EU wide monitoring survey on WWE samples recently published [21]. Comparatively to previous Portuguese findings, paracetamol was also found in WWIs at much higher concentration values than in WWEs [20,23], and the concentration range of ibuprofen, in WWIs was also similar to the present findings (ranging from 550 to 9102 ng L^{-1}) [20,23,157], nonetheless, higher values were reported for WWEs (ranging from 119 to 1250) [20,21,23].

Concerning lipid regulators, limited studies have examined the occurrence and fate of simvastatin and, on the contrary to our study, in which simvastatin presented an average concentration of 2652.1 ng L^{-1}, lower concentrations, below 10 ng L^{-1}, were reported, both for WWIs and WWEs [89]. Conversely to our study, comparable concentrations of gemfibrozil and bezafibrate, or even higher concentrations of gemfibrozil than bezafibrate were reported, in WWEs [21]. Nonetheless, our results are in good agreement with those found in other scientific literature [21,89,90,113], including the Portuguese available data [20,23].

In relation to antibiotics, concurring with our data, ciprofloxacin is usually reported at higher concentrations when compared to azithromycin [89,90,117,164,401]. In contrast to our findings (4373.6 ng L^{-1} and 1224.7 ng L^{-1}, in WWIs and WWEs, respectively), lower average concentrations of ciprofloxacin have been reported, 1600 ng L^{-1} and 860 ng L^{-1}, for WWI and WWE samples, respectively [89]. Antibiotics are the group that presents larger national differences. For instance, the measured concentrations of ciprofloxacin in the studied WWI and WWE samples were found at higher levels (up to 17500.0 and 9800.0 ng L^{-1}, respectively) than other previous findings (up to 667 and 369 ng L^{-1}, respectively) [6,20,21,23]. As for azithromycin, our results revealed lower concentrations than Sousa et al. [20] (600 and 700ng L^{-1}, respectively) and Santos et al. [23] (186 and 171 ng L^{-1}, respectively).

As for anxiolytics, results similar to ours were retrieved by other Portuguese and international studies, where low concentration values were found in WWIs and WWEs (up to 299 and 300 ng L^{-1}, respectively) [20,21,23,89,113,130]. The highest level found for lorazepam in a WWI

of a WWTP of a psychiatric hospital was 294 ng L^{-1} [160]. As in our research, lorazepam is found in higher frequencies and concentrations than alprazolam and zolpidem [113,160]. The EU WWE average concentrations of alprazolam and zolpidem, evaluated by Loos et al. [21], was also very low, 1 and 2 ng L^{-1}, with maximum concentrations of 33 and 43 ng L^{-1}, respectively.

III4.2.5. Removal efficiency

In the present study, the fate of the selected pharmaceuticals was determined in 15 Portuguese WWTPs employing different treatment processes (e.g. secondary and tertiary treatments, with UV). The WWTPs were operating normally during all sampling events, and generally achieved good removals on what concerns biochemical oxygen demand (BOD), chemical oxygen demand (COD) and total suspended solids (TSS) (Table 14, Supporting information).

As seen in Table 4, systems that use an activated sludge process are still widely employed for wastewater treatment, mostly because they produce an acceptable quality WWE at reasonable operating and maintenance costs. However, this type of treatment capability of removing pharmaceuticals is limited, depending on influents concentration and on the biological reactor configuration (sequence of anaerobic, aerobic and anoxic compartments) [372,383–385]. In fact, generally, despite some differences in the treatments applied, WWTPs were not able to completely remove these pharmaceuticals, exhibiting a comparable performance in their removal, in mean values of 94.5% (Table 12 and Table 17, Supporting information).

Nonetheless, it is noticeable a great variation in removal efficiencies among the different therapeutic groups (Figure 21), as well as within each group, going from not eliminated to 100%, and no association was established between the decreased BOD, COD and TSS in WWE and removal percentage (Table 12 and Table 14, Supporting information).

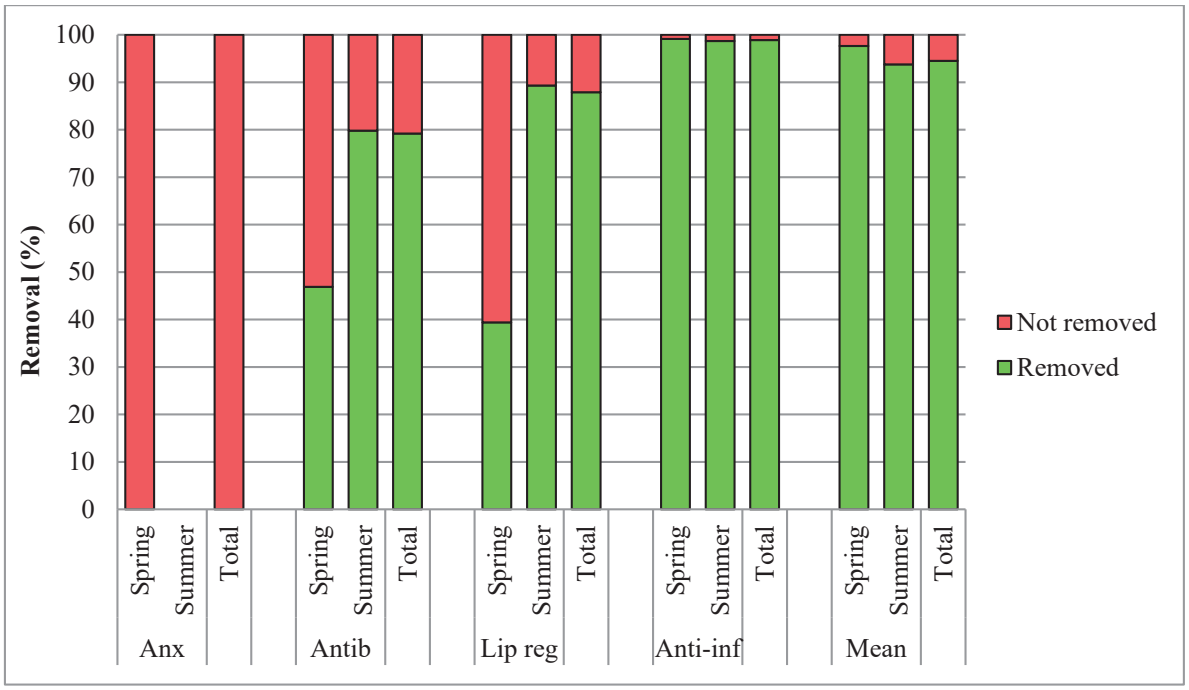


Figure 21. Removal efficiencies of the different therapeutic groups.
(Anx - anxiolytics and hypnotics; Antib - antibiotics; Lip reg - Lipid regulators; Anti-inf - anti-inflammatories and analgesics).

Anti-inflammatories were the group most efficiently removed (98.9%) (Figure 21), mostly due to the high removal rates of paracetamol, with an average of 99.9%. Diclofenac was the one with lower removal efficiency, with an average of 45.6% (Table 12). Considerable high removal efficiencies were observed for lipid regulators and antibiotics, 87.9% and 79.3%, respectively. As for anxiolytics, lorazepam was not eliminated, although it was the pharmaceutical with the lowest WWI mass loads. In some cases, lorazepam, azithromycin, ciprofloxacin, gemfibrozil, simvastatin and diclofenac had higher concentrations in WWEs than in WWIs (Table 17, Supporting information). Two possible explanations are that over the treatment process, conversion of their conjugated metabolites to the original substances takes place and also changes in the adsorption behavior to particles during the treatment process [11,20].

Our findings are in agreement with previous studies found in the scientific literature, where incomplete removal of a wide range of pharmaceuticals in conventional WWTPs has been described [8,20,23,96,98,121].

These results also allow evaluating which WWTPs release more pharmaceuticals into the aquatic environment (by multiplying the concentrations found by the daily flow rate) and inferring the possible risk for the receiving water. These data revealed that WWTP 11 released, per day, in the summer, 429 g of the selected pharmaceuticals in the surrounding aquatic environment, followed by WWTP 7 and 6, with 213 and 155 mg, respectively. It should also

be noted that WWTP 7 released 178 g per day of antibiotics, the group with higher contamination values, into the receiving aquatic compartment, with all the problems associated concerning the emergence of bacterial resistances. These results translate the consumption pattern and number of the population served by each WWTP and removal efficiencies of each WWTP and, as expected, higher values were obtained from WWTPs that serve higher populations.

Although pharmaceutical concentrations in sludge or suspended solids were not considered nor measured, one should note that good removal rates obtained in aqueous phase do not imply degradation to the same extent [96,402]. Moreover, the conversion of a given pharmaceutical to transformation products other than the analysed might lead to lower pharmaceutical levels in WWE samples, and to an apparent removal [96,113]. For instance, metabolites of diclofenac [38,80] and a phototransformation product, more toxic than the parent compound, were already detected in the environment [403].

III4.2.6. Seasonal variation

During summer, in some areas, like Algarve, the population increases and this reflects on the flow rate of some WWTPs (Table 14, Supporting information). However, in other regions, like Alentejo, the flow rate decreases, a fact that can be explained by the reduced precipitation typical of this period, this fact is explained by the combined sewer, sewage that includes both anthrophic discharges and rain water, that is common in Portuguese WWTPs, (IPMA, 2014). These facts might be responsible for the results obtained in our study, where the sum of mass loads in WWIs for summer was 7010.6 mg/day/1000 inhab., higher than that found during the spring season, 3472.3 mg/day/1000 inhab. (Figure 20). This pattern was observed not only in WWIs, but also in WWE samples, with 437.2 and 81.2 mg/day/1000 inhab., for spring and summer, respectively, and was similar to all therapeutic groups, with the exception of anxiolytics.

Regarding the obtained results for each pharmaceutical, our data are in agreement with other studies, where higher levels of some pharmaceuticals, such as diclofenac, ibuprofen, paracetamol, gemfibrozil, were found in summer [11,111,126,404]. Conversely, other authors observed no variation between seasons for diclofenac, ibuprofen, paracetamol, bezafibrate and gemfibrozil [113]; or even observed lower concentrations in the summer, for ibuprofen and bezafibrate [111,390].

Many factors, including solid retention time (SRT), organic load, microbial community, raw sewage temperature and pH, were shown to have pronounced effects on the efficiency of activated sludge treatments [367]. As such, seasonal variations may also affect the treatment efficiency of WWTPs, leading to concentration variations of pharmaceuticals in the WWEs. Generally, in spring the microbial activity and biological reactions are reduced due to lower temperatures and dilution effects, leading to a lower removal efficiency [89,367,390,391]. In fact, regarding lipid regulators and antibiotics, lower removal efficiencies were observed in spring (36.2% and 47.2%, respectively) than in summer (89.3% and 79.8%, respectively), corroborating the expected tendency (Figure 21). However, anti-inflammatories presented similar removal percentages, 99.1% and 98.7%, for spring and summer, respectively, that translated into a higher mean removal in spring when compared to summer (Figure 21). As for anxiolytics, they were only found in the spring season and in low concentrations, not providing enough data for any seasonal comparison.

Although the overall results indicate that removal efficiency was higher in the spring season, this is due to the small difference in the percentage of removal group of anti-inflammatories, that has mass loads exceptionally higher than the others do, strongly influencing the average removal results.

III4.2.7. Environmental risk assessment

The above-mentioned data about occurrence and fate of several pharmaceuticals is crucial in order to improve ERA in a way to evaluate health, ecological and economic consequences. Since pharmaceuticals concentration in water is low, ecotoxicological long-term data are preferred to short-term data. However, due to the lack of long-term toxicological studies, a widespread approach is the use of data from short-term studies (EC50 or LC50) to calculate PNECs [23,372]. It should be taken into account that the choice of data can obviously affect the outcome and that only 30 samples (15 WWTPs in each seasons) were used. The highest concentrations of pharmaceuticals in the WWE samples (to set in the worst-case scenario) [23,372], PNEC values (together with UFs) and RQs deemed for each analyte are shown in Table 13.

The low resulting PNEC values could be explained by these compounds high biological activity and bioconcentration, being detected in biota tissues in higher concentrations than in the aquatic environment.

Table 13. Maximum environmental concentrations (MECs) in WWEs. PNECs and RQs for algae, daphnids and fish for the studied pharmaceuticals.

Therapeutic group	**Pharmaceutical**	**MEC (ng L^{-1})**	**PNEC (ng L^{-1}) algae**	**RQ algae**	**PNEC (ng L^{-1}) daphnids**	**RQ daphnids**	**PNEC (ng L^{-1}) fish**	**RQ fish**
Anxiolytics and hypnotics	Lorazepam	399.8	1 683 [a, b]	0.238	44 712 [e, b]	0.009	49 008 [e, b]	0.008
Antibiotics	Azithromycin	200.0	1 874 [a, b]	0.107	3 023 [e, b]	0.066	21 945 [e, b]	0.009
	Ciprofloxacin	9 800.0	1 000 [c, d] [235]	9.800	180 000 [c, d] [234]	0.054	13 131 424 [e, b]	0.001
Lipid regulators	Bezafibrate	2 400.0	1 352 [a, b]	1.775	2 300 [c, d] [239]	1.043	26 435 [e, b]	0.091
	Gemfibrozil	1 500.0	312 500 [c, d] [239]	0.005	7 800 [c, d] [239]	0.192	150 [c, d] [289]	10
	Simvastatin	369.8	22 800 [b] [242]	0.016	3.2 [f] [262]	115.563	765 000 [e, b]	0.000
Anti-Inflammatories and/or analgesics	Diclofenac	670.0	1 000 000 [c, d] [245]	0.001	20 000 [c, d] [276]	0.034	50 [c, d] [299]	13.400
	Ibuprofen	1 370.0	4 010 [b] [254]	0.342	102 000 [c, d] [278]	0.013	41 561 [e, b]	0.033
	Paracetamol	530.7	134 000 [b] [257]	0.004	2 040 [b] [279]	0.260	378 000 [b] [257]	0.001

[a] EC50 was estimated with ECOSAR
[b] UF=1000
[c] long-term data
[d] UF=10
[e] LC50 was estimated with ECOSAR
[f] UF=50 (uncertainty factor used for LOEC in acute toxicity)

According to these results, the pharmaceuticals ciprofloxacin, bezafibrate, gemfibrozil, simvastatin and diclofenac showed RQs higher than one, in the range of 1.043 to 115.563, for at least one trophic level, posing a risk to algae, daphnids and fish. Although all the other RQs values were lower than 1, a certain risk could be expected for the substances with a RQ between 0.1 and 1, including, in this way, all the other pharmaceuticals that were detected in WWEs, regarding at least one trophic level [372] (Table 13).

In accordance with these findings, it could be concluded that due to the incomplete removal of pharmaceuticals in WWTPs, their WWEs would represent a threat to aquatic ecosystems and probably the dilution of wastewaters in receiving surface waters may not be enough to mitigate their ecotoxicological risk.

The approach followed in this work was only focused on the ecotoxicity that individual pharmaceuticals may cause to aquatic organisms. However, in the aquatic environment they are present as a mixture of different therapeutic groups, their metabolites and transformation products, which may have synergic or additive effects, exhibiting higher toxicities than single compounds, even at lower concentrations, as was shown by some authors, being the real hazard greater than the calculated [23,133,232,405].

This risk evaluation has its limitations, such as the lack of more long-term toxicological studies and the unfeasibility to carry out chronic studies during the lifespan of the organisms (especially in fishes).

III5. Conclusions

These findings allow concluding that pharmaceuticals are ubiquitous in Portuguese WWTPs, both in WWIs and WWEs, and their systematic prevalence in WWEs leads to a continuous exposure, even if in some cases at low levels, of the aquatic wildlife to these compounds.

With the exception of alprazolam and zolpidem, pharmaceuticals were found up to 66700.0 ng L^{-1} and 9800.0 ng L^{-1}, in WWIs and WWEs, respectively. Mass loads were found in WWIs, as following, in the decreasing order: anti-inflammatories, antibiotics, lipid regulators and anxiolytics. As for WWEs the order was: antibiotics, lipid regulators, anti-inflammatories and anxiolytics.

Some geographical differences were observed, mainly due to the increased population in Algarve during summer. In fact, during summer higher mass loads were observed, as a consequence of the increased number of tourists. Removal efficiencies were similar for all WWTPs, however, anti-inflammatories had higher removal efficiencies than the other

therapeutic groups, especially as a result of the high removal efficiency for paracetamol. As expected, excepting for anti-inflammatories, better removal efficiencies were observed in summer.

Environmental risk assessment, using worst-case scenario approach, showed that nine out of the eleven pharmaceuticals had RQ above 0.1, and five presented RQ over 1. Furthermore, ciprofloxacin, gemfibrozil, simvastatin and diclofenac exhibited RQs superior to 1, even when the average measured concentrations were used. These results underline that the aquatic ecosystem may be threatened.

As the use of pharmaceuticals cannot be avoided, these results highlight the importance of these monitoring studies, as required by the Directive 2013/39/EU, in order to minimize their aquatic environmental contamination and support future prioritization measures.

III6. Supporting information

Table 14. Characterization of WWTP parameters for the different sampling periods.

WWTP	Sampling date	Flow rate (m^3 day^{-1})	Flow rate (L/day/1000 inhab)	WWI				WWE			
				pH	BOD5 (mg O_2 L^{-1})	COD (mg O_2 L^{-1})	TSS (mg L^{-1})	pH	BOD5 (mg O_2 L^{-1})	COD (mg O_2 L^{-1})	TSS (mg L^{-1})
1	12-05-2013	3819	91026	8.3	480	768	453	7.3	10	47	18
	23-07-2013	4728	112692	8.0	700	1275	644	7.3	36	94	22
2	15-05-2013	134	13400	8.0	2600	3750	1460	8.2	6	23	8
	23-07-2013	225	22500	7.8	1700	4230	3777	7.3	12	68	9
3	14-05-2013	7236	125303	7.2	50	100	26	6.9	8	40	6
	23-07-2013	5673	98237	8.2	– [a]	– [a]	– [a]	8.0	– [a]	– [a]	– [a]
4	29-05-2013	6356	140442	– [a]	400	753	310	– [a]	45	114	51
	23-07-2013	5151	113817	– [a]	280	843	294	– [a]	45	112	72
5	28-05-2013	11216	43888	7.3	260	690	310	7.2	<4	47	<10
	22-07-2013	14884	58241	7.5	480	896	512	7.1	9	30	10
6	23-05-2013	31770	105900	7.5	260	580	250	7.4	6	47	18
	11-07-2013	29690	98967	7.2	680	720	470	7.5	6	42	13
7	22-05-2013	32258	151446	– [a]	640	1000	250	7.5	50	110	24
	14-08-2013	18180	85352	– [a]	370	510	200	7.5	50	110	42
8	07-05-2013	495	72263	7.4	208	512	150	7.4	13	88	10
	23-07-2013	634	92555	7.4	306	729	360	7.4	16	74	26
9	03-06-2013	60000 [b]	85714	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]
	22-07-2013	60000 [b]	85714	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]
10	03-06-2013	50000 [b]	232558	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]
	22-07-2013	50000 [b]	232558	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]
11	04-06-2013	140000 [b]	185185	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]
	23-07-2013	140000 [b]	185185	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]
12	21-05-2013	12305	205083	– [a]	220	510	158	7.5	8	41	54
	23-07-2013	6001	100017	– [a]	389	510	231	7.6	7	52	16
13	21-05-2013	980	112644	– [a]	177	580	116	7.6	<3	17	<10
	23-07-2013	822	94483	– [a]	848	1380	597	7.1	18	<3	<10
14	21-05-2013	7887	159182	7.4	400	800	300	7.8	<10	34	5
	23-07-2013	12554	253376	– [a]	– [a]	900	– [a]	– [a]	– [a]	39	– [a]
15	24-05-2013	4910	159592	7.7	260	470	220	8.2	<10	25	4
	19-07-2013	8146	264773	7.5	400	700	600	7.7	11	38	8

BOD – Biochemical oxygen demand; COD – Chemical oxygen demand; TSS – Total suspended solids; [a] Data not available; [b] Average loads

Table 15. MS/MS parameters for the analysis of target pharmaceuticals.

Therapeutic group	**Compound**	**R_t (min)**	**Precursor ion (m/z)**	**MS^2 product ion (m/z)**	**Ionization mode**	**R_t window (min)**	**Capillary voltage (V)**	**RF loading (%)**	**CID voltage I (V)**	**MS^3 product ion(s) (m/z)**	**CID voltage II (V)**
Anxiolytics and hypnotics	Alprazolam	7.89	309	281	ESI+	7.30–8.60	92	87	1.28	-	-
	Lorazepam	8.02	321	303	ESI+	7.30–8.60	65	88	0.85	275. 277	1.04
	Zolpidem	2.64	308	263	ESI+	2.35–5.50	102	87	1.35	-	-
Antibiotics	Azithromycin	3.72	375	591	ESI+	2.35–5.50	42	90	1.21	398. 416. 434. 573	1.52
	Ciprofloxacin	1.32	332	314	ESI+	0.00–2.35	72	87	0.65	-	-
Lipid regulators	Bezafibrate	9.31	360	274	ESI-	8.60–10.00	63	100	1.44	154	0.67
	Gemfibrozil	11.49	249	121	ESI-	11.00–11.70	48	67	0.80	106	2.20
	Simvastatin	12.19	441	325	ESI+	11.70–13.00	126	66	1.15	295. 310. 311	1.20
Anti-Inflammatories and/or analgesics	Diclofenac	10.54	294	250	ESI-	10.00–11.00	36	111	0.79	214	1.32
	Ibuprofen	10.71	205	161	ESI-	10.00–11.00	33	57	0.69	-	-
	Paracetamol	0.95	152	110	ESI+	0.00–2.35	54	62	1.00	65. 82. 92. 93	0.86

Table 16. Method detection limits (MDLs), method quantification limits (MQLs), recoveries and relative standard deviation (RSD) of target compounds.

Therapeutic group	**Compound**	**MDL (ng L^{-1})**		**MQL (ng L^{-1})**		**Recoveries (%)**		**RSD (%)**	
		WWI	**WWE**	**WWI**	**WWE**	**WWI**	**WWE**	**WWI**	**WWE**
Anxiolytics and hypnotics	Alprazolam	1.2	1.0	4.0	3.3	70.2	69.2	10.6	10.0
	Lorazepam	1.7	1.5	5.6	5.0	72.2	80.0	11.0	9.2
	Zolpidem	1.5	1.3	5.0	4.4	66.7	64.1	11.9	7.4
Antibiotics	Azithromycin	0.5	0.4	1.7	1.4	*	*	*	*
	Ciprofloxacin	6.0	5.0	20.0	16.7	111.8	82.9	10.4	7.1
Lipid regulators	Bezafibrate	6.7	6.0	22.2	20.0	84.0	88.2	8.2	6.4
	Gemfibrozil	8.6	7.5	28.6	25.0	65.2	78.0	14.2	6.7
	Simvastatin	1.0	0.8	3.3	2.5	75.2	84.7	23.0	22.0
Anti-Inflammatories and/or analgesics	Diclofenac	16.7	15.0	55.6	50.0	70.6	88.2	7.2	5.9
	Ibuprofen	61.2	60.0	204.1	200.0	73.6	72.5	12.5	11.1
	Paracetamol	10.0	9.4	33.3	31.3	*	68.6	*	13.0

* - High contaminated sample, not allowing the calculation of these parameters

Table 17. Occurrence, average, standard deviation, frequency results and removal for the selected pharmaceuticals in spring (A) and summer (B).

(A)	Anxiolytics and hypnotics								Antibiotics						Lipid regulators								Anti-Inflammatories and/or analgesics								Total average		
	Alprazolam ng L^{-1}		Lorazepam ng L^{-1}		Zolpidem ng L^{-1}		Average		Azithromycin ng L^{-1}		Ciprofloxacin ng L^{-1}		Average ng L^{-1}		Bezafibrate ng L^{-1}		Gemfibrozil ng.L^{-1}		Simvastatin ng L^{-1}		Average ng L^{-1}		Diclofenac ng L^{-1}		Ibuprofen ng L^{-1}		Paracetamol ng L^{-1}		Average ng L^{-1}				
	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	Removal (%)
WWTP 1	n.d..	n.d.	443.8	202.5	n.d.	n.d.	147.9	67.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	140.2	31.8	52.6	79.1	50.7	67.9	81.2	59.6	n.d.	n.d.	6810.0	n.d.	32856.0	n.d.	13222.0	n.d.	3668.5	34.7	99.1
WWTP 2	n.d.	n.d.	58.5	22.8	n.d.	n.d.	19.5	7.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	52.1	n.d.	49.4	42.3	57.5	55.0	53.0	32.4	n.d.	n.d.	394.3	n.d.	27172.0	39.5	9188.8	13.2	2525.8	14.5	99.4
WWTP 3	n.d.	n.d.	74.6	185.1	n.d.	n.d.	24.9	61.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	37.8	n.d.	121.3	29.9	65.5	31.3	74.9	20.4	75.7	n.d.	2244.0	334.3	9159.0	n.d.	3826.2	111.4	1070.7	52.8	95.1
WWTP 4	n.d.	n.d.	196.5	233.5	n.d.	n.d.	65.5	77.8	n.d.	n.d.	n.d.	608.6	n.d.	304.3	55.4	n.d.	53.2	41.6	52.6	32.1	53.7	24.6	97.3	n.d.	2337.0	995.4	25053.0	n.d.	9162.4	331.8	2531.4	173.7	93.1
WWTP 5	n.d.	n.d.	38.4	399.8	n.d.	n.d.	12.8	133.3	n.d.	n.d.	792.7	n.d.	396.4	n.d.	98.7	32.3	119.0	71.5	45.7	53.4	87.8	52.4	59.7	62.0	1811.3	n.d.	1347.7	235.4	1072.9	99.1	392.1	77.7	80.2
WWTP 6	n.d.	n.d.	97.7	29.3	n.d.	n.d.	32.6	9.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	152.8	n.d.	160.0	32.5	76.4	59.3	129.7	30.6	150.8	n.d.	1235.0	n.d.	25623.0	n.d.	9002.9	n.d.	2499.6	11.0	99.6
WWTP 7	n.d.	n.d.	65.5	306.7	n.d.	n.d.	21.8	102.2	547.9	n.d.	n.d.	246.4	274.0	123.2	76.9	58.4	59.4	74.1	51.4	81.3	62.6	71.3	92.7	73.0	5733.0	684.8	27393.0	530.7	11072.9	429.5	3092.7	186.9	94.0
WWTP 8	n.d.	n.d.	330.2	246.8	n.d.	n.d.	110.1	82.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	79.1	25.1	133.1	126.0	76.5	369.8	96.2	173.6	108.4	78.5	3738.0	352.8	22620.0	n.d.	8822.1	143.8	2462.3	109.0	95.6
WWTP 9	n.d.	n.d.	58.5	154.2	n.d.	n.d.	19.5	51.4	n.d.	n.d.	n.d.	165.7	n.d.	82.9	107.8	50.4	68.2	133.0	50.7	38.4	75.6	73.9	63.3	n.d.	3212.0	n.d.	20944.0	n.d.	8073.1	n.d.	2227.7	49.2	97.8
WWTP 10	n.d.	n.d.	62.4	211.7	n.d.	n.d.	20.8	70.6	n.d.	n.d.	84.3	178.9	42.2	89.5	85.4	24.1	52.1	55.4	62.8	62.9	66.8	47.5	n.d.	70.5	1424.7	n.d.	21599.0	n.d.	7674.6	23.5	2124.6	54.9	97.4
WWTP 11	n.d.	n.d.	294.1	158.3	n.d.	n.d.	98.0	52.8	719.3	n.d.	n.d.	n.d.	359.7	n.d.	138.1	73.3	53.7	29.2	65.8	38.1	85.9	46.9	232.7	n.d.	5944.0	n.d.	17625.0	n.d.	7933.9	n.d.	2279.3	27.2	98.8
WWTP 12	n.d.	n.d.	109.3	45.2	n.d.	n.d.	36.4	15.1	n.d.	n.d.	330.2	n.d.	165.1	n.d.	138.0	n.d.	187.9	72.4	76.3	99.5	134.1	57.3	n.d.	n.d.	4796.0	n.d.	22492.0	n.d.	9096.0	n.d.	2557.2	19.7	99.2
WWTP 13	n.d.	n.d.	475.8	182.3	n.d.	n.d.	158.6	60.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	35.6	n.d.	64.6	56.7	53.0	58.6	51.1	38.4	67.5	90.1	305.2	n.d.	30329.0	n.d.	10233.9	30.0	2848.2	35.2	98.8
WWTP 14	n.d.	n.d.	59.2	107.3	n.d.	n.d.	19.7	35.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	47.5	n.d.	52.8	51.6	56.4	26.3	52.2	26.0	n.d.	n.d.	1846.0	n.d.	48878.0	n.d.	16908.0	n.d.	4630.9	16.8	99.6
WWTP 15	n.d.	n.d.	58.5	55.1	n.d.	n.d.	19.5	18.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	65.8	n.d.	53.3	42.0	70.9	105.3	63.3	49.1	62.9	n.d.	2904.0	n.d.	14681.0	n.d.	5882.6	n.d.	1626.9	18.4	98.9
Average	0.0	0.0	161.5	169.4	0.0	0.0	53.8	56.5	84.5	0.0	80.5	80.0	82.5	40.0	87.4	19.7	85.4	62.5	60.8	78.6	77.9	53.6	67.4	24.9	2982.3	157.8	23184.8	53.7	8744.8	78.8	1807.7	55.6	96.9
SD	0.0	0.0	145.3	103.0	0.0	0.0	114.6	100.7	217.6	0.0	207.7	162.2	216.4	123.5	38.6	24.2	44.6	30.7	10.2	81.1	37.0	58.3	63.4	35.7	1964.3	296.9	10433.6	140.3	12103.8	201.3	6393.2	117.6	5.0
Frequency	0%	0%	100%	100%	0%	0%	33%	33%	13%	0%	20%	27%	17%	13%	100%	47%	100%	100%	100%	100%	100%	82%	67%	33%	100%	27%	100%	20%	89%	27%	100%	100%	

n.d. - not detected

Table 17. Occurrence, average, standard deviation, frequency results and removal for the selected pharmaceuticals in spring (A) and summer (B). *(continued)*

(B)	Anxiolytics and hypnotics								Antibiotics						Lipid regulators								Anti-Inflammatories and/or analgesics								Total average		
	Alprazolam ng L^{-1}		Lorazepam ng L^{-1}		Zolpidem ng L^{-1}		Average		Azithromycin ng L^{-1}		Ciprofloxacin ng L^{-1}		Average ng L^{-1}		Bezafibrate ng L^{-1}		Gemfibrozil ng.L^{-1}		Simvastatin ng L^{-1}		Average ng L^{-1}		Diclofenac ng L^{-1}		Ibuprofen ng L^{-1}		Paracetamol ng L^{-1}		Average ng L^{-1}				
	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	Removal
WWTP 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1800.0	1200.0	900.0	600.0	5400.0	1600.0	910.0	1500.0	5100.0	n.d.	3803.3	1033.3	n.d.	79.0	8600.0	370.0	60700.0	n.d.	23100.0	149.7	7500.9	431.7	94.2
WWTP 2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6000.0	40.0	n.d.	n.d.	n.d.	n.d.	2000.0	13.3	n.d.	n.d.	n.d.	n.d.	9680.0	n.d.	3226.7	n.d.	1425.5	3.6	99.7
WWTP 3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4200.0	n.d.	2100.0	n.d.	1000.0	190.0	280.0	240.0	4200.0	n.d.	1826.7	143.3	n.d.	240.0	4700.0	n.d.	20600.0	n.d.	8433.3	80.0	3180.0	60.9	98.1
WWTP 4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	17500.0	2800.0	8750.0	1400.0	800.0	470.0	85.0	150.0	5000.0	n.d.	1961.7	206.7	56.0	63.0	3500.0	620.0	23800.0	n.d.	9118.7	227.7	4612.8	373.0	91.9
WWTP 5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	200.0	10400.0	5000.0	5200.0	2600.0	550.0	250.0	220.0	n.d.	8500.0	n.d.	3090.0	83.3	n.d.	490.0	3600.0	950.0	20900.0	n.d.	8166.7	480.0	4015.5	626.4	84.4
WWTP 6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6800.0	3100.0	3400.0	1550.0	2200.0	850.0	570.0	730.0	5900.0	n.d.	2890.0	526.7	n.d.	170.0	3400.0	380.0	26700.0	n.d.	10033.3	183.3	4142.7	475.5	88.5
WWTP 7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	9600.0	9800.0	4800.0	4900.0	880.0	560.0	120.0	n.d.	6900.0	n.d.	2633.3	186.7	n.d.	n.d.	6000.0	1370.0	37200.0	n.d.	14400.0	456.7	5518.2	1066.4	80.7
WWTP 8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6700.0	3400.0	3350.0	1700.0	790.0	590.0	n.d.	1400.0	7900.0	n.d.	2896.7	663.3	63.0	n.d.	2200.0	850.0	4200.0	n.d.	2154.3	283.3	1986.6	567.3	71.4
WWTP 9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8500.0	760.0	4250.0	380.0	5100.0	180.0	250.0	170.0	4950.0	n.d.	3433.3	116.7	n.d.	100.0	4300.0	n.d.	17500.0	n.d.	7266.7	33.3	3690.9	110.0	97.0
WWTP 10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11500.0	2500.0	5750.0	1250.0	5800.0	340.0	390.0	n.d.	3500.0	n.d.	3230.0	113.3	72.0	n.d.	2900.0	n.d.	31200.0	n.d.	11390.7	n.d.	5032.9	258.2	94.9
WWTP 11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13200.0	1200.0	6600.0	600.0	3200.0	930.0	280.0	420.0	6200.0	n.d.	3226.7	450.0	n.d.	n.d.	3500.0	530.0	24500.0	n.d.	9333.3	176.7	4625.5	280.0	93.9
WWTP 12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7900.0	4000.0	3950.0	2000.0	4100.0	2400.0	n.d.	1400.0	5200.0	n.d.	3100.0	1266.7	n.d.	n.d.	3700.0	n.d.	21800.0	n.d.	8500.0	n.d.	3881.8	709.1	81.7
WWTP 13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4400.0	n.d.	2200.0	n.d.	930.0	150.0	270.0	n.d.	7200.0	n.d.	2800.0	50.0	97.0	n.d.	4700.0	n.d.	43900.0	n.d.	16232.3	n.d.	5590.6	13.6	99.8
WWTP 14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13800.0	1600.0	6900.0	800.0	2600.0	40.0	n.d.	n.d.	8100.0	n.d.	3566.7	13.3	64.0	510.0	5000.0	n.d.	66700.0	n.d.	23921.3	170.0	8751.3	195.5	97.8
WWTP 15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13700.0	180.0	6850.0	90.0	420.0	180.0	4300.0	170.0	n.d.	n.d.	1573.3	116.7	2400.0	670.0	2700.0	n.d.	20900.0	n.d.	8666.7	223.3	4038.2	109.1	97.3
Average	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	13.3	8666.7	2369.3	4333.3	1191.3	2651.3	584.7	511.7	412.0	5243.3	0.0	2802.1	332.2	183.5	154.8	3920.0	338.0	28685.3	0.0	10929.6	164.3	4275.5	461.3	89.2
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	49.9	4694.3	2505.2	5551.9	2164.1	2039.6	630.7	1040.2	546.0	2490.3	0.0	2780.1	546.6	593.3	215.5	1832.7	427.0	16624.4	0.0	16095.9	312.3	8395.8	1060.3	8.4
Frequency	0%	0%	0%	0%	0%	0%	0%	0%	0%	7%	93%	80%	47%	43%	100%	100%	73%	60%	87%	0%	87%	53%	40%	53%	93%	47%	100%	0%	78%	33%	100%	100%	

n.d. - not detected

Chapter IV – Assessing environmental risk of pharmaceuticals in Portugal: an approach for the selection of the Portuguese monitoring stations in line with Directive 2013/39/EU

This publication covered all the four sampling campaigns, not only addressing the occurrence, spatial and temporal variation and environmental risk assessment (ERA) of the selected pharmaceuticals, but also, in line with the Directive 2013/39/EU, selected the most impacted surface waters.

The reason why selective serotonin re-uptake inhibitors (SSRIs) were not included in this approach regarded the fact that they presented much lower frequencies and concentrations when compared to the rest of the selected pharmaceuticals.

The work presented and discussed in this chapter resulted in the following publication:
PEREIRA A.M.P.T., SILVA L.J.G., LINO C.M., MEISEL L.M., PENA A.. Assessing environmental risk of pharmaceuticals in Portugal: an approach for the selection of the Portuguese monitoring stations in line with Directive 2013/39/EU. Chemosphere, 144, 2507- 2515, 2016 (DOI: 10.1016/j.chemosphere.2015.10.100).

IV1. Abstract

In line with the Directive 2013/39/EU, the most representative surface waters, regarding pharmaceuticals contamination, were selected based on a Portuguese nationwide monitoring exercise. To meet this purpose, and given that wastewater treatment plants (WWTPs) are regarded as the major point sources of pharmaceuticals environmental contamination, the occurrence, fate and environmental risk assessment (ERA) of eleven of the most consumed pharmaceuticals, belonging to several therapeutic classes were assessed in 15 WWTPs (influents (WWIs) and effluents (WWEs)), from five different regions during one year (4 sampling campaigns).

Results showed that all samples were contaminated with at least 1, and up to 8 from the 11 targeted pharmaceuticals. The highest concentrations observed were 150 and 33 μg L^{-1} for WWIs and WWEs, respectively.

Regarding temporal and spatial influence, the winter season, Alentejo, Algarve and Center regions presented higher mass loads. The ERA posed by 7 of the selected pharmaceuticals presented a risk quotient higher than 1 to the three trophic levels. Our findings highlighted that the rivers Mondego, Tagus, Ave, Trancão, Fervença and Xarrama should be selected as surface water monitoring stations.

This study gives a good overview on pharmaceuticals contamination in WWTPs and its impact on surface waters in Portugal. Thus, a more integrative approach to rank and prioritize pharmaceuticals, based on an integrated assessment of ERA and exposure of surface water, was provided to support the future selection of the 6 most representative monitoring stations in Portugal, as required by the above mentioned directive.

Keywords

Environmental contaminants, pharmaceuticals, municipal wastewaters, environmental risk assessment

IV2. Introduction

Pharmaceuticals are designed to perform a biological effect, having different characteristics and, consequently, producing different environmental exposure profiles. As its use cannot be avoided, a sound risk assessment of their presence in the environment is a key issue that must be tackled to meet the European Union (EU) Water Framework Directive (WFD) [2,406]. As a result, 3 pharmaceuticals became part of the WFD watch list established by the recent Directive 2013/39/EU. This list is dynamic, changing with the awareness on the persistence in the water cycle and its validity in time is limited. Therefore, identifying and prioritizing new pharmaceuticals are important goals to be accomplished for future updates [14].

High-quality monitoring data, along with data on ecotoxicological and toxicological effects, are crucial for the environmental risk assessment (ERA) associated to their impact on aquatic mesocosm and human health, that will support the selection of possible new priority substances [1,17,18]. Overall, European water bodies still disregard the pharmaceutical data on this issue and Portugal is a good example of this fact since only a few isolated data on pharmaceuticals occurrence are available [20,21,23,27,131]. A systematic monitoring embracing several therapeutic groups and encompassing temporal and spatial representativeness is necessary in order to provide a clear insight on pharmaceuticals contamination of the water compartment [25].

Wastewaters are regarded as the main route of entry of pharmaceuticals into the environment [30]. Indeed, several studies argue that wastewater treatment plants (WWTPs) are not able to completely remove pharmaceuticals, continuously releasing residues excreted in urine and faeces, either as unchanged compounds or metabolites [12,96,98,101,113,115,131,230].

As a part of the strategy implemented by the Directive 2013/39/EU, all member states shall monitor each substance in the watch list at selected surface waters representative monitoring stations, which in the case of Portugal regards 6 sampling locations [190].

In line with this directive, a monitoring based exercise is proposed, providing scientific evidence of the most impacted surface waters, and updating the information needed for prioritization of pharmaceuticals. In this way, the occurrence of the most consumed pharmaceuticals in Portugal [373]: alprazolam, lorazepam and zolpidem, azithromycin and ciprofloxacin, simvastatin, bezafibrate and gemfibrozil, and ibuprofen, diclofenac and paracetamol (Table 19, Supporting information), in 15 WWTPs, from 5 different regions of Portugal, during 4 seasons, was assessed.

A more realistic water quality assessment contributed for a more integrative approach to rank and prioritize pharmaceuticals, based on an integrated assessment of ERA and exposure of surface water, providing support for the future selection of the 6 most representative monitoring stations in Portugal, as required by the above mentioned directive.

IV3. Material and methods

IV3.1. Sampling site and collection

Influents (WWIs) and effluents (WWEs) of 15 different WWTPs, located in 5 Portuguese regions, North, Center, Lisbon and Tagus Valley, Alentejo and Algarve, were collected. These WWTPs are designed for 6850 to 756,000 population equivalents, representing 26.3% of the national population (10.457,300, in 2013), with average loads ranging between 349 and 140,000 m^3 per day, having their discharge points in the main Portuguese rivers and Atlantic Ocean. They are designed to treat domestic, hospital and industrial wastewaters, operating with secondary or tertiary treatments (Table 20, Supporting information).

WWI and WWE sampling campaigns, carried out in 2013 and 2014, were performed during a one year follow-up study, embracing four sampling periods: between 14 May/04 June (2013) – spring, 11 July/14 August (2013) – summer, 24 October/7 November (2013) - autumn and 30 January/11 February (2014) – winter. WWI and WWE parameters of each WWTP, for the different sampling periods, are shown in Table 21 (Supporting information). For each plant, samples were collected, in high-density polyethylene containers previously rinsed with bi-distilled water, as time proportional 24-h composite influent and effluent samples. Samples were kept refrigerated (±4 °C) during the transport to the laboratory. Upon reception, samples were frozen and stored at −20 °C until analysis.

IV3.1.1. Standards and Chemicals

All pharmaceutical standards, with purity degree ≥ 98%, were purchased from Fluka, Sigma and Riedel-de-Haen (Sigma-Aldrich, Spain), except alprazolam, lorazepam and zolpidem that were acquired from LGC Standards (Barcelona, Spain).

J.T. Baker (Deventer, Netherlands) supplied Baker-analyzed methanol for LC-MS and ultrapure Milli-Q water was obtained from a Milli-Q apparatus from Millipore (Molsheim, France). Formic acid (50%) and hydrochloric acid (37%) were obtained from Fluka, Sigma and

Riedel-de-Haen (Sigma-Aldrich, Spain). Glass microfiber filters (1.0 μm, 934-AH) and 0.45 and 0.2 μm polyamide membrane filters were acquired from Whatman Schleicher and Schuell (USA) and from Whatman (Dassel, Germany), respectively. Oasis MAX (500mg, 6mL) cartridges, from Waters Corporation (Milford, Massachusetts, USA), were used for solid phase extraction (SPE).

IV3.1.2. Experimental Procedure

The analytical procedure was based on a previously reported and revalidated method for the identification and quantification of these pharmaceuticals in WWI and WWE samples from WWTPs [20,115].

Briefly, after defrosting and reaching room temperature, samples were acidified with hydrochloric acid (37%) to pH 2 and filtered. Solid phase extraction (SPE) was performed through Oasis MAX (500 mg, 6 mL) cartridges.

Instrumentation analysis was performed in a liquid chromatography with tandem mass detection (LC/MSn) system equipped with a Varian 500 MS ion trap mass spectrometer (Table 22, Supporting information) at Instituto da Água da Região do Norte (IAREN), a NORMAN network laboratory. The system was assembled with an analytical column of short dimensions, Pursuit UPS C18 (2.1mm i.d.x50 mm, 2.4 mm) from Varian and a guard column of the same characteristics (2.1mm i.d.x10 mm, 3 mm). Chromatographic separation was achieved using a flow rate of 300 μL min^{-1} and a gradient of methanol and 10 mM formic acid in Milli Q water as follows. The gradient programme started with 25% methanol, rising to 75% methanol in 8 min, then to 100% methanol at 10 min and holding until 13 min.

IV3.1.3. Mass loading estimations

Mass loadings of all pharmaceuticals were calculated for each sampling period by multiplying the measured concentration of each pharmaceutical by the mean daily flow rate of the wastewater as provided by each WWTP (Table 21, Supporting information). The WWTPs loads were normalized by the population equivalent (Table 21, Supporting information).

IV3.1.4. Ecotoxicological risk assessment

The evaluation of the potential ecotoxicological risk posed for the aquatic compartment was based on a dual approach. Following the guideline on the ERA of medicinal products for human

use [353], the risk evaluation was performed calculating the risk quotient (RQ), using 3 different trophic levels representatives of the aquatic ecosystem (algae, daphnids and fish), between measured environmental concentration (MEC) and predicted no-effect concentration (PNEC), where the maximum individual concentrations of pharmaceuticals found in WWEs were used as MEC to set a worst-case scenario approach [23,115,372]. Moreover, we also used a second approach, using the mean concentrations for each pharmaceutical as MEC, instead of the maximum individual concentrations.

PNEC values were calculated by applying an uncertainty factor (UF) of 10 to the long-term no-observed-effect-concentration (NOEC) and values of 50 and 1000 to the short-term lowest-observed-effect-concentration (LOEC) and lethal (effective) concentration L(E)C50 values, respectively [115,131]. The UF is an expression of the degree of uncertainty in the extrapolation from the test data on a limited number of species to the actual environment [353]. When no experimental values were available, L(E)C50 values estimated with ECOSAR 1.11 were used. If the calculated RQ was equal or above 1 there is a potential environmental risk situation, whereas when values were lower than 1, no risk is expected.

IV3.1.5. Selection of the most representative WWTPs and most impacted surface waters

The selection of the most representative WWTPs was calculated by multiplying the concentrations found in WWEs by the respective flow rate, for every WWTP in each season, obtaining the amount of pharmaceuticals released by each WWTP in the aquatic surroundings, thus assessing the most impacted surface waters. These values were also refined with the dilution attributed to the different river flows, provided by the Portuguese Environment Agency (APA). The average surface water contamination was also evaluated, using the standard deviations of the contamination levels.

IV3.1.6. Statistical analysis

Complete statistical analysis was performed using GraphPad Prism (6.01, GraphPad Software, Inc., San Diego, USA). To test whether the datasets were of Gaussian distribution, D'Agostino–Pearson normality test was used. Since most of the data sets were not normally distributed, with non-homogeneous variances, nonparametric tests were applied. Kruskal–Wallis test with Dunns post-test were used for the comparison between each and the total of pharmaceuticals in the different sampling locations. The statistical significance level was set to $p<0.05$ [340].

IV4. Results and discussion

IV4.1. Occurrence

Table 23 (Supporting information) outlines a summary on analytical methodology validation: method detection limits (MDLs), method quantification limits (MQLs), recoveries and relative standard deviations of each compound.

Generally, the results showed that, as expected, the frequencies of contamination, concentration levels and mass loads were higher in WWI samples, although some exceptions were observed. As can be seen in Table 18 and Table 24 (Supporting information), from the 11 targeted pharmaceuticals, only two were not present, alprazolam and zolpidem, being all samples contaminated with at least one, and up to 8 pharmaceuticals.

Regarding the individual frequency of contamination, paracetamol and bezafibrate were detected in all of the WWI analysed samples, as for WWEs, bezafibrate was the one with higher values. Concerning the frequency of each therapeutic group, although higher values in WWIs, the decreasing order, both in WWIs and WWEs was: lipid regulators; anti-inflammatories; antibiotics and anxiolytics (Table 24, Supporting information).

Mean concentrations (mass loads) by therapeutic group in increasing order were as following: anxiolytics, lipid regulators, antibiotics and anti-inflammatories with 13.5 (1.6), 3223.1 (335.9), 3346.3 (515.5) and 15,584.9 ng L^{-1} (2238.2 mg/day/1000 inhab.), in WWI samples and 14.1 (1.7), 693.5 (107.5), 886.9 (113.8) and 1806.6 ng L^{-1} (120.9 mg/day/1000 inhab.) in WWE samples. The comparison between the therapeutic groups presented statistically significant differences, with the exception of the comparison between lipid regulators and anti-inflammatories in WWIs and of antibiotics and anti-inflammatories in WWEs (Table 18).

Concentration levels ranged from not detected to 150,000.0 ng L^{-1} (23,580.3 mg/day/1000 inhab.) and from not detected to 32,000.0 ng L^{-1} (4056.3 mg/day/1000 inhab.), in WWIs and WWEs, respectively (Table 18). Paracetamol was the pharmaceutical compound with the highest average concentration and the highest level, 41,022.5 ng L^{-1} (5815.2 mg/day/1000 inhab.) and 150,000.0 (23,580.3), respectively.

These results are consistent with those previously reported by other authors in wastewater samples worldwide where the concentration found in WWIs and WWEs were up to 292 µg L^{-1} and 24.6 µg L^{-1}, respectively [89,90]. Concerning the EU, similar results were also observed, with concentrations in WWIs and WWEs in the range of ng L^{-1} and µg L^{-1} [21,23,113,117,124].

Table 18. Mass loads (mg/day/1000inhab.) and concentrations (ng L^{-1}) of the selected pharmaceuticals in the four seasons concerning the 15 WWTPs.

Therapeutic group	WWI										WWE									
	Spring		Summer		Autumn		Winter		Total		Spring		Summer		Autumn		Winter		Total	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean*	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean*
Anxiolytics and hypnotics	0.8-54.5 (38.4-475.8)	6.5 (53.8)	-	-	-	-	-	-	n.d.-54.5 (n.d.-475.8)	1.6 ^{C} (13.5)	0.3-49.2 (22.8-399.8)	6.8 (56.5)	-	-	-	-	-	-	0.3-49.2 (22.8-399.8)	1.7 ^{A} (14.1)
Alprazolam	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lorazepam	0.8-54.5 (38.4-475.8)	19.5 (161.5)	-	-	-	-	-	-	n.d.-54.5 (n.d.-475.8)	4.9 (40.4)	0.3-49.2 (22.8-399.8)	20.5 (169.4)	-	-	-	-	-	-	0.3-49.2 (22.8-399.8)	5.1 (41.9)
Zolpidem	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Antibiotics	n.d.-133.2 (n.d.-719.3)	11.3 (82.5)	n.d.-3627.4 (n.d.-17500.0)	650.1 (4333.4)	n.d.-3328.5 (n.d.-28800.0)	404.7 (3842.4)	n.d.-10439.1 (n.d.-32100.0)	995.8 (5126.7)	n.d.-10439.1 (n.d.-32100.0)	515.5 ^{D} (3346.3)	n.d.-85.5 (n.d.-608.6)	6.0 (40.0)	n.d-836.5 (n.d-9800.0)	131.3 (1191.3)	n.d.-753.8 (n.d.-7400.0)	99.8 (929.7)	n.d.-1871.3 (n.d.-10200.0)	217.9 (1386.7)	n.d.-1871.3 (n.d.-10200.0)	113.8 ^{B,C} (886.9)
Azithromycin	n.d.-133.2 (n.d.-719.3)	14.4 (84.5)	-	-	-	-	-	-	n.d.-133.2 (n.d.-719.3)	3.6 (21.1)	-	-	n.d.-11.7 (n.d.-200.0)	0.8 (13.3)	-	-	-	-	n.d.-11.7 (n.d.-200.0)	0.2 (3.3)
Ciprofloxacin	n.d.-67.7 (n-d—792.7)	8.1 (80.5)	n.d.-3627.4 (n.d.-17500.0)	1300.2 (8666.7)	n.d.-3328.5 (n.d.-28800.0)	809.3 (7684.7)	n.d.-10439.1 (n.d.-32100.0)	1991.5 (10253.3)	n.d.-10439.1 (n.d.-32100.0)	1027.3 (6671.3)	n.d.-85.5 (n.d.-608.6)	11.9 (80.0)	n.d-836.5 (n.d-9800.0)	261.7 (2369.3)	n.d.-753.8 (n.d.-7400.0)	199.5 (1859.3)	n.d.-1871.3 (n.d.-10200.0)	435.8 (2773.3)	n.d.-1871.3 (n.d.-10200.0)	227.2 (1770.5)
Lipid regulators	0.7-38.5 (49.4-187.9)	9.9 (77.9)	n.d.-2052.4 (n.d.-8500.0)	358.2 (2802.1)	n.d.-1391.0 (n.d.-9000.0)	230.6 (5961.4)	n.d.-7444.4 (n.d.-40200.0)	744.7 (4051.1)	n.d.-7444.4 (n.d.-40200.0)	335.9 ^{A,C} (3223.1)	n.d.-26.7 (n.d.-369.8)	6.0 (63.6)	n.d.-240.0 (n.d.-2400.0)	38.3 (332.2)	n.d.- 1683.8 (n.d.-9200)	123.0 (922.5)	n.d.-3777.8 (n.d.-20400.0)	262.7 (1455.6)	n.d.-3777.8 (n.d.-20400.0)	107.5 ^{E} (693.5)
Bezafibrate	0.7-28.3 (35.6-152.8)	11.3 (87.4)	32.0-1348.8 (420.0-6000.0)	331.8 (2651.3)	0.7-916.9 (40.0-9000.0)	157.5 (1425.3)	66.4-7444.4 (1200.0-40200.0)	1568.3 (8486.7)	0.7-7444.4 (35.6-40200.0)	517.2 (3162.7)	n.d.-13.6 (n.d.-73.3)	2.6 (19.7)	0.9-240.0 (40.0-2400.0)	68.9 (584.7)	1.1-1683.8 (63-9200)	300.6 (2238.2)	27.7-3777.8 (500.0-20400.0)	775.4 (4266.7)	0.7.-3777.8 (n.d.-20400.0)	286.9 (1777.3)
Gemfibrozil	0.7-38.5 (49.4-187.9)	10.6 (85.4)	n.d.-1138.5 (n.d.-4300.0)	103.2 (511.7)	1.9-102.3 (49.0-920.0)	25.1 (216.8)	-	-	n.d.-1138.5 (n.d.-4300.0)	34.7 (203.5)	0.6-14.9 (29.2-133.9)	7.3 (62.5)	n.d.-169.0 (n.d.-1500.0)	45.9 (412.0)	n.d.-337.7 (n.d.-1720.0)	66.8 (513.3)	-	-	n.d.-337.7 (n.d.-1720.0)	30.0 (247.0)
Simvastatin	0.8-15.7 (45.7-76.5)	7.8 (60.8)	n.d.-2052.4 (n.d.-8500.0)	639.6 (5243.3)	n.d.-1391.0 (n.d.-7900.09	509.1 (4319.3)	n.d.-2243.9 (n.d.-8900.0)	665.9 (3666.7)	n.d.-2243.9 (n.d.-8900.0)	455.6 (3322.5)	0.7-26.7 (26.3-369.8)	9.1 (78.6)	-	-	n.d.-26.0 (n.d.-240.0)	1.7 (16.0)	n.d.-190.8 (n.d.-1500)	12.7 (100.0)	n.d.-190.8 (n.d.-1500,0)	5.9 (48.7)
Anti-inflammatories and/or analgesics	n.d.-7780.5 (n.d.-48878.0)	1133.5 (8744.8)	n.d.-16900.2 (n.d.-66700.0)	1545.3 (10929.4)	n.d.-10668.7 (n.d.-77800.0)	1993.7 (17265.5)	n.d.-23580.3 (n.d.-150000.0)	4280.3 (25400.0)	n.d.-23580.3 (n.d.-150000.0)	2238.2 ^{A,B} (15584.9)	n.d.-139.8 (n.d.-995.4)	10.0 (78.8)	n.d.-177.4 (n.d.-670.0)	20.0 (164.3)	n.d.-2416.1 (n.d.-19700.0)	181.6 (1376.6)	n.d.-4056.3 (n.d.-32000.0)	271.9 (5606.6)	n.d.-4056.3 (n.d.-32000.0)	120.9 ^{B,C} (1806.6)
Diclofenac	n.d.-43.1 (n.d.-232.7)	8.7 (67.4)	n.d.-635.5 (n.d.-2400.0)	46.0 (183.0)	n.d.-585.7 (n.d.-3500.0)	36.3 (235.1)	n.d.-1137.5 (n.d.-6200.0)	75.8 (413.3)	n.d.-1137.5 (n.d.-6200.0)	41.7 (224.7)	n.d.-16.4 (n.d.-90.1)	3.1 (24.9)	n.d.-177.4 (n.d.-670.0)	26.7 (154.8)	n.d.-584.7 (n.d.-3200.0)	56.7 (371.1)	n.d.-91.4 (n.d.-500)	6.1 (33.3)	n.d.-584.7 (n.d.-3200.0)	23.2 (146.0)
Ibuprofen	5.3-1100.7 (305.2-6810.0)	404.4 (2982.3)	n.d.-1266.9 (n.d.-8600.0)	505.2 (3920.0)	n.d.-2160.9 (n.d.-16100.0)	610.2 (5341.3)	n.d.-9398.5 (n.d.-28900.0)	1910.7 (9786.7)	n.d.-9398.5 (n.d.-28900.0)	857.6 (5507.6)	n.d.-139.8 (n.d.-995.4)	20.7 (157.8)	n.d.-116.9 (n.d.-1370.0)	33.3 (338.0)	n.d.-805.3 (n.d.-4400.0)	187.6 (1465.3)	n.d.-1365.9 (n.d.-6200.0)	327.0 (1840.0)	n.d.-1365.9 (n.d.-6200.0)	142.2 (950.3)
Paracetamol	59.2-7780.5 (1347.7-48878.0)	2987.4 (23184.8)	217.8-16900.2 (4200.0-66700.0)	4084.6 (28685.3)	726.3-10668.7 (15500.0-77800.0)	5334.5 (46220.0)	2815.2-23580.3 (25000.0-150000.0)	10854.4 (66000.0)	59.2-23580.3 (1347.7-150000.0)	5815.2 (41022.5)	n.d.-80.4 (n.d.-530.7)	6.1 (53.7)	-	-	n.d.-2416.1 (n.d.-19700.0)	300.5 (2293.3)	n.d.-4056.3 (n.d.-32000.0)	482.5 (3733.3)	n.d.-4056.3 (n.d.-32000.0)	197.3 (1520.1)
All pharmaceuticals	n.d.-7780.5 (n.d.-48878.0)	231.5 (1786.3)	n.d.-16900.2 (n.d.-66700.0)	467.4 (3324.1)	n.d.-10668.7 (n.d.-77800.0)	680.2 (7033.2)	n.d.-23580.3 (n.d.-150000.0)	1551.5 (8964.2)	n.d.-23580.3 (n.d.-150000.0)	796.2 (5741.6)	n.d.-139.8 (n.d.-995.4)	5.4 (43.1)	n.d-836.5 (n.d-9800.0)	29.2 (258.1)	n.d.-2416.1 (n.d.-19700.0)	101.2 (796.1)	n.d.-4056.3 (n.d.-32000.0)	185.4 (2178.2)	n.d.-4056.3 (n.d.-32000.0)	83.4 (846.9)

* - In each therapeutic group, the means followed by different upper case letters are significantly different (p<0.05). Comparisons were made separately for the WWI and WWE samples.

IV4.2. Spatial and temporal variation

Although some research has been made for understanding the fate of pharmaceuticals in Portuguese WWTPs [20,23,131], this approach should be performed at national level covering different geographical regions, that might have discrepancies due to the level of pharmaceuticals use, population demographics, cultural practices, environmental and climatic characteristics and infrastructure related to wastewater treatment [407].

Although no statistical significance was found in the data between the total and each therapeutic group mass loads in the different regions, in WWIs Alentejo and Algarve presented higher values (Figure 22). The increased mass loads in Alentejo can be explained by the fact that this region has the higher aging index in Portugal and, consequently, a higher pharmaceutical consumption. Algarve is a well-known vacation destination and in summer the number of inhabitants triplicates. The population-equivalent served during this period is much higher, increasing the overall flow rates and consequently the mass loads (Table 21, Supporting information). Concerning WWEs, Center and Algarve obtained higher mass loads than the other regions.

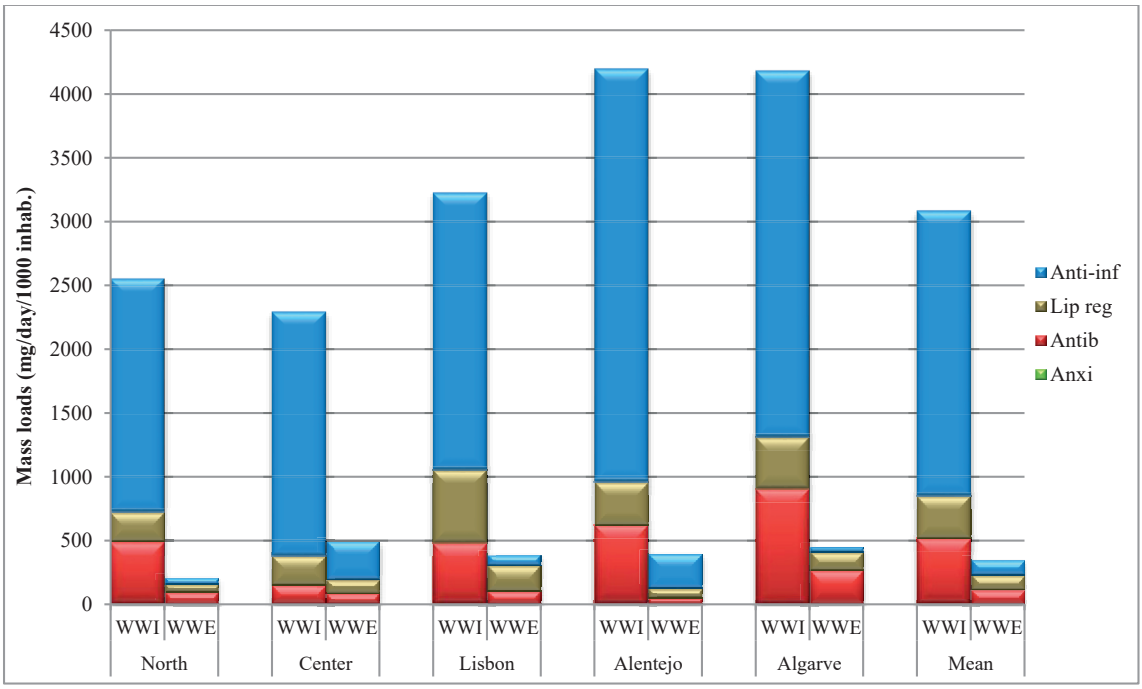


Figure 22. Spatial influence.
(Anx - anxiolytics and hypnotics; Antib - antibiotics; Lip reg - Lipid regulators; Anti-inf - anti-inflammatories and analgesics).

On what regards temporal influence, winter was clearly the season with superior mass loads, both in WWIs and WWEs (Figure 23), followed by autumn, summer and spring. Since most of these compounds easily degrade with high temperatures, it would be predictable that lower concentrations were to be found in summer, both in WWIs and WWEs. However, mass loads were higher in summer when compared with spring season as a result of tourism increase in the summer months. Furthermore, some pharmaceuticals like antibiotics and anti-inflammatories have higher consumption rates during winter, leading to contamination differences between winter and the other seasons.

Although the differences, no statistical significance was found between seasons, neither in each therapeutic group, nor in the sum of all pharmaceuticals per season. These results provide useful information for management purposes and for an environmental-oriented monitoring [400].

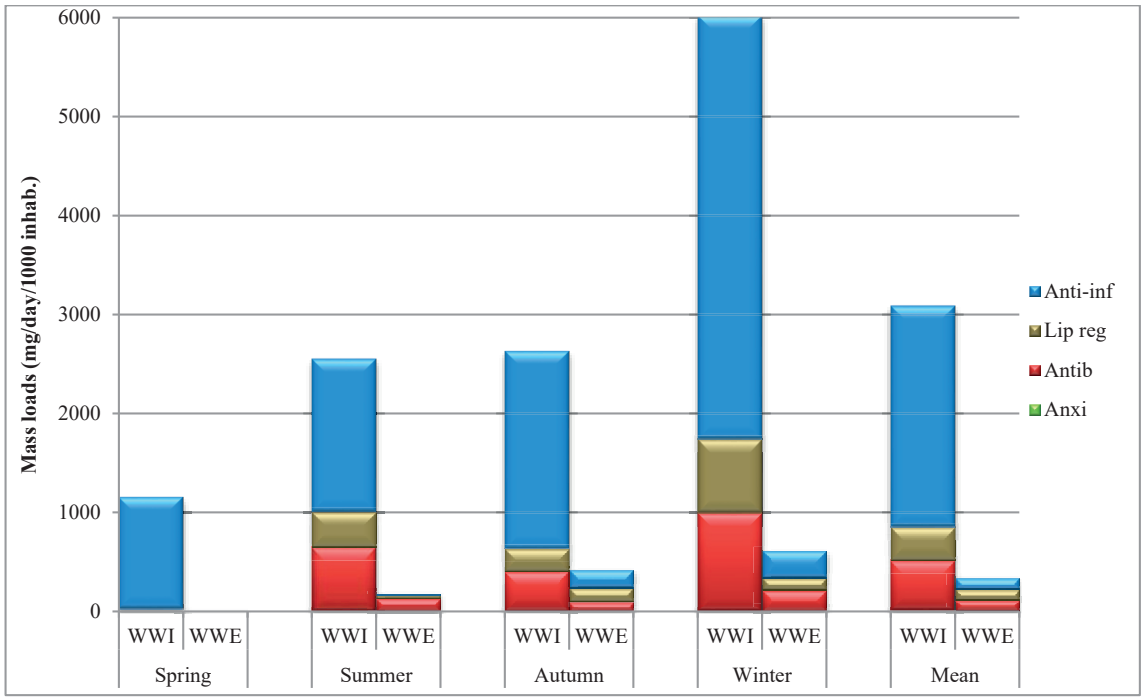


Figure 23. Temporal influence.
(Anx - anxiolytics and hypnotics; Antib - antibiotics; Lip reg - Lipid regulators; Anti-inf - anti-inflammatories and analgesics).

IV4.3. Environmental risk assessment (ERA)

Nowadays prioritization lists of pharmaceuticals are based on the concept of ERA, which takes into account the potential effect of a given pharmaceutical and its exposure level. Although it is very difficult to estimate if pharmaceuticals adverse effects to non target organisms will occur at low environmental levels, the RQs could be a useful measure tool that improves ERA in a way to evaluate health, ecological and economic consequences [386].

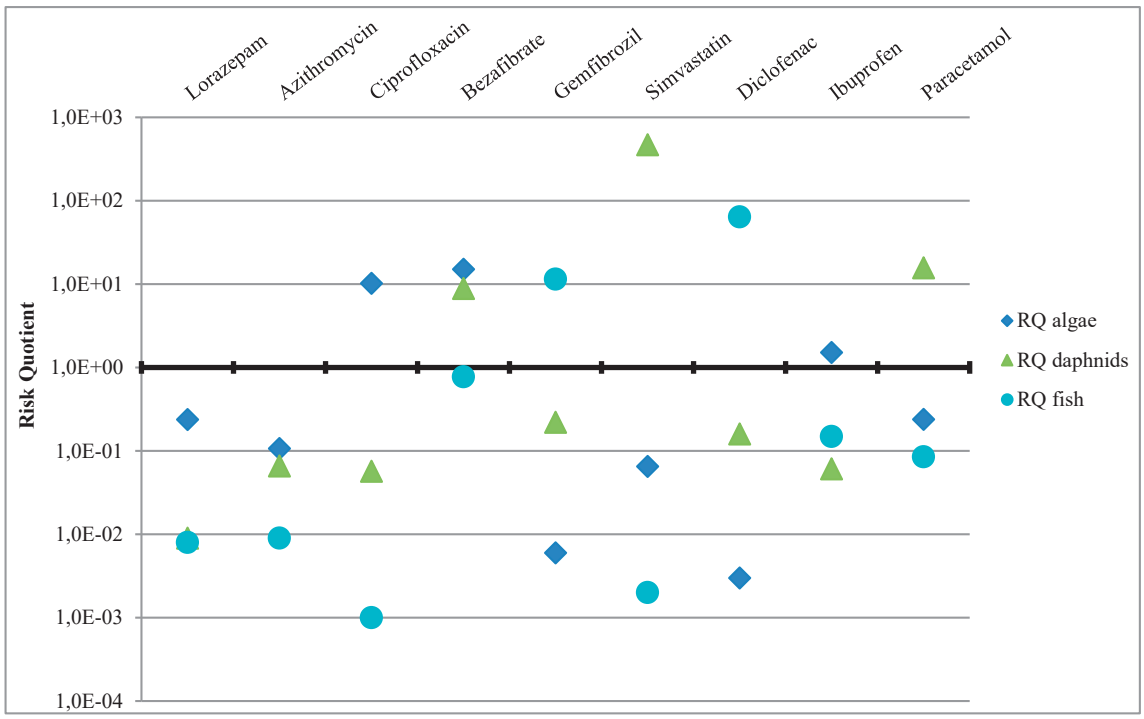
Using the approach recommended by EMA [353], RQs were provided by dividing the highest concentration of pharmaceuticals in WWE samples (MECs) by the PNECs values, considering the above mentioned UFs (Figure 24 (A)). From the 9 pharmaceuticals found in WWEs, 7 presented RQs superior to 1 for at least one trophic level, posing a risk to algae, daphnids and fish. The RQs values found ranged from 469 for simvastatin to zero for alprazolam and zolpidem, being anxiolytics the only therapeutic group that did not present environmental risk. Nevertheless, a certain risk could be expected for the substances with a RQ between 0.1 and 1, including, in this way, all the pharmaceuticals that were detected in WWEs. Moreover, even for RQs higher than 10, the predicted dilution effect of 10 in the receiving water bodies does not mitigate possible environmental hazards [353].

Using a less conservative approach, we also assessed the ERA with the mean concentrations as MECs (Figure 24 (B)). Although this evaluation presented lower values for RQs, as expected, 5 pharmaceuticals still had RQs superior to 1, highlighting the fact that it poses a risk to the 3 trophic levels considered.

Both approaches, did not allow to observe a clear pattern regarding the most sensitive trophic levels. It should also be noted that, given the mixture of these compounds, in some cases with the same pharmacological mechanisms, additive or even synergistic effects could be expected, being the real hazard greater than the calculated [23,133,230,232,405].

The lack of toxicological studies, namely long-term studies and long-term studies across the lifespan of the organisms, points out that this risk evaluation has its limitations [17]. Nonetheless, this is a contribution to assess the ecotoxicological risk posed by these pharmaceuticals to aquatic organisms.

(A)



(B)

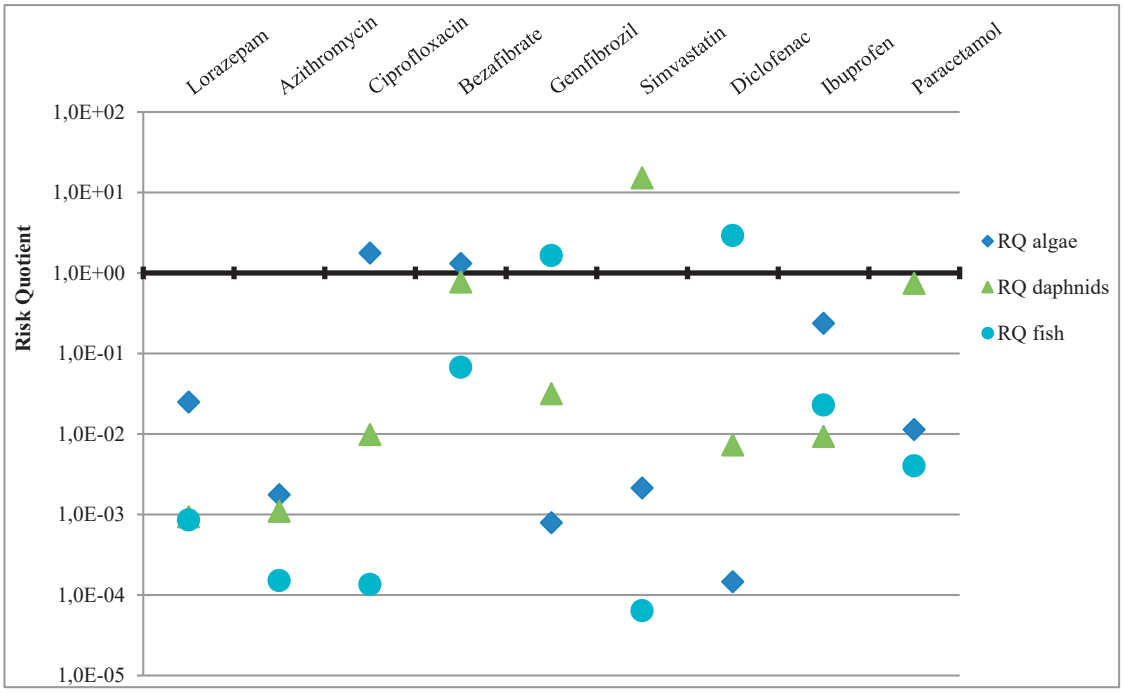


Figure 24. Environmental risk assessment. (A) Using worst-case scenario; (B) Using the average.

IV4.4. Selection of the most representative WWTPs and most impacted surface waters

WWTPs are the main source of pharmaceuticals in the aquatic environment and WWE contamination ought to be considered in the selection of the 6 representative monitoring stations for surface waters in Portugal, as required by the Directive 2013/39/EU.

To ensure the representativeness of the samples and viewing the implementation of the Directive 2013/39/EU, the proposed monitoring stations should be located 500 m downstream the WWTPs discharge points, thus enabling complete homogenization of WWEs and receiving surface waters.

This assessment was performed multiplying the concentration found in the WWEs by the flow rate for each WWTP (Figure 25 (A)). These values were then refined, to predict the surface water contamination, taking into account the flow and, consequently, the dilution factor of the receiving rivers (Figure 25 (B)). According to the APA, Mondego, Tagus and Guadiana rivers have a flow average of 100, 500 and 500 $m^3 s^{-1}$, respectively, as for the others it is approximately 50 $m^3 s^{-1}$.

Figure 25 compares the pharmaceuticals released by WWEs. As expected, with minor exceptions, the WWTPs with higher population equivalent have higher amount of pharmaceuticals released into the receiving surface waters. Overall and by decreasing order, WWTPs 11, 7, 6, 10, 5, 14 and 1 release the higher amounts of pharmaceuticals.

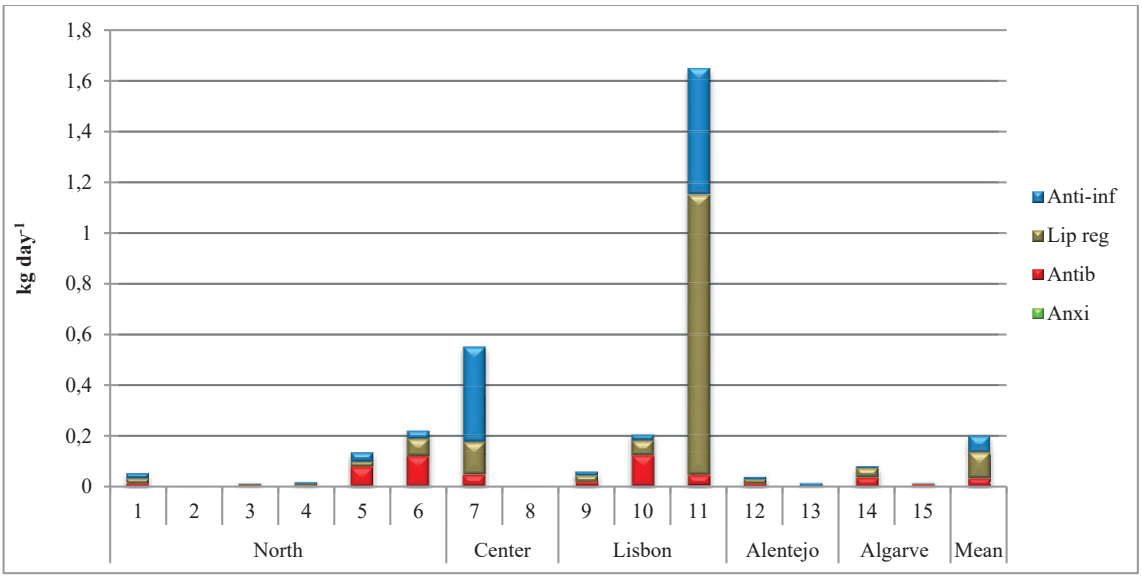
Regarding the surface water contamination, excluding the ones discharging in the Atlantic Ocean, we were able to predict that the most contaminated rivers are those impacted by the WWTPs 7, 6, 11, 5, 9 and 12. These results suggest that the rivers Mondego, Tagus, Ave, Trancão, Fervença and Xarrama should be selected for surface water monitoring stations (Figure 25). One should also bear in mind that more than one WWTP can discharge their effluents in one river basin and additive effects could be observed.

We are aware of the fact that the selection of the surface water monitoring stations should consider not only the most contaminated surface waters but also their average contamination. However, the previously selected representative monitoring stations already included the average contaminated rivers, Fervença and Xarrama.

The obtained results for the predicted average surface water contamination ranged from 0.1 to 64.2 ng L^{-1} concerning the sum of the 11 pharmaceuticals, being the anti-inflammatories and lipid regulators the therapeutic groups with higher impact on the surface waters, with averages of 6.5 and 5.4 ng L^{-1}, respectively. Although slightly lower, these values are in agreement with

other studies, where concentrations up to 1014 ng L^{-1} and average concentrations, usually under 100 ng L^{-1}, were observed [124,191,210]. These values were also similar to the ones predicted in a modelling exercise performed in England for ibuprofen and diclofenac, 24 and 14 ng L^{-1}, respectively [18].

(A)



(B)

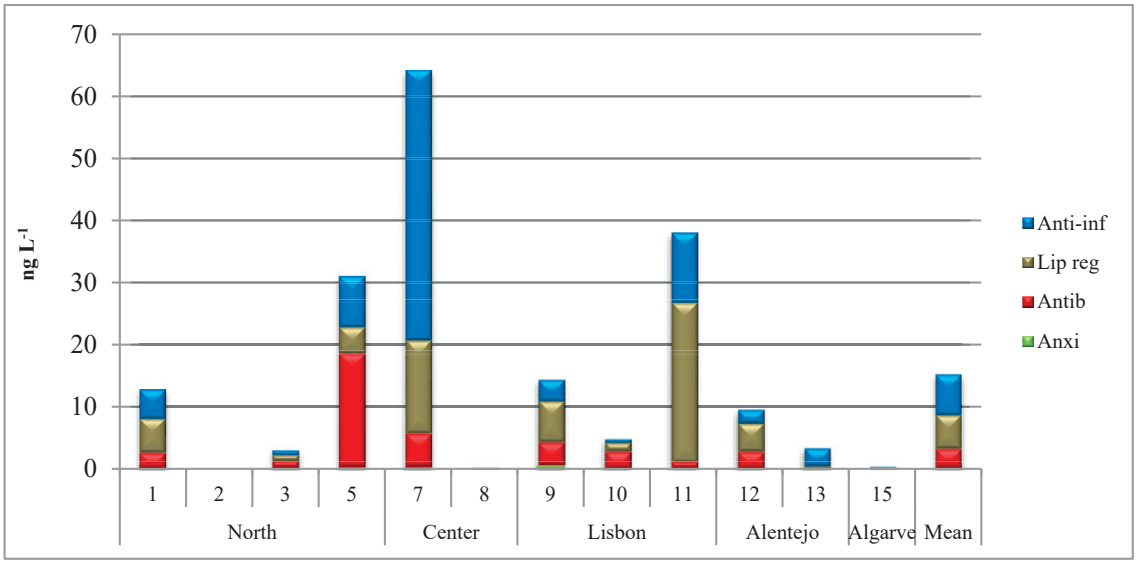


Figure 25. Aquatic contamination. (A) Amount released by each WWTP; (B) Predicted surface water concentrations.
(Anx - anxiolytics and hypnotics; Antib - antibiotics; Lip reg - Lipid regulators; Anti-inf - anti-inflammatories and analgesics).

IV5. Conclusions

This monitoring based exercise, developed in 15 WWTPs, throughout four sampling campaigns during one year, evidences that the selected pharmaceuticals are ubiquitous in the Portuguese aquatic environment, and this fact should be recognized as a priority issue in the environmental policies, both as national and European level.

Overall, the results showed that, as expected, the frequencies of contamination, concentration levels and mass loads were higher in WWI samples. All samples were contaminated with at least one and up to 8 from the 11 targeted pharmaceuticals. Only alprazolam and zolpidem were not detected. The highest concentrations observed were 150 and 32 μg L^{-1} for WWIs and WWEs, respectively.

Concerning the temporal influence, winter was the season with higher values, both in WWIs and WWEs. As for the spatial influence in WWIs, Alentejo and Algarve had superior mass loads than the other regions, as for WWEs, Center and Algarve regions were the ones that presented higher mass loads.

After evaluating the potential ecotoxicological risk posed by the selected pharmaceuticals, we concluded that 7 pharmaceuticals had RQs higher than 1 and up to 469, posing possible risk to all the three different trophic levels. Moreover, even when the averages concentrations were used for ERA, 5 pharmaceuticals still had RQs superior to 1.

Finally, based upon our results, and in line with the Directive 2013/39/EU, the rivers Mondego, Tagus, Ave, Trancão, Fervença and Xarrama should be selected as monitoring stations, since they are hotspots of contamination for pharmaceuticals in Portuguese surface waters.

A global picture of pharmaceuticals contamination in Portugal was achieved, an important input to the Directive 2013/39/EU, tackling the concern towards the aquatic contamination by pharmaceuticals, setting prioritizing measures and sustainable strategies, for minimizing its impact in the aquatic environment.

IV6. Supporting information

Table 19. Therapeutic groups, characteristics, CAS number and national sales for the selected pharmaceuticals.

Therapeutic group	Pharmaceutical	Molecular formula	Molecular weight	CAS no.	National sales by package
Anxiolytics and hypnotics	Alprazolam	$C_{17}H_{13}ClN_4$	308.8	28981-97-7	2 384 299
	Lorazepam	$C_{15}H_{10}N_2Cl_2O_2$	321.2	846-49-1	1 947 305
	Zolpidem	$C_{19}H_{21}N_3O$	307.4	82626-48-0	1 089 029
Antibiotics	Azithromycin	$C_{38}H_{72}N_2O_{12}$	749	83905-01-5	944 513
	Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	331.4	85721-33-1	618 465
Lipid regulators	Bezafibrate	$C_{19}H_{20}ClNO_4$	361.8	41859-67-0	41 450
	Gemfibrozil	$C_{15}H_{22}O_3$	250.3	25812-30-0	n.a.
	Simvastatin	$C_{25}H_{38}O_5$	418.6	79902-63-9	3 440 703
Anti-Inflammatories and/or analgesics	Diclofenac	$C_{14}H_{10}Cl_2NNaO_2$	318.1	15307-79-6	1 295 809
	Ibuprofen	$C_{13}H_{18}O_2$	206.3	15687-27-1	2 063 414
	Paracetamol	$C_8H_9NO_2$	151.2	103-90-2	3 239 035

n.a. – Not available

Table 20. Codes of the sampling points and characteristics of the wastewater treatment plants (WWTPs).

WWTP Code	Region	Population equivalent	Type of wastewater treated	Discharging points	Average loads (m3/d)	Type of treatment	Process
WWTP 1	North	41955	Domestic. industrial (residual)	Fervença River	5685	Secondary	Activated Sludge with conventional aeration
WWTP 2	North	10000	Domestic. industrial (mainly)	Tua River	349	Tertiary with UV	Activated Sludge with extended aeration
WWTP 3	North	57748	Domestic. industrial (residual)	Tâmega River	8069	Tertiary with UV	Activated Sludge with extended aeration
WWTP 4	North	45257	Domestic. hospital and industrial	Atlantic Ocean	8580	Tertiary with UV	Activated Sludge with medium load aeration
WWTP 5	North	255557	Domestic and industrial	Ave River	15000	Tertiary with UV	Activated Sludge with conventional and extended aeration
WWTP 6	North	300000	Domestic	Atlantic Ocean	66718	Tertiary with UV	Activated Sludge with extended aeration
WWTP 7	Center	213000	Domestic and industrial	Mondego River	36000	Secondary	Trickling Filters
WWTP 8	Center	6850	Domestic. hospital and industrial	Mondego River	600	Secondary	Activated Sludge
WWTP 9	Lisbon and Tagus Valley	700000	Domestic and industrial	Trancão River	60000	Secondary with biofiltration	Activated Sludge
WWTP 10	Lisbon and Tagus Valley	215000	Domestic and industrial	Tagus River	50000	Tertiary with UV	Activated Sludge
WWTP 11	Lisbon and Tagus Valley	756000	Domestic	Tagus River	140000	Tertiary with UV	Biofiltration
WWTP 12	Alentejo	60000	Domestic. hospital and industrial	Xarrama River	13720	Tertiary with UV	Activated Sludge with medium load aeration
WWTP 13	Alentejo	8700	Domestic	Álamo Brook	1239	Tertiary with UV	Activated Sludge with extended aeration
WWTP 14	Algarve	49547	Domestic	Atlantic Ocean	9239	Tertiary with UV	Activated Sludge with extended aeration
WWTP 15	Algarve	30766	Domestic	Guadiana River	6141	Tertiary with UV	Lagoons with extended aeration

Table 21. Characterization of WWTPs parameters for the different sampling periods.

WWTP	Sampling date	Flow rate (m^3 day^{-1})	Flow rate (L/day/1000 inhab)	WWTP influent				WWTP effluent			
				pH	BOD5 (mg O_2 L^{-1})	COD (mg O_2 L^{-1})	TSS (mg L^{-1})	pH	BOD5 (mg O_2 L^{-1})	COD (mg O_2 L^{-1})	TSS (mg L^{-1})
1	12-05-2013	3819	91026	8.3	480	768	453	7.3	10	47	18
	23-07-2013	4728	112692	8.0	700	1275	644	7.3	36	94	22
	29-10-2013	5631	134215	8.2	440	722	331	7.5	8	20	11
	05-02-2014	13644	325206	7.1	260	483	277	7.6	16	32	21
2	15-05-2013	134	13400	8.0	2600	3750	1460	8.2	6	23	8
	23-07-2013	225	22500	7.8	1700	4230	3777	7.3	12	68	9
	30-10-2013	175	17500	7.8	1800	2825	556	7.2	8	72	17
	05-02-2014	553	55300	8.1	1585	950	226	7.6	39	11	8
3	14-05-2013	7236	125303	7.2	50	100	26	6.9	8	40	6
	23-07-2013	5673	98237	8.2	– [a]	– [a]	– [a]	8.0	– [a]	– [a]	– [a]
	30-10-2013	6591	114134	7.5	195	286	150	7.5	7	11	12
	05-02-2014	7204	124749	8.2	44	160	96	7.3	4	14	16
4	29-05-2013	6356	140442	– [a]	400	753	310	– [a]	45	114	51
	23-07-2013	5151	113817	– [a]	280	843	294	– [a]	45	112	72
	30-10-2013	5031	111165	– [a]	250	375	110	– [a]	17	56	16
	06-02-2014	10235	226153	7.1	40	62	39	6.8	<10	41	<10
5	28-05-2013	11216	43888	7.3	260	690	310	7.2	<4	47	<10
	22-07-2013	14884	58241	7.5	480	896	512	7.1	9	30	10
	30-10-2013	12136	47488	7.7	225	334	200	7.4	6	67	11
	06-02-2014	16351	63982	7.4	210	273	146	7.1	5	34	8
6	23-05-2013	31770	105900	7.5	260	580	250	7.4	6	47	18
	11-07-2013	29690	98967	7.2	680	720	470	7.5	6	42	13
	07-11-2013	32530	108433	7.4	250	810	590	7.5	8	76	<10
	06-02-2014	38160	127200	7.4	17	169	73	7.2	5	31	15
7	22-05-2013	32258	151446	– [a]	640	1000	250	7.5	50	110	24
	14-08-2013	18180	85352	– [a]	370	510	200	7.5	50	110	42
	24-10-2013	30299	142249	– [a]	340	490	210	7.4	29	80	20
	06-02-2014	36000[a]	169014	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]	– [a]
8	07-05-2013	495	72263	7.4	208	512	150	7.4	13	88	10
	23-07-2013	634	92555	7.4	306	729	360	7.4	16	74	26
	01-10-2013	996	145401	7.4	22	94	33	7.4	10	68	10
	06-02-2014	1351	197226	7.1	503	1070	1060	7.4	10	40	13
9	03-06-2013	48650	69500	7.7	330	760	300	7.7	11	46	19
	22-07-2013	47129	67327	7.4	260	680	290	7.9	<6	46	19
	04-11-2013	48246	68923	7.6	210	680	320	7.7	<6	66	7
	10-02-2014	60000[b]	85714	7.6	83	270	130	7.4	<6	<30	<3

Table 21. Characterization of WWTPs parameters for the different sampling periods. *(continued)*

WWTP	Sampling date	Flow rate (m^3 day^{-1})	Flow rate (L/day/1000 inhab)	WWTP influent				WWTP effluent			
				pH	BOD5 (mg O_2 L^{-1})	COD (mg O_2 L^{-1})	TSS (mg L^{-1})	pH	BOD5 (mg O_2 L^{-1})	COD (mg O_2 L^{-1})	TSS (mg L^{-1})
10	03-06-2013	45890	213442	7.5	280	640	260	7.5	<6	51	8
	22-07-2013	42270	196605	7.7	180	500	280	7.6	<6	<30	4
	04-11-2013	42210	196326	7.5	190	440	150	7.6	<6	35	4
	10-02-2014	50000[b]	232558	7.7	92	260	130	7.7	<6	<30	<3
11	04-06-2013	137761	182223	7.4	290	580	280	7.0	10	67	17
	23-07-2013	119272	157767	7.5	280	650	250	7.3	18	120	18
	05-11-2013	138366	183024	7.7	350	770	560	7.5	15	43	22
	11-02-2014	140000[b]	185185	7.9	29	94	44	7.3	<6	<30	7
12	21-05-2013	12305	205083	– [a]	220	510	158	7.5	8	41	54
	23-07-2013	6001	100017	– [a]	389	510	231	7.6	7	52	16
	24-10-2013	6113	101883	– [a]	132	430	158	6.7	6	44	14
	30-01-2014	12980	216333	7.8	112	340	74	7.4	19	45	18
13	21-05-2013	980	112644	– [a]	177	580	116	7.6	<3	17	<10
	23-07-2013	822	94483	– [a]	848	1380	597	7.1	18	<3	<10
	24-10-2013	1067	122644	– [a]	70	610	73	6.7	<3	28	<10
	30-01-2014	865	99425	7.9	261	560	28	7.2	5	31	14
14	21-05-2013	7887	159182	7.4	400	800	300	7.8	<10	34	5
	23-07-2013	12554	253376	– [a]	– [a]	900	– [a]	– [a]	– [a]	39	– [a]
	05-11-2013	5047	101863	7.5	379	1100	460	7.7	3	30	6
	06-02-2014	9090	183462	7.3	300	570	260	7.7	<10	28	3
15	24-05-2013	4910	159592	7.7	260	470	220	8.2	<10	25	4
	19-07-2013	8146	264773	7.5	400	700	600	7.7	11	38	8
	01-11-2013	4755	154554	7.6	146	800	220	8.0	<10	22	5
	07-02-2014	5625	182832	7.5	260	470	310	8.0	<10	30	9

[a]- Data not available

Table 22. MS/MS parameters for the analysis of target pharmaceuticals.

Therapeutic group	**Compound**	**R_t (min)**	**Precursor ion (m/z)**	**MS^2 product ion (m/z)**	**Ionization mode**	**R_t window (min)**	**Capillary voltage (V)**	**RF loading (%)**	**CID voltage I (V)**	**MS^3 product ion(s) (m/z)**	**CID voltage II (V)**
Anxiolytics and hypnotics	Alprazolam	7.89	309	281	ESI+	7.30–8.60	92	87	1.28	-	-
	Lorazepam	8.02	321	303	ESI+	7.30–8.60	65	88	0.85	275, 277	1.04
	Zolpidem	2.64	308	263	ESI+	2.35–5.50	102	87	1.35	-	-
Antibiotics	Azithromycin	3.72	375	591	ESI+	2.35–5.50	42	90	1.21	398, 416, 434, 573	1.52
	Ciprofloxacin	1.32	332	314	ESI+	0.00–2.35	72	87	0.65	-	-
Lipid regulators	Bezafibrate	9.31	360	274	ESI-	8.60–10.00	63	100	1.44	154	0.67
	Gemfibrozil	11.49	249	121	ESI-	11.00–11.70	48	67	0.80	106	2.20
	Simvastatin	12.19	441	325	ESI+	11.70–13.00	126	66	1.15	295, 310, 311	1.20
Anti-Inflammatories and/or analgesics	Diclofenac	10.54	294	250	ESI-	10.00–11.00	36	111	0.79	214	1.32
	Ibuprofen	10.71	205	161	ESI-	10.00–11.00	33	57	0.69	-	-
	Paracetamol	0.95	152	110	ESI+	0.00–2.35	54	62	1.00	65, 82, 92, 93	0.86

Table 23. Method detection limits (MDLs), method quantification limits (MQLs), recoveries and relative standard deviation (RSD) of target compounds.

Therapeutic group	**Compound**	**MDL (ng L^{-1})**		**MQL (ng L^{-1})**		**Recoveries (%)***		**RSD (%)***	
		WWI	**WWE**	**WWI**	**WWE**	**WWI**	**WWE**	**WWI**	**WWE**
Anxiolytics and hypnotics	Alprazolam	1.2	1.0	4.0	3.3	70.2	69.2	10.6	10.0
	Lorazepam	1.7	1.5	5.6	5.0	72.2	80.0	11.0	9.2
	Zolpidem	1.5	1.3	5.0	4.4	66.7	64.1	11.9	7.4
Antibiotics	Azithromycin	0.5	0.4	1.7	1.4	**	**	**	**
	Ciprofloxacin	6.0	5.0	20.0	16.7	111.8	82.9	10.4	7.1
Lipid regulators	Bezafibrate	6.7	6.0	22.2	20.0	84.0	88.2	8.2	6.4
	Gemfibrozil	8.6	7.5	28.6	25.0	65.2	78.0	14.2	6.7
	Simvastatin	1.0	0.8	3.3	2.5	75.2	84.7	23.0	22.0
Anti-Inflammatories and/or analgesics	Diclofenac	16.7	15.0	55.6	50.0	70.6	88.2	7.2	5.9
	Ibuprofen	61.2	60.0	204.1	200.0	73.6	72.5	12.5	11.1
	Paracetamol	10.0	9.4	33.3	31.3	**	68.6	**	13.0

* - Validation assays were performed using spiked samples at 100 ng L^{-1}

** - High contaminated sample, not allowing the calculation of these parameters

Table 24. Occurrence, average, standard deviation, frequency results and removal for the selected pharmaceuticals in spring (A), summer (B), autumn (C) and winter (D).

(A)	Anxiolytics and hypnotics								Antibiotics						Lipid regulators								Anti-Inflammatories and/or analgesics								Total average	
	Alprazolam ng L^{-1}		Lorazepam ng L^{-1}		Zolpidem ng L^{-1}		Average		Azithromycin ng L^{-1}		Ciprofloxacin ng L^{-1}		Average ng L^{-1}		Bezafibrate ng L^{-1}		Gemfibrozil ng.L^{-1}		Simvastatin ng L^{-1}		Average ng L^{-1}		Diclofenac ng L^{-1}		Ibuprofen ng L^{-1}		Paracetamol ng L^{-1}		Average ng L^{-1}			
	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE
WWTP 1	n.d..	n.d.	443.8	202.5	n.d.	n.d.	147.9	67.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	140.2	31.8	52.6	79.1	50.7	67.9	81.2	59.6	n.d.	n.d.	6810.0	n.d.	32856.0	n.d.	13222.0	n.d.	3668.5	34.7
WWTP 2	n.d.	n.d.	58.5	22.8	n.d.	n.d.	19.5	7.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	52.1	n.d.	49.4	42.3	57.5	55.0	53.0	32.4	n.d.	n.d.	394.3	n.d.	27172.0	39.5	9188.8	13.2	2525.8	14.5
WWTP 3	n.d.	n.d.	74.6	185.1	n.d.	n.d.	24.9	61.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	37.8	n.d.	121.3	29.9	65.5	31.3	74.9	20.4	75.7	n.d.	2244.0	334.3	9159.0	n.d.	3826.2	111.4	1070.7	52.8
WWTP 4	n.d.	n.d.	196.5	233.5	n.d.	n.d.	65.5	77.8	n.d.	n.d.	n.d.	608.6	n.d.	304.3	55.4	n.d.	53.2	41.6	52.6	32.1	53.7	24.6	97.3	n.d.	2337.0	995.4	25053.0	n.d.	9162.4	331.8	2531.4	173.7
WWTP 5	n.d.	n.d.	38.4	399.8	n.d.	n.d.	12.8	133.3	n.d.	n.d.	792.7	n.d.	396.4	n.d.	98.7	32.3	119.0	71.5	45.7	53.4	87.8	52.4	59.7	62.0	1811.3	n.d.	1347.7	235.4	1072.9	99.1	392.1	77.7
WWTP 6	n.d.	n.d.	97.7	29.3	n.d.	n.d.	32.6	9.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	152.8	n.d.	160.0	32.5	76.4	59.3	129.7	30.6	150.8	n.d.	1235.0	n.d.	25623.0	n.d.	9002.9	n.d.	2499.6	11.0
WWTP 7	n.d.	n.d.	65.5	306.7	n.d.	n.d.	21.8	102.2	547.9	n.d.	n.d.	246.4	274.0	123.2	76.9	58.4	59.4	74.1	51.4	81.3	62.6	71.3	92.7	73.0	5733.0	684.8	27393.0	530.7	11072.9	429.5	3092.7	186.9
WWTP 8	n.d.	n.d.	330.2	246.8	n.d.	n.d.	110.1	82.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	79.1	25.1	133.1	126.0	76.5	369.8	96.2	173.6	108.4	78.5	3738.0	352.8	22620.0	n.d.	8822.1	143.8	2462.3	109.0
WWTP 9	n.d.	n.d.	58.5	154.2	n.d.	n.d.	19.5	51.4	n.d.	n.d.	n.d.	165.7	n.d.	82.9	107.8	50.4	68.2	133.0	50.7	38.4	75.6	73.9	63.3	n.d.	3212.0	n.d.	20944.0	n.d.	8073.1	n.d.	2227.7	49.2
WWTP 10	n.d.	n.d.	62.4	211.7	n.d.	n.d.	20.8	70.6	n.d.	n.d.	84.3	178.9	42.2	89.5	85.4	24.1	52.1	55.4	62.8	62.9	66.8	47.5	n.d.	70.5	1424.7	n.d.	21599.0	n.d.	7674.6	23.5	2124.6	54.9
WWTP 11	n.d.	n.d.	294.1	158.3	n.d.	n.d.	98.0	52.8	719.3	n.d.	n.d.	n.d.	359.7	n.d.	138.1	73.3	53.7	29.2	65.8	38.1	85.9	46.9	232.7	n.d.	5944.0	n.d.	17625.0	n.d.	7933.9	n.d.	2279.3	27.2
WWTP 12	n.d.	n.d.	109.3	45.2	n.d.	n.d.	36.4	15.1	n.d.	n.d.	330.2	n.d.	165.1	n.d.	138.0	n.d.	187.9	72.4	76.3	99.5	134.1	57.3	n.d.	n.d.	4796.0	n.d.	22492.0	n.d.	9096.0	n.d.	2557.2	19.7
WWTP 13	n.d.	n.d.	475.8	182.3	n.d.	n.d.	158.6	60.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	35.6	n.d.	64.6	56.7	53.0	58.6	51.1	38.4	67.5	90.1	305.2	n.d.	30329.0	n.d.	10233.9	30.0	2848.2	35.2
WWTP 14	n.d.	n.d.	59.2	107.3	n.d.	n.d.	19.7	35.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	47.5	n.d.	52.8	51.6	56.4	26.3	52.2	26.0	n.d.	n.d.	1846.0	n.d.	48878.0	n.d.	16908.0	n.d.	4630.9	16.8
WWTP 15	n.d.	n.d.	58.5	55.1	n.d.	n.d.	19.5	18.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	65.8	n.d.	53.3	42.0	70.9	105.3	63.3	49.1	62.9	n.d.	2904.0	n.d.	14681.0	n.d.	5882.6	n.d.	1626.9	18.4
Average	0.0	0.0	161.5	169.4	0.0	0.0	53.8	56.5	84.5	0.0	80.5	80.0	82.5	40.0	87.4	19.7	85.4	62.5	60.8	78.6	77.9	53.6	67.4	24.9	2982.3	157.8	23184.8	53.7	8744.8	78.8	1807.7	55.6
SD	0.0	0.0	145.3	103.0	0.0	0.0	114.6	100.7	217.6	0.0	207.7	162.2	216.4	123.5	38.6	24.2	44.6	30.7	10.2	81.1	37.0	58.3	63.4	35.7	1964.3	296.9	10433.6	140.3	12103.8	201.3	6393.2	117.6
Frequency	0%	0%	100%	100%	0%	0%	33%	33%	13%	0%	20%	27%	17%	13%	100%	47%	100%	100%	100%	100%	100%	82%	67%	33%	100%	27%	100%	20%	89%	27%		

n.d. - not detected

Table 24. Occurrence, average, standard deviation, frequency results and removal for the selected pharmaceuticals in spring (A), summer (B), autumn (C) and winter (D). *(continued)*

	Anxiolytics and hypnotics								Antibiotics						Lipid regulators								Anti-Inflammatories and/or analgesics								Total average	
(B)	Alprazolam ng L^{-1}		Lorazepam ng L^{-1}		Zolpidem ng L^{-1}		Average		Azithromycin ng L^{-1}		Ciprofloxacin ng L^{-1}		Average ng L^{-1}		Bezafibrate ng L^{-1}		Gemfibrozil ng.L^{-1}		Simvastatin ng L^{-1}		Average ng L^{-1}		Diclofenac ng L^{-1}		Ibuprofen ng L^{-1}		Paracetamol ng L^{-1}		Average ng L^{-1}			
	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE
WWTP 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1800.0	1200.0	900.0	600.0	5400.0	1600.0	910.0	1500.0	5100.0	n.d.	3803.3	1033.3	n.d.	79.0	8600.0	370.0	60700.0	n.d.	23100.0	149.7	7500.9	431.7
WWTP 2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6000.0	40.0	n.d.	n.d.	n.d.	n.d.	2000.0	13.3	n.d.	n.d.	n.d.	n.d.	9680.0	n.d.	3226.7	n.d.	1425.5	3.6
WWTP 3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4200.0	n.d.	2100.0	n.d.	1000.0	190.0	280.0	240.0	4200.0	n.d.	1826.7	143.3	n.d.	240.0	4700.0	n.d.	20600.0	n.d.	8433.3	80.0	3180.0	60.9
WWTP 4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	17500.0	2800.0	8750.0	1400.0	800.0	470.0	85.0	150.0	5000.0	n.d.	1961.7	206.7	56.0	63.0	3500.0	620.0	23800.0	n.d.	9118.7	227.7	4612.8	373.0
WWTP 5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	200.0	10400.0	5000.0	5200.0	2600.0	550.0	250.0	220.0	n.d.	8500.0	n.d.	3090.0	83.3	n.d.	490.0	3600.0	950.0	20900.0	n.d.	8166.7	480.0	4015.5	626.4
WWTP 6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6800.0	3100.0	3400.0	1550.0	2200.0	850.0	570.0	730.0	5900.0	n.d.	2890.0	526.7	n.d.	170.0	3400.0	380.0	26700.0	n.d.	10033.3	183.3	4142.7	475.5
WWTP 7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	9600.0	9800.0	4800.0	4900.0	880.0	560.0	120.0	n.d.	6900.0	n.d.	2633.3	186.7	n.d.	n.d.	6000.0	1370.0	37200.0	n.d.	14400.0	456.7	5518.2	1066.4
WWTP 8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6700.0	3400.0	3350.0	1700.0	790.0	590.0	n.d.	1400.0	7900.0	n.d.	2896.7	663.3	63.0	n.d.	2200.0	850.0	4200.0	n.d.	2154.3	283.3	1986.6	567.3
WWTP 9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8500.0	760.0	4250.0	380.0	5100.0	180.0	250.0	170.0	4950.0	n.d.	3433.3	116.7	n.d.	100.0	4300.0	n.d.	17500.0	n.d.	7266.7	33.3	3690.9	110.0
WWTP 10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11500.0	2500.0	5750.0	1250.0	5800.0	340.0	390.0	n.d.	3500.0	n.d.	3230.0	113.3	72.0	n.d.	2900.0	n.d.	31200.0	n.d.	11390.7	n.d.	5032.9	258.2
WWTP 11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13200.0	1200.0	6600.0	600.0	3200.0	930.0	280.0	420.0	6200.0	n.d.	3226.7	450.0	n.d.	n.d.	3500.0	530.0	24500.0	n.d.	9333.3	176.7	4625.5	280.0
WWTP 12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7900.0	4000.0	3950.0	2000.0	4100.0	2400.0	n.d.	1400.0	5200.0	n.d.	3100.0	1266.7	n.d.	n.d.	3700.0	n.d.	21800.0	n.d.	8500.0	n.d.	3881.8	709.1
WWTP 13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4400.0	n.d.	2200.0	n.d.	930.0	150.0	270.0	n.d.	7200.0	n.d.	2800.0	50.0	97.0	n.d.	4700.0	n.d.	43900.0	n.d.	16232.3	n.d.	5590.6	13.6
WWTP 14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13800.0	1600.0	6900.0	800.0	2600.0	40.0	n.d.	n.d.	8100.0	n.d.	3566.7	13.3	64.0	510.0	5000.0	n.d.	66700.0	n.d.	23921.3	170.0	8751.3	195.5
WWTP 15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13700.0	180.0	6850.0	90.0	420.0	180.0	4300.0	170.0	n.d.	n.d.	1573.3	116.7	2400.0	670.0	2700.0	n.d.	20900.0	n.d.	8666.7	223.3	4038.2	109.1
Average	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	13.3	8666.7	2369.3	4333.3	1191.3	2651.3	584.7	511.7	412.0	5243.3	0.0	2802.1	332.2	183.5	154.8	3920.0	338.0	28685.3	0.0	10929.6	164.3	4275.5	461.3
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	49.9	4694.3	2505.2	5551.9	2164.1	2039.6	630.7	1040.2	546.0	2490.3	0.0	2780.1	546.6	593.3	215.5	1832.7	427.0	16624.4	0.0	16095.9	312.3	8395.8	1060.3
Frequency	0%	0%	0%	0%	0%	0%	0%	0%	0%	7%	93%	80%	47%	43%	100%	100%	73%	60%	87%	0%	87%	53%	40%	53%	93%	47%	100%	0%	78%	33%		

n.d. - not detected

Table 24. Occurrence, average, standard deviation, frequency results and removal for the selected pharmaceuticals in spring (A), summer (B), autumn (C) and winter (D). *(continued)*

(C)	Anxiolytics and hypnotics								Antibiotics						Lipid regulators								Anti-Inflammatories and/or analgesics								Total average	
	Alprazolam ng L^{-1}		Lorazepam ng L^{-1}		Zolpidem ng L^{-1}		Average		Azithromycin ng L^{-1}		Ciprofloxacin ng L^{-1}		Average ng L^{-1}		Bezafibrate ng L^{-1}		Gemfibrozil ng.L^{-1}		Simvastatin ng L^{-1}		Average ng L^{-1}		Diclofenac ng L^{-1}		Ibuprofen ng L^{-1}		Paracetamol ng L^{-1}		Average ng L^{-1}			
	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE
WWTP 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	24800.0	1700.0	12400.0	850.0	750.0	3100.0	120.0	1100.0	7900.0	n.d.	2923.3	1400.0	n.d.	380.0	16100.0	3600.0	67800.0	n.d.	27966.7	1326.7	10679.1	898.2
WWTP 2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	10900.0	620.0	5450.0	310.0	40.0	63.0	110.0	n.d.	7200.0	n.d.	2450.0	21.0	n.d.	100.0	4800.0	n.d.	41500.0	n.d.	15433.3	33.3	5868.2	71.2
WWTP 3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4010.0	840.0	2005.0	420.0	980.0	560.0	150.0	280.0	4500.0	n.d.	1876.7	280.0	n.d.	120.0	9600.0	n.d.	31200.0	n.d.	13600.0	40.0	4585.5	163.6
WWTP 4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1600.0	n.d.	800.0	n.d.	390.0	480.0	920.0	1670.0	7200.0	n.d.	2836.7	716.7	n.d.	620.0	5800.0	n.d.	48100.0	n.d.	17966.7	206.7	5819.1	251.8
WWTP 5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11500.0	6000.0	5750.0	3000.0	1900.0	1800.0	220.0	1150.0	n.d.	n.d.	706.7	983.3	n.d.	n.d.	3500.0	3900.0	46900.0	n.d.	16800.0	1300.0	5820.0	1168.2
WWTP 6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3900.0	4400.0	1950.0	2200.0	900.0	1800.0	540.0	730.0	5100.0	240.0	2180.0	923.3	26.0	76.0	5300.0	850.0	42400.0	n.d.	15908.7	308.7	5287.8	736.0
WWTP 7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	39.0	n.d.	19.5	40.0	4200.0	50.0	210.0	n.d.	2390.0	30.0	2266.7	n.d.	n.d.	n.d.	4200.0	75000.0	14700.0	25000.0	6300.0	6826.4	2339.9
WWTP 8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2500.0	150.0	49.0	n.d.	n.d.	n.d.	849.7	50.0	n.d.	n.d.	n.d.	n.d.	15500.0	n.d.	5166.7	n.d.	1640.8	13.6
WWTP 9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5700.0	200.0	2850.0	100.0	1600.0	130.0	220.0	n.d.	3200.0	n.d.	1673.3	43.3	n.d.	n.d.	5600.0	n.d.	31200.0	n.d.	12266.7	n.d.	4320.0	30.0
WWTP 10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3900.0	3100.0	1950.0	1550.0	610.0	1200.0	410.0	1720.0	6000.0	n.d.	2340.0	973.3	n.d.	n.d.	4800.0	890.0	26000.0	n.d.	10266.7	296.7	3792.7	628.2
WWTP 11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7200.0	n.d.	3600.0	n.d.	800.0	9200.0	114.0	540.0	7600.0	n.d.	2838.0	3246.7	n.d.	3200.0	7000.0	4400.0	41000.0	n.d.	16000.0	2533.3	5792.2	1576.4
WWTP 12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	28800.0	790.0	14400.0	395.0	9000.0	1800.0	200.0	n.d.	4500.0	n.d.	4566.7	600.0	n.d.	210.0	13600.0	760.0	77800.0	n.d.	30466.7	323.3	12172.7	323.6
WWTP 13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	40.0	1300.0	50.0	300.0	n.d.	2800.0	30.0	1466.7	n.d.	n.d.	n.d.	2600.0	68000.0	19700.0	22666.7	7433.3	6190.0	2427.3
WWTP 14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7560.0	7400.0	3780.0	3700.0	610.0	7600.0	50.0	n.d.	6400.0	n.d.	2353.3	2533.3	n.d.	640.0	3600.0	780.0	37800.0	n.d.	13800.0	473.3	5092.7	1492.7
WWTP 15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5400.0	2800.0	2700.0	1400.0	1220.0	190.0	49.0	n.d.	n.d.	n.d.	423.0	63.3	3500.0	220.0	420.0	n.d.	43100.0	n.d.	15673.3	73.3	4880.8	291.8
Average	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7684.7	1859.3	3842.3	929.6	1425.3	2238.2	216.8	513.3	3973.3	362.0	1871.8	1037.8	235.1	371.1	5341.3	1465.3	46220.0	2293.3	17265.5	1376.6	5101.7	869.2
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8284.3	2314.6	7125.3	1914.5	2134.0	2683.0	232.7	598.0	3063.2	881.1	2697.6	1892.4	872.6	784.7	4632.8	1681.7	17735.4	5917.7	23408.6	3707.6	12530.2	2096.3
Frequency	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	80%	73%	40%	37%	100%	100%	100%	60%	67%	20%	89%	60%	13%	60%	80%	60%	100%	13%	64%	40%		

n.d. - not detected

Table 24. Occurrence, average, standard deviation, frequency results and removal for the selected pharmaceuticals in spring (A), summer (B), autumn (C) and winter (D). *(continued)*

(D)	Anxiolytics and hypnotics								Antibiotics						Lipid regulators								Anti-Inflammatories and/or analgesics								Total average	
	Alprazolam ng L^{-1}		Lorazepam ng L^{-1}		Zolpidem ng L^{-1}		Average		Azithromycin ng L^{-1}		Ciprofloxacin ng L^{-1}		Average ng L^{-1}		Bezafibrate ng L^{-1}		Gemfibrozil ng.L^{-1}		Simvastatin ng L^{-1}		Average ng L^{-1}		Diclofenac ng L^{-1}		Ibuprofen ng L^{-1}		Paracetamol ng L^{-1}		Average ng L^{-1}			
	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE	WWI	WWE
WWTP 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	32100.0	2200.0	16050.0	1100.0	8500.0	4000.0	n.d.	n.d.	6900.0	n.d.	5133.3	1333.3	n.d.	n.d.	28900.0	4200.0	70000.0	n.d.	32966.7	1400.0	13309.1	945.5
WWTP 2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5200.0	n.d.	2600.0	n.d.	1200.0	500.0	n.d.	n.d.	5400.0	n.d.	2200.0	166.7	n.d.	n.d.	6900.0	1200.0	58000.0	n.d.	21633.3	400.0	6972.7	154.5
WWTP 3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3600.0	2200.0	1800.0	1100.0	2100.0	1200.0	n.d.	n.d.	3900.0	n.d.	2000.0	400.0	n.d.	n.d.	12400.0	1200.0	42000.0	n.d.	18133.3	400.0	5818.2	418.2
WWTP 4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4200.0	n.d.	2100.0	n.d.	2200.0	1200.0	n.d.	n.d.	3200.0	n.d.	1800.0	400.0	n.d.	n.d.	10400.0	1600.0	60000.0	n.d.	23466.7	533.3	7272.7	254.5
WWTP 5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	23000.0	10000.0	11500.0	5000.0	4200.0	2100.0	n.d.	n.d.	n.d.	n.d.	1400.0	700.0	n.d.	n.d.	8200.0	4500.0	44000.0	n.d.	17400.0	1500.0	7218.2	1509.1
WWTP 6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7100.0	6200.0	3550.0	3100.0	3600.0	2600.0	n.d.	n.d.	6200.0	1500.0	3266.7	1366.7	n.d.	n.d.	8200.0	2100.0	65000.0	n.d.	24400.0	700.0	8190.9	1127.3
WWTP 7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	16400.0	8200.0	n.d.	n.d.	n.d.	n.d.	5466.7	2733.3	n.d.	n.d.	n.d.	n.d.	100000.0	24000.0	33333.3	8000.0	10581.8	2927.3
WWTP 8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4500.0	900.0	2250.0	450.0	2200.0	1100.0	n.d.	n.d.	n.d.	n.d.	733.3	366.7	n.d.	n.d.	n.d.	n.d.	25000.0	n.d.	8333.3	n.d.	2881.8	181.8
WWTP 9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4200.0	n.d.	2100.0	n.d.	2500.0	1200.0	n.d.	n.d.	4400.0	n.d.	2300.0	400.0	n.d.	n.d.	10900.0	900.0	50000.0	n.d.	20300.0	300.0	6545.5	190.9
WWTP 10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8900.0	4200.0	4450.0	2100.0	4200.0	2100.0	n.d.	n.d.	6800.0	n.d.	3666.7	700.0	n.d.	n.d.	9200.0	1100.0	50000.0	n.d.	19733.3	366.7	7190.9	672.7
WWTP 11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3200.0	n.d.	1600.0	n.d.	40200.0	20400.0	n.d.	n.d.	8900.0	n.d.	16366.7	6800.0	n.d.	n.d.	16800.0	6200.0	65000.0	n.d.	27266.7	2066.7	12190.9	2418.2
WWTP 12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	26400.0	1500.0	13200.0	750.0	14200.0	3200.0	n.d.	n.d.	4100.0	n.d.	6100.0	1066.7	n.d.	n.d.	25200.0	2500.0	109000.0	n.d.	44733.3	833.3	16263.6	654.5
WWTP 13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5200.0	2600.0	n.d.	n.d.	n.d.	n.d.	1733.3	866.7	n.d.	n.d.	n.d.	n.d.	150000.0	32000.0	50000.0	10666.7	14109.1	3145.5
WWTP 14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	16200.0	10200.0	8100.0	5100.0	18200.0	12400.0	n.d.	n.d.	5200.0	n.d.	7800.0	4133.3	n.d.	n.d.	7200.0	1200.0	42000.0	n.d.	16400.0	400.0	8072.7	2163.6
WWTP 15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	15200.0	4200.0	7600.0	2100.0	2400.0	1200.0	n.d.	n.d.	n.d.	n.d.	800.0	400.0	6200.0	500.0	2500.0	900.0	60000.0	n.d.	22900.0	466.7	7845.5	618.2
Average	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10253.3	2773.3	5126.7	1386.7	8486.7	4266.7	0.0	0.0	3666.7	100.0	4051.1	1455.6	413.3	33.3	9786.7	1840.0	66000.0	3733.3	25400.0	1868.9	7797.5	1228.7
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9689.4	3416.9	8703.5	2833.4	10041.9	5290.8	0.0	0.0	2917.2	374.2	7044.9	3692.3	1546.6	124.7	8251.7	1749.6	30598.5	9629.6	34656.9	5915.7	18485.0	3435.9
Frequency	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	87%	60%	43%	30%	100%	100%	0%	0%	67%	7%	56%	36%	7%	7%	80%	80%	100%	13%	62%	29%		

n.d. - not detected

Chapter V – A critical evaluation of different parameters for estimating pharmaceutical exposure seeking an improved environmental risk assessment

Since 2006, the guideline of the European Medicines Agency (EMA) on environmental risk assessment (ERA) for human medicinal products, with procedures to evaluate the ERA for new marketing authorizations, came into force. This publication, based on the previous work performed on the occurrence of five therapeutic groups in wastewaters, critically evaluates the procedures on EMA guideline, especially the calculation of the predicted environmental concentrations (PECs) and respective risk quotient, suggesting improvements to the referred guideline.

Some parts of this publications were included in chapter one since they mainly focused the theoretical background.

The work presented and discussed in this chapter resulted in the following publication:
PEREIRA A.M.P.T., SILVA L.J.G., LINO C.M., MEISEL L.M., PENA A.. A critical evaluation of different parameters for estimating pharmaceutical exposure seeking an improved environmental risk assessment. Submitted to *Chemosphere*.

V1. Abstract

A critical evaluation of the European Medicines Agency (EMA) Guideline on Environmental Risk Assessment (ERA) was performed on 16 of Portugal's most consumed pharmaceuticals in wastewater effluents (WWEs), the main route for aquatic contamination. The predicted environmental concentrations (PECs) were formulated based on the Guideline, after incorporating several refinements. The best approach was selected by comparing the measured environmental concentrations (MECs) to the PECs in WWEs. Finally, risk was assessed by comparing PECs to predicted no-effect concentrations (PNECs).

The results showed that the default value of the penetration factor (Fpen) used by the EMA (0.01) was surpassed and that national consumption and excretion data were the two most important parameters for PEC calculations. The risk quotient between PECs and PNECs was higher than 1 for 12 pharmaceuticals, indicating a risk to all three trophic levels of aquatic organisms (algae, daphnids and fish).

To improve the current ERA framework, suggestions were made for incorporating consumption and excretion data, changing the default value of Fpen to 0.04 and adding a safety factor of 10. Moreover, this evaluation should be performed for pharmaceuticals already on the market, and future ERAs should incorporate a risk-benefit analysis, an important risk-management step.

Keywords

Environmental contaminants, pharmaceuticals, environmental risk assessment, predicted environmental concentrations, measured environmental concentrations, wastewater treatment plant effluent.

V2. Introduction

The presence of human pharmaceuticals in the environment has raised concerns worldwide. Due to their increased consumption and their pharmacokinetic properties, pharmaceuticals can be excreted in their parent form or as metabolites and enter into aquatic systems mainly through wastewater treatment plant (WWTP) effluents. Due to their physicochemical and biological properties, as well as their low removal efficiencies in WWTPs, several hundred types of pharmaceuticals have been found in sewage water, surface water, groundwater and tap water in concentrations from sub-ng L^{-1} to more than μg L^{-1}, which has led to concerns about their potential to affect non-target species [38,123,349–351].

Despite this awareness, legal limits have not yet been set for pharmaceuticals in surface water, although a "watch list" that includes 7 pharmaceuticals has been created recently [39,189,190]. The Guideline on the environmental risk assessment (ERA) of medicinal products for human use, previously discussed, and the predicted environmental concentrations (PEC) calculation, in particular, have been debated by scholars, some of whom argue that other parameters should also be incorporated, such as consumption data and excretion rates [349–352].

The aim of the present work was to introduce, rationalize and discuss a general tiered approach for estimating the PECs based on the European Medicines Agency (EMA) Guideline, taking into account the Portuguese scenario for 16 of the most consumed pharmaceuticals [373]. We also aimed to critically evaluate uncertainties in PEC calculations, compare the measured environmental concentrations (MECs) with the appropriate PECs, adopt the best-suited model, assess which parameters included in the model are more crucial and suggest solutions to strengthen the European Union (EU) legislation to improve the environmental exposure estimations.

V3. Assessing the predicted environmental concentrations (PECs) of pharmaceuticals in wastewater effluents (WWEs) using different formulas

In the scope of the present manuscript, 16 pharmaceuticals, namely, alprazolam (ALP), lorazepam (LOR) and zolpidem (ZOL) (anxiolytics and hypnotics), azithromycin (AZI) and ciprofloxacin (CIP) (antibiotics), simvastatin (SIM), bezafibrate (BEZ) and gemfibrozil (GEM)

(lipid regulators), citalopram (CIT), escitalopram (ESC), fluoxetine (FLU), paroxetine (PAR) and sertraline (SER) (selective serotonin reuptake inhibitors (SSRIs)), and ibuprofen (IBU), diclofenac (DIC) and paracetamol (PARA) (non-steroidal anti-inflammatories and analgesics) (Table 26, Supporting information) were selected for the assessments of the environmental exposure based on data regarding their national consumption rates [373]. These consumption data were supported by two extensive Portuguese studies [116,131]. To perform this evaluation, the PECs were assessed in WWEs, by considering several different approaches, because, according to the Guideline, the PECs for surface water are derived from the PECs in WWE after considering a dilution factor of 10 [353]. The first approach used to calculate the PECs for human pharmaceuticals was that advocated by the EMA Guideline for the ERA [353], which derives the initial crude wastewater PEC for pharmaceuticals using a simple formula that multiplies the maximum daily dose (DOSEai) (mg day^{-1}) with a default penetration factor (Fpen) and dividing by the amount of wastewater per inhabitant per day (WASTEWinhab) (L inh^{-1} d^{-1}) (Equation 4) [57,350,353]. This estimation of exposure uses certain default values: a Fpen of 0.01; the DOSEai, obtained from the Summaries of Product Characteristics; and the WASTEWinhab of 200 L inh^{-1} d^{-1}, not factoring in any human metabolism or removal by the WWTPs [350].

Equation 4. EMA guideline for PEC calculation.

$$PEC = \frac{DOSEai * Fpen}{WASTEWinhab}$$

Our second approach replaced the DOSEai and the Fpen with data regarding the Portuguese consumption (PortCons) of the selected pharmaceuticals (2013) divided by the Portuguese population (PortPop) (2013) (Equation 5).

Equation 5. PECs calculation adding national consumption.

$$PEC = \frac{PortCons}{WASTEWinhab * PortPop}$$

As pharmaceuticals are metabolized in the human body, the third equation considered the percentage of excretion of the parent compound (or conjugates) (Fexcreta). This equation has previously been used [35] to develop a prioritization approach for antibiotics (Equation 6).

Equation 6. PECs calculation adding human excretion.

$$PEC = \frac{Fexcreta * PortCons}{WASTEWinhab * PortPop}$$

In the fourth equation, besides the human excretion rates, another refinement was made by incorporating WWTPs removal efficiencies (WASTWremo) [57,58] (Equation 7).

Equation 7. PECs calculation adding removal efficiencies.

$$PEC = \frac{Fexcreta * PortCons * WASTEWremo}{WASTEWinhab * PortPop}$$

In the final refinement, using the WASTEWinhab data from the Portuguese population, the default value of 200 L inh^{-1} d^{-1} was replaced by the true volume of wastewater produced by the Portuguese population (PORTWASTEWinhab) [116,131,408,409] (Equation 8).

Equation 8. PECs calculation adding the volume of wastewater produced by the Portuguese population.

$$PEC = \frac{Fexcreta * PortCons * WASTEWremo}{PORTWASTEWinhab * PortPop}$$

To quantify the uncertainty in these calculations, two PEC values were obtained for Equations 6, 7 and 8. We took the highest excretion and lowest removal efficiency for each pharmaceutical as a worst-case scenario and also considered the average values found in the literature to predict the concentrations in WWEs for these two settings [354].

V3.1. Pharmaceuticals consumption

The presence of pharmaceuticals in the environment generally correlates well with the amount used in human medicine. Therefore, these data can be used to identify pharmaceuticals that may pose a risk to the environment [35]. An accurate estimate of the extent of drug exposure in a population is difficult in most countries, as precise consumption data are often lacking.

Considering consumption values in calculating PECs, the results can be misleading because 100% compliance with the therapeutic and a correct disposal is assumed. However, it is known that the compliance for cardiovascular pharmaceuticals is 71% [40], for example.

As previously discussed, the EMA Guideline for ERA in Phase I assumes a market penetration factor of 0.01 (95th percentile of 800 pharmaceuticals evaluated in Germany in 2001) as the worst-case scenario, which translates to 1% of the population consumes the defined daily dose (DDD). Concerning the selected pharmaceuticals and observing the consumption data in Figure 3, 9 of the 16 pharmaceuticals had penetration factors over 0.01 and up to 0.0394 (SIM), which was expected since they are the most consumed in Portugal, with a total average of 0.0135 (Table 27, Supporting information). Accordingly, as reported in Norway [36], three pharmaceuticals (SIM, ALP and PARA) exceeded this default penetration factor value with values of 0.03, 0.022 and 0.014, respectively. In this way, the first evaluation of the EMA Guideline might underestimate the PECs; therefore, the penetration factor should be reviewed. As there are no consumption data regarding newly authorized active substances, the highest penetration factor registered in the EU (0.04) should be used as a default value instead of the reference value of 0.01, disallowing false negatives. However, as these data can differ over time, risk assessments for pharmaceuticals that are already available on the market should also be reassessed. This could be performed with real consumption data every five years and after new therapeutic indications or spikes in consumption; this method would foster a more accurate and up-to-date ERA.

V3.2. Excretion rates

To determine the excretion rate, the proportion of the unchanged active molecule excreted in urine and/or in faeces and the proportion of the parent molecule excreted as conjugates (glucuronide and sulphate) was included, which assumes that the conjugates are cleaved in WWTPs and in the environment into the parent compound [57,58].

This pharmacokinetic feature, in addition to the consumption data, contributes to either a greater or lesser environmental impact and is related to the reported occurrence of the parent compound and its metabolites in the aquatic compartment [35]. Therefore, the excretion features were revised and are presented in Figure 26 and Table 28 (Supporting information).

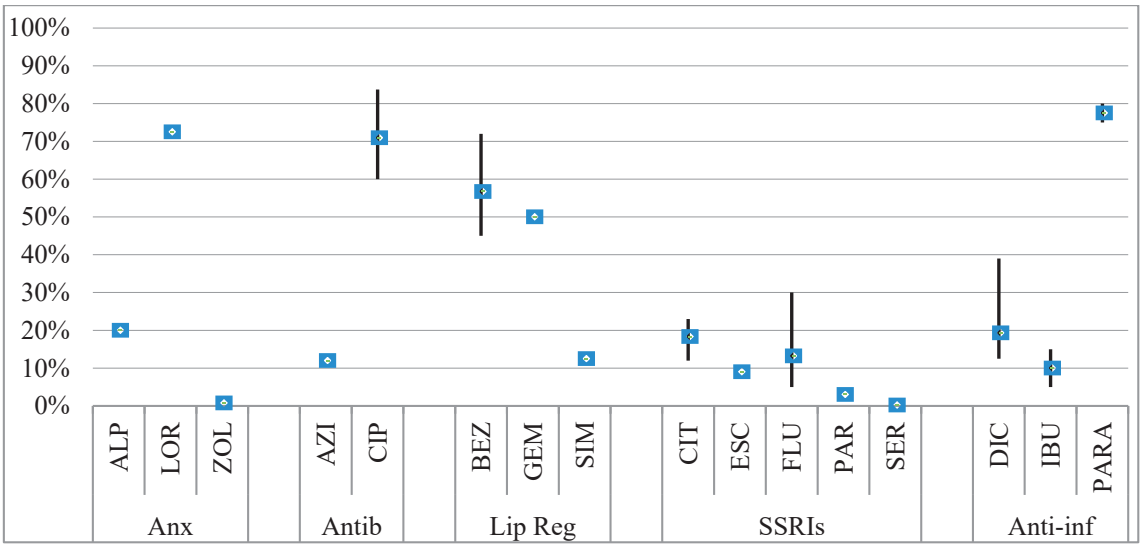


Figure 26. Minimum, maximum and average excretion rates (%).
References available in Table 28.(Anx - anxiolytics and hypnotics; Antib - antibiotics; Lip reg - Lipid regulators; Anti-inf - anti-inflammatories and analgesics).

While several publications are available on the metabolism of pharmaceuticals, the results of these studies can vary. The observed differences are probably explained by genomically distinct metabolizing capacities, as well as differences in race, sex, age and health status of the studied subjects, which are all known to affect the route and rate of metabolism [18]. Although it is suggested that metabolites with excretion rates superior to 10% should also be assessed, it is not necessary to perform toxicity tests, which would not clarify whether environmentally relevant concentrations can affect both aquatic and terrestrial environments [357].

SSRIs were clearly the therapeutic group with lower excretion rates, ranging from 0.2 to 30%, whereas the other groups presented higher variability. The compounds with higher excretion rates were CIP (84%), PARA (80%), LOR (73%), BEZ (72%) and GEM (50%).

Therefore, discrepancies involving metabolism and excretion can also bias the PECs, resulting in differences in the observed concentrations of pharmaceuticals in WWEs.

V3.3. Removal efficiencies of wastewater treatment plants (WWTPs)

For further PEC refinement, the recorded removal efficiencies for full-scale working WWTPs were collated from several published works (Figure 27 and Table 29, Supporting information). These removal efficiencies were collected from different types of WWTPs, encompassing

distinct regions, population equivalents, types of wastewater, average loads and type of treatments and processes.

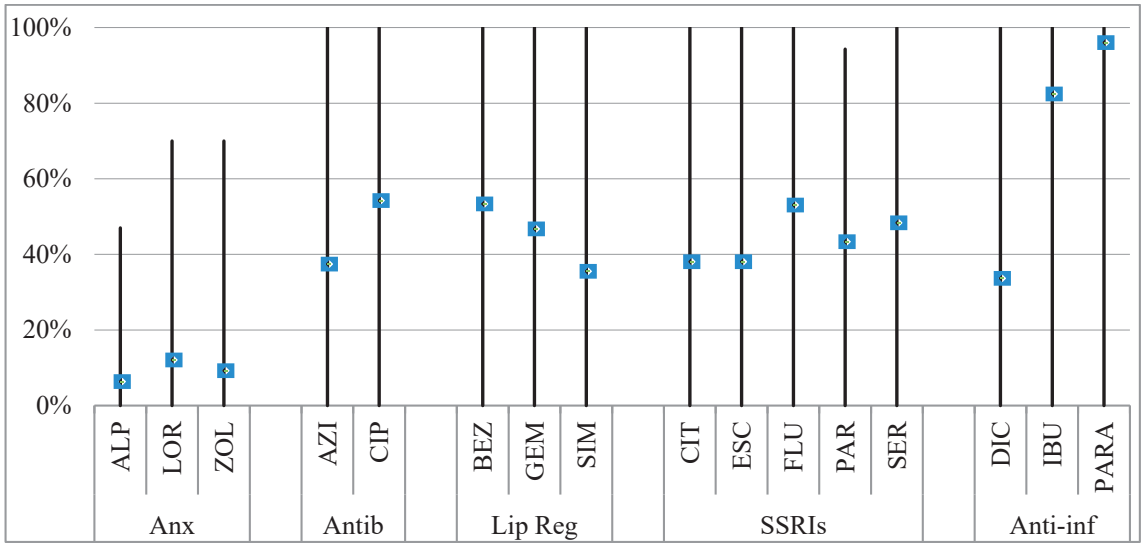


Figure 27. Minimum, maximum and average removal efficiencies in WWTPs (%).
References available in Table 29. (Anx - anxiolytics and hypnotics; Antib - antibiotics; Lip reg - Lipid regulators; Anti-inf - anti-inflammatories and analgesics).

Concerning the average removal rates, the therapeutic anxiolytics group had lower average removal values (ranging from 6 to 12%) than the other pharmaceuticals. From the selected pharmaceuticals, only 31% had removal efficiencies over 50%. Nonetheless, assuming the lowest values found in the literature, all the selected pharmaceuticals did not present any removal in WWTPs. The variations observed in the reported removal efficiencies can be explained by differences in location, differences in the served population, sampling methods (grab or composite), sampling seasons, WWTP capacity, types of treatments and treatment configurations, operating parameters, hydraulic retention times and solid retention times, which shows the inherent variability associated with these processes. Some metabolites may also be re-converted back to the parent compound during wastewater treatment [18,57].

Although pharmaceutical concentrations in sludge or suspended solids were neither considered nor measured, it is notable that good removal rates obtained in the aqueous phase do not imply degradation to the same extent [96,402]. In fact, the Guideline states that for compounds with K_{oc} values greater than 10.000 L Kg^{-1}, an environmental risk assessment should also be conducted for the terrestrial compartment [353]. Moreover, the conversion of a given pharmaceutical to transformation products other than those analysed might lead to lower

pharmaceutical levels in WWE samples and to an apparent removal. In addition, some metabolites and transformation products can be more toxic than the parent compound; therefore, those over 10% of the mass balance should be identified through aerobic and anaerobic transformation in aquatic sediment systems (OECD 308). However, as with the human metabolites, no toxicity tests are requested under the ERA Guideline [115,357].

V3.4. Volume of wastewater produced by the Portuguese population

The EMA Guideline [353] provides a default value for the amount of wastewater produced by each person (200 L); however, this value is not specific to any country. This default value is higher than the actual value for the Portuguese population, where 133 L of wastewater per day are produced by each inhabitant [116,131]. This value was obtained during a one-year follow-up study with four sampling periods [116,131] by using the average amount of water from 15 WWTPs designed to treat domestic, hospital and industrial wastewaters, operating with secondary or tertiary treatments, and located in 5 Portuguese regions.

Although only a few publications addressed this issue, we found that the Portuguese values are lower than other countries, such as Iraq, Iran and Canada, in which volumes of 156, 186 and 500 L inh^{-1} d^{-1} were reported, respectively [375,409,410].

This difference can, once again, underestimate the PECs, increasing the probability of false negatives when performing ERA of pharmaceuticals.

V3.5. Predicted environmental concentrations (PECs) calculation

Given the scope of the present paper, different PECs in WWE were calculated based on the 5 equations discussed above (Table 25). The issue on possible refinements to the PECs are contentious; nonetheless, it is simpler and safer to apply the worst-case scenario approach as used in MEC calculations. However, when available, data regarding excretion and removal rates averages were also included.

When using Equation 4, all pharmaceuticals, with the exception of ALP, have PECs greater than 0.1 μg L^{-1} (Table 25); due to the dilution factor, they would have PECs in the surface water over 0.01 μg L^{-1} and would enter Phase II of the ERA.

Adding national consumption (Equation 5) to the first formula, the predicted concentration is reduced for the majority of the pharmaceuticals, with the only exceptions being PARA, SIM

and ALP, resulting in possible false negatives. In the case of ALP, its predicted concentration in water surpasses 0.1 μg L^{-1} and for this reason alone, would move to the Phase II assessment. As none of the selected pharmaceuticals truly have 100% excretion rates of the parent compound or conjugates, the values for the third formula (Equation 6) are lower than the previous values. As observed for DIC and FLU, some variation can occur between the values obtained using the average or the worst-case scenario. For these compounds, the PECs double when the higher excretion values were used.

Table 25. Average predicted and measured environmental concentrations (worst-case scenario) in Portuguese WWEs (ng L^{-1}).

Therapeutic group	Pharmaceutical	PECs					MECs				
		Equation 4	Equation 5	Equation 6	Equation 7	Equation 8	[115,116,131][a]	[20][b]	[23][a]	[21][c]	[22][b]
Anx	ALP	75.0	167.3	33.5 (33.5)	31.3 (33.5)	47.1 (50.4)	n.d.	n.d.	27.5 (33.5)	(33.0)	-
	LOR	500.0	297.2	215.5 (215.5)	195.7 (215.5)	294.3 (324.1)	41.9 (399.8)	(438)	294 (347)	-	-
	ZOL	500.0	398.7	3.0 (3.0)	2.7 (3.0)	4.1 (4.5)	n.d.	n.d.	-	(0.8)	-
Antib	AZI	50 000.0	2304.3	276.5 (276.5)	173.1 (276.5)	260.3 (415.8)	3.3 (200.0)	(836)	171 (297)		-
	CIP	75 000.0	6319.6	4482.2 (5289.5)	2052.9 (5289.5)	3087.1 (7954.1)	1770.5 (10 200.0)	n.d.	369 (1396)	(119.0)	-
Lip Reg	BEZ	30 000.0	1363.2	772.9 (981.5)	361.0 (981.5)	541.4 (1475.9)	1777.3 (20 400.0)	(1797)	409 (635)	(103.0)	-
	GEM	60 000.0	1829.6	914.8 (914.8)	487.6 (914.8)	733.2 (1375.6)	247.0 (1720.0)	(1183)	n.d.	(975.9)	-
	SIM	2000.0	5916.2	739.5 (739.5)	477.0 (739.5)	717.3 (1112.0)	48.7 (1500.0)	(1255)		-	-
SSRIs	CIT	3000.0	159.1	47.5 (59.6)	36.9 (59.6)	55.5 (89.6)	7.5 (95.6)	-	34.0 (49.1)	(47.8)	-
	ESC	1000.0	653.3	58.8 (58.8)	36.5 (58.8)	54.9 (88.4)	-	-	-	-	-
	FLU	3000.0	1494.2	197.2 (448.3)	73.7 (448.3)	110.8 (674.1)	n.d.	n.d.	n.d.	(21.5)	n.d.
	PAR	3000.0	755.9	22.7 (22.7)	12.9 (22.7)	19.4 (34.1)	1.4 (81.1)	(240)	n.d.	n.d.	(3367)
	SER	10 000.0	4572.0	9.1 (9.1)	4.7 (9.1)	7.1 (13.7)	n.d.	-	n.d.	n.d.	-
Anti-inf	DIC	7500.0	7269.2	1403.0 (2835.0)	931.6 (2835.0)	1400.9 (4263.2)	146.0 (3200.0)	(1429)	42.9 (83.1)	(102.5)	(1612)
	IBU	120 000.0	108 767.0	10 876.7 (16 315.1)	1914.3 (16 315.1)	2878.6 (24 534.0)	950.3 (6200.0)	(1527)	119 (639)	(1889.0)	(43 653)
	PARA	200 000.0	278 611.3	215 923.8 (222 889.1)	8637.0 (222 889.1)	12 988.0 (130 381.7)	1520.1 (32 000.0)	n.d.	96.1 (106)	-	-

n.d. – not detected
[a] –Time proportional composite samples (24 h)
[b] – Grab samples
[c] – Type of sampling not specified

When the removal efficiencies for the WWTPs were introduced, no changes were observed in the results for the worst-case scenario (Equation 7), which is explained by the fact that all the selected compounds had 0% removal efficiency in at least one of the reviewed publications. When using average removal efficiencies CIP, BEZ, FLU, DIC, IBU and PARA had their PEC values considerably reduced.

For the last PEC refinement, the wastewater produced by each Portuguese inhabitant per day was used. Since lower volumes (133 L inh^{-1} d^{-1}) were produced than the default value (200 L inh^{-1} d^{-1}), PECs were increased approximately 50% over the last equation.

Comparing the fourth and the eighth equations, the PECs calculated from the EMA Phase I approach were always higher, indicating that the fourth formula complied with the precautionary principle. Subsequent refinements led to significant reductions in the predicted levels due to modifications of the parent compound within the human body and/or the WWTP processes. Nonetheless, using Equation 8, the concentrations for LOR, AZI, CIP, BEZ, GEM, SIM, FLU, DIC, IBU and PARA still exceeded the 0.1 μg L^{-1} concentration threshold and would therefore trigger further investigation.

V4. Measured environmental concentrations (MECs) compared to predicted environmental concentrations (PECs)

V4.1. Measured environmental concentrations (MECs)

To evaluate the performance of the different approaches to calculating PECs in WWEs, the obtained values were then compared with MECs in WWEs reported in six Portuguese studies, where the occurrence of the selected pharmaceuticals was assessed in 20 Portuguese WWTPs (81 samples). Three of these studies used 24-h time proportional composite samples [23,115,131], two used grab samples [20,22] and one study did not specify the type of sampling [21]. These studies, which were conducted between 2009 and 2013, focused on different therapeutic pharmaceutical groups in different WWEs, which varied in terms of the population served and in the type of wastewater treatment technologies employed. These studies not only represent a snapshot of WWE contamination by pharmaceuticals but a complete overview of the Portuguese context.

The variability in these results emerged as a result of the heterogeneous populations served, differences in removal efficiencies and possible changes in consumption patterns. However,

Portuguese contamination levels agree with those found in several other WWEs reported worldwide [89,90,98,102,113,117,122,185]. Notably, because the liquid chromatography methodologies used are unable to separate enantiomers, the observed CIT concentrations corresponded to the sum of both CIT and ESC. Nevertheless, even the MECs have a certain degree of uncertainty, mainly due to sampling procedures. For example, for compounds detected in very low concentrations or with concentrations that exhibit great fluctuations, the sampling mode and frequency can induce uncertainties over 30%. Additionally, the chemical analysis procedure can contribute to a degree of uncertainty from 2 to 15%, evaluated by the validation procedures [39,411]. Therefore, the maximum individual concentrations of pharmaceuticals found in the different WWEs were used as MECs to set a worst-case scenario approach [18,123].

V4.2. Ratio between measured environmental concentration (MECs) and predicted environmental concentrations (PECs)

One approach to overcome problems with the parameter selection process for PEC calculation was to use monitoring data alongside of inverse modelling to derive the model input parameters. A comparison of MECs and the crude and refined PECs of the investigated compounds on WWEs was performed using the ratio MEC/PEC, to establish whether the predicted equations used tend to underestimate or overestimate the measured values [39]. As mentioned previously, crude PECs were obtained, supported by the EMA Phase I, and these PECs were further refined using worst-case scenario assumptions [408]. Finally, these values were then compared with the highest concentrations measured in Portugal, as reported in the scientific literature.

The ratio between the MECs and PECs for different pharmaceuticals are presented in Figure 28. The values for Equation 4 show that all the PECs were higher than the MECs, and the average standard deviation was highest when comparing all five different equations (Table 30, Supporting information). With regard to the PEC results obtained with Equation 5, there was a slight improvement in the average standard deviation, with only three (21%) pharmaceuticals (ZOL, BEZ and FLU) exceeding a factor of 10, which illustrates the potential of using sales data to predict concentrations in the aquatic environment [40,412].

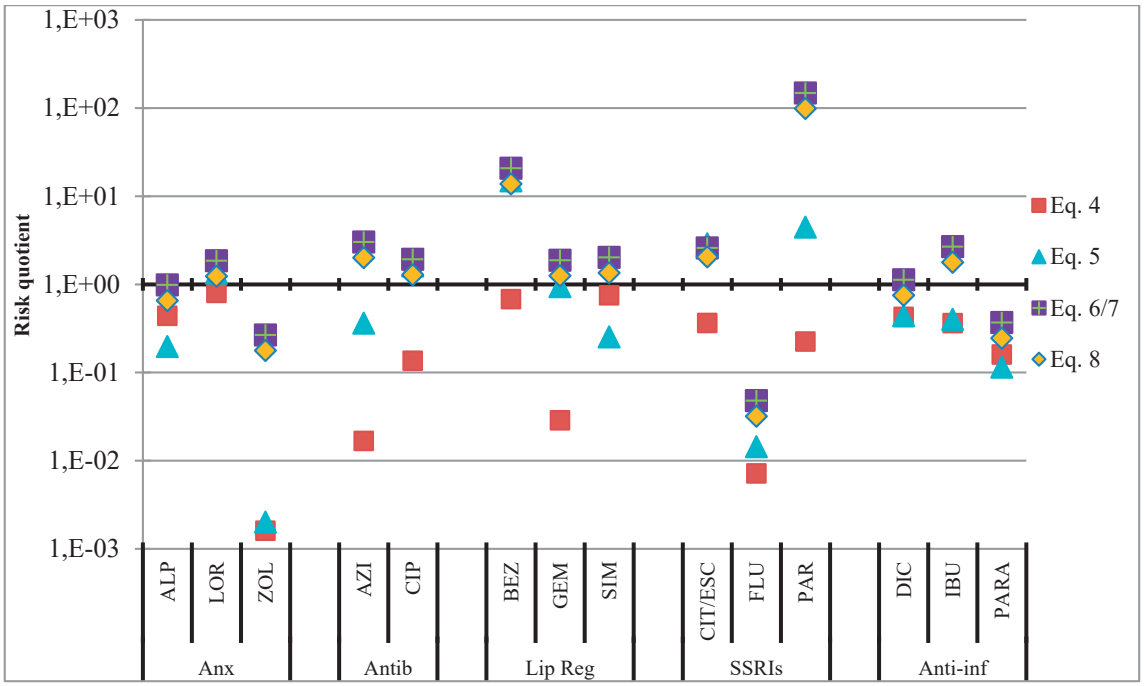


Figure 28. The ratio between MECs and PECs in WWEs (worst-case scenario).
(Anx - anxiolytics and hypnotics; Antib - antibiotics; Lip reg - Lipid regulators; Anti-inf - anti-inflammatories and analgesics).

The values calculated using Equation 6 were in close agreement with the MECs for 6 (43%) of the 14 pharmaceuticals with factors lower than 2 (0.5<MEC/PEC<2) [39]; however, the predictions for BEZ, FLU and PAR were not as accurate with factors greater than 10. However, this equation presented the lowest standard deviation average, showing the usefulness of including the excretion data of pharmaceuticals in PEC calculations. Because the worst-case scenario approach was used, no variations were obtained with Equation 7 after adding the WWTP removal efficiency as a variable. When the volume of wastewater produced by the Portuguese population was introduced as a variable in Equation 8, there was a 50% increase in the PECs for the selected pharmaceuticals; however, there was a higher standard deviation average.

The PECs should always err on the side of caution and produce false positives that lead to further investigation rather than false negatives that might leave a potential risk unexplored [352]. Only BEZ and PAR had MEC/PEC ratios higher than 10 using Equation 6. With regard to BEZ, these discrepancies were already observed by another author and are related to its high persistence [39]. As for PAR, this result is a consequence of a very high concentration detected by one of the published works [22], which is much different from the ratio reported by the other authors [20,21,23,131]. These results suggest that a safety factor of 10 should be implemented

in Equation 6 to prevent false negatives from occurring. This safety factor would offset the deviations originated by the lower WASTEWinhab values observed, by incorrect disposal of unused pharmaceuticals, by consumption patterns variations and by possibly transforming the parent compound to active metabolites and transformation products that can be toxic to the environment.

Nonetheless, on the basis of this sensitive analysis, considering all the different factors, it is perhaps unsurprising that the selection of the input parameters for exposure modelling for pharmaceuticals is challenging and that, while some exposure modelling of this type has been successful for some contaminants, predictions do not always agree with observed measurements in the field [18]. Nevertheless, it might be said that the most influential parameters in predictive models of pharmaceutical concentrations in WWEs are national consumption (when available) and excretion rates data that should be used in PEC refinements when performing the ERA. The refinement calculations, including the removal efficiencies, did not improve the obtained results. Thus, a very simple mass balance (Equation 6) can predict WWE concentrations with relative accuracy, despite all the uncertainties. Therefore, this approach might be useful when no monitoring data are available, thus improving the selection of relevant pharmaceuticals for monitoring programmes in each country and supporting the accuracy of theoretical models to predict concentrations of many pharmaceuticals [18,25,39,40,354].

These models offer valuable insight for the prioritization of pharmaceuticals by highlighting their potential to enter the aquatic environment. When a drug has yet to be released onto the market, it is not possible to make environmental observations; therefore, a prediction is the only way to assess the potential risks presented by that drug [352].

V5. Risk calculation PECs/PNECs

The risk assessment for the aquatic compartment was also based on the EMA Guideline on the Environmental Risk Assessment of Medicinal Products for Human Use [353]. Following this Guideline, the risk quotients (RQs) associated with the selected pharmaceuticals were calculated by the ratio of PEC and predicted no-effect concentrations (PNEC), which is the traditional approach to an environmental risk assessment.

V5.1. Predicted no-effect concentrations (PNECs) estimation

The calculation of PNECs on non-target organisms was performed using an uncertainty factor (UF) of 10 to validate the chronic no-observed-effect-concentration (NOEC) values. Additionally, UFs of 50 and 1000 were applied to the values available in the literature for the acute lowest-observed-effect-concentration (LOEC) and lethal (effective) concentration for 50% of the population (L(E)C50), respectively. The UF is an expression of the degree of uncertainty in the extrapolation from the test data to the actual environment on a limited number of species [353]. When no experimental data were available, L(E)C50 values were estimated using ECOSAR 1.11. If the RQ is equal to or above 1, there is a potential environmental risk situation, whereas when values are less than 1, no risk is expected [115,350,354].

V5.2. Risk assessment

The majority of prioritization lists of pharmaceuticals are based on the ERA concept, which takes into account the potential effect of a given pharmaceutical and its PEC in surface water [202]. For this determination, RQs might be a useful tool, as has been found previously [202,413,414]. RQs were calculated by dividing the PECs in WWEs that were obtained from Equation 6 and including the proposed safety factor (10), by the PNEC values, considering the above mentioned UFs. It should be taken into account that the choice of data obviously affects the outcome. The PNEC value (together with the UFs used) [115,131,415] and RQ calculated for each analyte are shown in Figure 29. As discussed above, because CIT and ESC are enantiomers, the PECs of both SSRIs were added and compared with the sum of the PNECs.

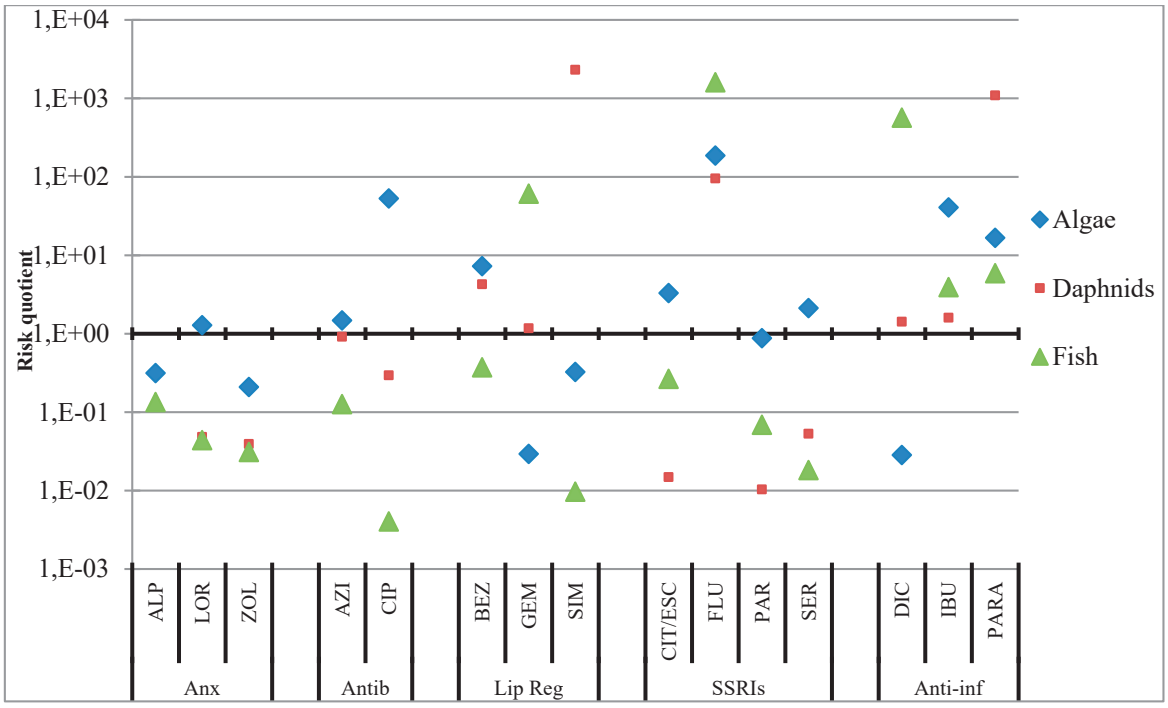


Figure 29. The risk quotients for pharmaceuticals, calculated as the ratio between PECs in WWEs and PNECs.
(Anx - anxiolytics and hypnotics; Antib - antibiotics; Lip reg - Lipid regulators; Anti-inf - anti-inflammatories and analgesics).

When the PEC/PNEC ratio exceeds 1, this compound poses an unacceptable risk to the aquatic population, triggering further investigation [18,352]. According to the results presented in Figure 29, 12 pharmaceuticals (LOR, AZI, CIP, BEZ, GEM, SIM, CIT/ESC, FLU, SER, DIC, IBU and PARA) presented RQs greater than 1 for species in at least one trophic level. RQ values up to 2311 were found for SIM, and anxiolytics had a lower environmental risk than the other pharmaceuticals tested. In addition, a certain risk might be expected for the remaining pharmaceuticals, ALP, ZOL and PAR, with RQs between 0.1 and 1, determined for exposure to algae. Moreover, even when using the EMA Guideline default value for dilution (10) to obtain the PEC in surface water, the possible environmental hazard is not mitigated when the RQ is greater than 10 (CIP, GEM, SIM, FLU, DIC IBU and PARA) [353]. It is also notable that the threshold advocated by the EMA Guideline (10 ng L^{-1} for surface water) to enter into Phase II of ERA is a low value for most of the selected pharmaceuticals. Nevertheless, SIM and FLU have PNECs lower than this value, meaning that at concentrations of less than 10 ng L^{-1}, some compounds can negatively impact the aquatic environment.

To date, scarce information is available on the individual ecotoxicity of these compounds. Their exposure effects during multiple life stages or even multiple generations of aquatic organisms

are lacking. However, it is notable that, given their environmental presence in mixtures and given their similar pharmacological mechanisms, additive or even synergistic effects may occur; therefore, the real hazard may be greater than that calculated [17,40,416]. Additionally, because the emergence of bacterial resistance is a major concern involving the presence of pharmaceuticals in the aquatic environment, the evaluation of the risk for developing antibiotic resistance should be implemented [349,417].

Nonetheless, this evaluation should include the risk-benefit analysis for the authorization or re-evaluation of human pharmaceuticals as is considered for the approval of marketing authorizations for veterinarian medicines. Additionally, this information should be made publicly available.

V6. Conclusions

Several different factors were considered for the development of an equation that best predicts real WWE concentrations. Concerning the consumption data, 9 out of the 16 pharmaceuticals had penetration rates higher than the default value suggested by EMA (0.01) and up to 0.04, enabling false negative results. The selected pharmaceuticals have a wide range (0.2 to 84%) on what regards excretion rates, being SSRIs the therapeutic group with lower values. Regarding the removal efficiencies of WWTPs and, using the worst-case scenario results, all of the selected pharmaceuticals did not present any removal. Therefore, from the five equations assessed, both 6 and 7 gave the best results, showing concentrations closer to the MECs. Since Equation 6 has fewer refinements (national consumption and excretion data) we suggest that these parameters, using worst-case scenario, should be taken into account when performing the evaluation of PECs for the ERA.

Observing these results for pharmaceuticals already in the market, we can suggest improvements for the calculation of PECs for the new active substances approvals. Viewing the precautionary principle, the default value of Fpen, should be updated regularly to comply with the worst scenario approach, and for now, it should be 0.04. Also a safety factor of 10 should also be added to Equation 6, ensuring that no false negatives can arise from this evaluation.

Additionally each five years, after new therapeutic indications or increased consumption the ERA should be carefully reviewed. In this assessment, the real consumption date can be used, replacing the Fpen default value. Since this information will significantly differ between

countries, ERA must be performed for each. The Fexcreta and PNECs can be also updated if there is new relevant information. Moreover, toxicity evaluation ought to be performed for metabolites or transformation products above 10% and ERA should incorporate the risk-benefit analysis.

Comparing our PECs with PNECs, a RQ higher than 1 was found for 12 of the 15 pharmaceuticals and was found up to 2311. Moreover, even when using the dilution factor, obtaining the PECs in surface water, 7 still have RQs higher than 1.

Theoretical models can provide valuable PECs; however, we believe that the available models would benefit from the careful consideration of our recommendations, to strengthen the protection of the environment from pharmaceutical contamination.

V7. Supporting information

Table 26. Physicochemical properties of the selected pharmaceuticals (adapted from Silva et al. [3] and ECOSARv1.11).

		Cas number	MW	pKa	Log K_{ow}	Solubility (mg L^{-1})
Anx	ALP	28981-97-7	308.77	5.1/18.3	3.87	13.1
	LOR	846-49-1	321.16	13.0	2.41	80.0
	ZOL	82626-48-0	307.40	6.2	3.85	0.9
Antib	AZI	83905-01-5	749.00	8.7	3.24	2.4
	CIP	85721-33-1	331.35	6.1	0.01	30,000.0
Lip Reg	BEZ	41859-67-0	361.83	3.83	4.25	0.4
	GEM	25912-30-0	250.34	4.42	4.77	10.9
	SIM	79902-63-9	418.58	14.91	5.19	0.1
SSRIs	CIT	59729-33-8	324.16	9.6	1.39	31.1
	FLU	54910-89-3	309.13	10.1	1.22	60.3
	PAR	61869-08-7	329.14	10.3	1.37	35.3
	SER	87857-41-8	305.07	9.5	1.37	3.52
Anti-inf	DIC	15307-86-5	296.15	4.2	4.02	2.4
	IBU	15687-27-1	206.29	4.9	3.80	21.0
	PARA	103-90-2	151.17	9.4	0.269	14,000.0

Table 27. Penetration factors (Fpens) of the selected pharmaceuticals.

		ATC code	DDD	Fpen*
Anx	ALP	N05BA12	1	0.0335
	LOR	N05BA06	2.5	0.0238
	ZOL	N05CF02	10	0.0080
Antib	AZI	J01FA10	500	0.0009
	CIP	J01MA02	1000	0.0013
Lip Reg	BEZ	C10AB02	600	0.0005
	GEM	C10AB04	1200	0.0003
	SIM	C10AA01	30	0.0394
SSRIs	CIT	N06AB04	20	0.0026
	ESC	N06AB10	10	0.0131
	FLU	N06AB03	20	0.0149
	PAR	N06AB05	20	0.0076
	SER	N06AB06	50	0.0183
Anti-inf	DIC	M01AB05	100	0.0145
	IBU	C01EB16	1200	0.0181
	PARA	N02BE01	3000	0.0186
	Average			0.0135

ATC - Anatomical Therapeutic Chemical

DDD – defined daily dose (mg) [418]

* $Fpen = \frac{consumption\ (mg\ year^{-1})}{DDD(mg\ d^{-1}\ inhab).inhabitants.365(d.year^{-1})}$

Table 28. Data concerning the percentage excretion of parent compound and conjugates.

Therapeutic group	Pharmaceutical	Excretion results	References
Anx	ALP	20	[59]
	LOR	72.5	[60]
	ZOL	0.75	[61]
Antib	AZI	12	[60]
	CIP	70	[8]
		60	[1]
		83.7	[1]
		70	[60]
Lip Reg	BEZ	69	[8]
		47.5	[1]
		72	[64]
		50	[65]
		45	[66]
	GEM	50	[67]
	SIM	12.5	[1]
		12.5	[66]
SSRIs	CIT	23	[60]
		12	[69]
		20	[69]
	ESC	9	[70]
	FLU	10	[3]
		11	[69]
		5	[69]
		10	[69]
	SER	0.2	[60]
		0.2	[3]
		0.2	[69]
	PAR	3	[60]
		3	[3]
		3	[69]
Anti-inf	DIC	39	[8]
		15	[1]
		15	[67]
		15	[64]
		12.5	[66]
	IBU	5	[1]
		10	[72]
		15	[71]
		10	[65]
	PARA	80	[73]
		75	[60]

Table 29. Removal efficiencies (%) of pharmaceuticals in wastewater treatment plants.

Pharmaceutical	Country	Year	Number of WWTPs	Number of samples	Sampling type	Type of Wastewater treatment	Removal Efficiencies (Range)	Removal Efficiencies (Average)	References
Anxiolytics									
ALP	Spain	2008-2009	3	84/na	t.p.	2º	na	NE	[113]
	Spain	2009	3	28/na	t.p.	2º	na	NE	[113]
	Portugal	2011	1	14/na	t.p.	2º	NE-47	19	[23]
							NE-47	6.3	
LOR	Spain	2007-2009	3	72	t.p.	2º,3º	na	25	[96]
	Spain	2008-2009	3	84/na	t.p.	2º	na	NE	[113]
	Spain	2009	3	28/na	t.p.	2º	na	NE	[113]
	Portugal	2009	2	4	g.s.	na	NE	NE	[20]
	Italy	2010	1	8	t.p.	3º	na	52	[114]
	Portugal	2011	1	14/na	t.p.	2º	NE-26	7	[23]
	Portugal	2013-2014	60	15	t.p.	2º, 3º	NE-70	NE	[115,116]
							NE-70	12.0	
ZOL							NE-70	9.2	*
Antibiotics									
AZI	Spain/Croatia	na	5	10	g.s.	na	na	12.5	[117]
	International	1997-2007	117 pub	na	na	2º,3º	na	46.9	[90]
	Portugal	2009	2	4	g.s.	na	NE-7.1	NE	[20]
	Italy	2010	1	8	t.p.	3º	na	86	[114]
	Italy	2011	na	na	na	na	na	33	[8]
	Portugal	2011	1	14/na	t.p.	2º	NE-67	6	[23]
	USA	2013	1	66	g.s.	3º	na	47.9	[91]
	Portugal	2013-2014	15	60	t.p.	2º, 3º	NE-100	66.7	[115,116]
							NE-100	37.4	
CIP	Taiwan	na	1	na	na	3º	na	100	[118]

Table 29. Removal efficiencies (%) of pharmaceuticals in wastewater treatment plants. *(continued)*

Pharmaceutical	Country	Year	Number of WWTPs	Number of samples	Sampling type	Type of Wastewater treatment	Removal Efficiencies (Range)	Removal Efficiencies (Average)	References
	International	1997-2007	117 pub	na	na	2º,3º	na	82.5	[90]
	USA	2001-2002	7	20	f.p./g.s.	2º	22.2-100	60	[119]
	Sweden	2002-2003	5	20	f.p.	2º	58.3-100	86.3	[120]
	Italy	2004	6	14	t.p.	2º	45-78	61.5	[121]
	Portugal	2007	1	4	t.p.	2º	54-76	64.8	[6]
	Korea	2008	12	64	g.s.	na	na	NE	[122]
	Spain	2009	3	28/na	t.p.	2º	na	71.4	[113]
	Italy	2010	1	8	t.p.	3º	na	91	[114]
	Italy	2011	na	na	na	na	na	83	[8]
	Portugal	2011	1	14/na	t.p.	2º	NE-28	NE	[23]
	USA	2013	1	66	g.s.	3º	na	NE	[91]
	Greece	2013-2014	1	48	t.p.	2º	NE-20	0	[24]
	Portugal	2013-2014	15	60	t.p.	2º, 3º	NE-100	58.4	[115,116]
							NE-100	54.2	
Lipid regulators									
BEZ	International	na	na	na	na	na	na	41.2	[93]
	International	1997-2007	117 pub	na	na	2º,3º	na	66.6	[90]
	Italy	2004	6	14	t.p.	2º	NE-98	51.0	[121]
	Spain	2007-2009	3	72	t.p.	2º,3º	na	41.2	[96]
	Spain	2008-2009	3	84/na	t.p.	2º	na	56.3	[113]
	Spain	2009	3	28/na	t.p.	2º	na	25.0	[113]
	Portugal	2009	2	4	g.s.	na	NE-93.4	67.4	[20]
	Italy	2010	1	8	t.p.	3º	na	71	[114]
	Greece	2010-2011	8	64	t.p.	2º	65-80	72	[123]
	Portugal	2011	1	14/na	t.p.	2º	NE-75	28	[23]
	Spain	2012	3	42	t.p.	3º	na	67.9	[124]

Table 29. Removal efficiencies (%) of pharmaceuticals in wastewater treatment plants. *(continued)*

Pharmaceutical	Country	Year	Number of WWTPs	Number of samples	Sampling type	Type of Wastewater treatment	Removal Efficiencies (Range)	Removal Efficiencies (Average)	References
	Portugal	2013-2014	15	60	t.p.	2º, 3º	NE-100	51.8	[115,116]
							NE-100	53.3	
GEM	Sweden	na	na	na	na	na	na	69	[67]
	International	na	na	na	na	na	na	73.3	[93]
	International	1997-2007	117 pub	na	na	2º,3º	na	65.4	[90]
	Sweden	2002	1	2	f.p.	na	na	75	[67]
	Canada	2004	8	16	t.p.	na	na	26.9	[125]
	Spain	2007-2009	3	72	t.p.	2º,3º	na	31.7	[96]
	Spain	2008-2009	3	84/na	t.p.	2º	na	51.4	[113]
	Spain	2009	3	28/na	t.p.	2º	na	NE	[113]
	Portugal	2009	2	4	g.s.	na	NE	NE	[20]
	Korea	2010	5	10	t.p.	2º	na	92.3	[98]
	Italy	2010	1	8	t.p.	3º	na	58	[114]
	Greece	2010-2011	8	64	t.p.	2º	70-75	71	[123]
	Spain	2012	3	42	t.p.	3º	na	NE	[124]
	China	2012-2013	1	8	g.s.	3º	na	100	[126]
	USA	2013	1	66	g.s.	3º	na	5	[91]
	Portugal	2013-2014	15	60	t.p.	2º,3º	NE-100	28.8	[115,116]
							NE-100	46.7	
SIM	Portugal	2009	2	4	g.s.	na	NE-79.8	NE	[20]
	Greece	2010-2011	8	64	t.p.	2º	65-80	70	[123]
	Greece	2013-2014	1	48	t.p.	2º	NE	NE	[24]
	Portugal	2013-2014	15	60	t.p.	2º,3º	NE-100	72	[115,116]
							NE-100	35.5	
SSRIs									
CIT	Denmark	na	na	na	na	na	97.7-98.5	98.1	[127]

Table 29. Removal efficiencies (%) of pharmaceuticals in wastewater treatment plants. *(continued)*

Pharmaceutical	Country	Year	Number of WWTPs	Number of samples	Sampling type	Type of Wastewater treatment	Removal Efficiencies (Range)	Removal Efficiencies (Average)	References
	Norway	2005	3	6	f.p./g.s.	na	29.2-57.2	41.3	[128]
	Norway	2007	3	6	f.p./g.s.	na	19.7-60.4	39.0	[129]
	Canada	2007	1	4	t.p.	na	NE-11.2	5.6	[77]
	Portugal	2011	1	14/na	t.p.	2º	NE-28	NE	[23]
	China	2011	3	30	g.s.	2º	NE	NE	[130]
	Portugal	2013	15	60	t.p.	2º,3º	34.6-100	82.1	[131]
							NE-100	38.0	
ESC							NE-100	38.0	
FLU	Portugal	na	1	8	t.p.	3º	80-90	84	[92]
	Denmark	na	na	na	na	na	76.8-81.6	79.2	[127]
	England	na	7	168	g.s.	2º	na	48	[132]
	Norway	2005	3	6	f.p./g.s.	na	7.7-100	51.2	[128]
	Canada	2007	1	4	t.p.	na	NE-35.5	17.8	[77]
	Norway	2007	3	6	f.p./g.s.	na	11.1-55.1	37.2	[129]
	Italy	2010	1	8	t.p.	3º	na	58	[114]
	Portugal	2011	1	14/na	t.p.	2º	NE-100	NE	[23]
	USA	2013	1	66	g.s.	3º	na	23.1	[91]
	Greece	2013-2014	1	48	t.p.	2º	60-95	85	[24]
	Portugal	2013-2014	15	60	t.p.	2º,3º	100	100	[131]
							NE-100	53.0	
PAR	Norway	2005	3	6	f.p./g.s.	na	16.6-94.3	51.3	[128]
	Norway	2007	3	6	f.p./g.s.	na	9.3-67.0	36.9	[129]
	Canada	2007	1	4	t.p.	na	NE-18.9	9.4	[77]
	Italy	2010	1	8	t.p.	3º	na	100	[114]
	Portugal	2011	1	14/na	t.p.	2º	NE-78	19	[23]
							NE-94.3	43.3	

Table 29. Removal efficiencies (%) of pharmaceuticals in wastewater treatment plants. *(continued)*

Pharmaceutical	Country	Year	Number of WWTPs	Number of samples	Sampling type	Type of Wastewater treatment	Removal Efficiencies (Range)	Removal Efficiencies (Average)	References
SER	Denmark	na	na	na	na	na	55.3-62.2	58.8	[127]
	Norway	2005	3	6	f.p./g.s.	na	11.1-55.0	28.7	[128]
	Canada	2007	1	4	t.p.	na	4.9-15.0	10.0	[77]
	Norway	2007	3	6	f.p./g.s.	na	12.8-60.1	33.4	[133]
	Portugal	2011	1	14/na	t.p.	2º	43-100	59	[23]
	Portugal	2013-2014	15	60	t.p.	2º,3º	100	100	[131]
							NE-100	48.3	
Anti-inflammatories									
DIC	International	na	na	na	na	na	na	35.8	[93]
	Portugal	na	1	8	t.p.	3º	NE-100	45	[92]
	Spain, Belgium, Germany and Slovenia	na	25	39	t.p.	na	na	40	[134]
	Spain, Belgium, Germany and Slovenia	na	25	39	t.p.	na	na	40	[134]
	Spain/Croatia	na	5	10	g.s.	na	na	NE	[117]
	Sweden	na	na	na	na	na	na	40	[67]
	Taiwan	na	4	8	g.s.	2º	NE-80	42.8	[135]
	Taiwan	na	1	na	na	3º	na	78.2	[118]
	International	1997-2007	117 pub	na	na	2º,3º	na	49.3	[90]
	Sweden	2002	1	2	f.p.	na	na	22	[67]

Table 29. Removal efficiencies (%) of pharmaceuticals in wastewater treatment plants. *(continued)*

Pharmaceutical	Country	Year	Number of WWTPs	Number of samples	Sampling type	Type of Wastewater treatment	Removal Efficiencies (Range)	Removal Efficiencies (Average)	References
	Spain	2003-2004	1	20	t.p.	2º	na	40	[71]
	Switzerland	2003-2004	3	86	f.p.	2º,3º	NE	NE	[136]
	Canada	2004	8	16	t.p.	na	na	35.3	[125]
	United Kingdom	2004	1	6	t.p.	na	na	71	[102]
	Spain	2007-2009	3	72	t.p.	2º,3º	na	29.8	[96]
	Korea	2008	12	64	g.s.	na	na	19.4	[122]
	Spain	2008-2009	3	84/na	t.p.	2º	na	41.1	[113]
	Portugal	2009	2	4	g.s.	na	NE-10.5	NE	[20]
	Spain	2009	3	28/na	t.p.	2º	na	35.8	[113]
	Italy	2010	1	8	t.p.	3º	na	38	[114]
	Korea	2010	5	10	t.p.	2º	na	81.4	[98]
	Greece	2010-2011	8	64	t.p.	2º	NE-70	18	[123]
	Italy	2011	na	na	na	na	na	34	[8]
	Portugal	2011	1	14/na	t.p.	2º	NE-87	NE	[23]
	Spain	2012	3	42	t.p.	3º	na	NE	[124]
	China	2012-2013	1	8	g.s.	3º	na	20	[126]
	Portugal	2013-2014	15	60	t.p.	2º,3º	NE-100	49.7	[115,116]
							NE-100	33.6	
IBU	Spain/Croatia	na	5	10	g.s.	na	na	50.0	[117]
	Sweden	na	na	na	na	na	na	90	[67]
	Spain, Belgium, Germany and Slovenia	na	25	39	t.p.	na	45-75	60	[134]
	Taiwan	na	4	8	g.s.	2º	56-85	75.8	[135]

Table 29. Removal efficiencies (%) of pharmaceuticals in wastewater treatment plants. *(continued)*

Pharmaceutical	Country	Year	Number of WWTPs	Number of samples	Sampling type	Type of Wastewater treatment	Removal Efficiencies (Range)	Removal Efficiencies (Average)	References
	Portugal	na	1	8	t.p.	3º	75-100	83	[92]
	International	na	na	na	na	na	na	91.4	[93]
	Taiwan	na	1	na	na	3º	na	90.9	[118]
	International	1997-2007	117 pub	na	na	2º,3º	na	86.6	[90]
	Japan	2001-2003	5	80	t.p.	2º	84.3-99.7	95.8	[72]
	Sweden	2002	1	2	f.p.	na	na	96	[67]
	Spain	2003-2004	1	20	t.p.	2º	na	91.5	[71]
	Switzerland	2003-2004	3	86	f.p.	2º,3º	NE-93	44.5	[136]
	Canada	2004	8	16	t.p.	na	na	95.4	[125]
	Italy	2004	6	14	t.p.	2º	NE-100	65.5	[121]
	United Kingdom	2004	1	6	t.p.	na	na	89.0	[102]
	Spain	2008-2009	3	84/na	t.p.	2º	na	100	[113]
	Spain	2009	3	28/na	t.p.	2º	na	100	[113]
	Portugal	2009	2	4	g.s.	na	23.3-69.7	46.8	[20]
	Korea	2010	5	10	t.p.	2º	na	98.2	[98]
	Italy	2010	1	8	t.p.	3º	na	94	[114]
	Greece	2010-2011	8	64	t.p.	2º	80	80	[123]
	Portugal	2011	1	14/na	t.p.	2º	NE-99	52	[23]
	Spain	2012	3	42	t.p.	3º	na	100	[124]
	China	2012-2013	1	8	g.s.	3º	na	80	[126]
	USA	2013	1	66	g.s.	3º	na	99.7	[91]
	Portugal	2013-2014	15	60	t.p.	2º,3º	NE-100	85.1	[115,116]
							NE-100	82.4	
PARA	Spain/Croatia	na	5	10	g.s.	na	na	61.1	[117]
	International	na	na	na	na	na	na	99.8	[93]

Table 29. Removal efficiencies (%) of pharmaceuticals in wastewater treatment plants. *(continued)*

Pharmaceutical	Country	Year	Number of WWTPs	Number of samples	Sampling type	Type of Wastewater treatment	Removal Efficiencies (Range)	Removal Efficiencies (Average)	References
	Taiwan	na	1	na	na	3º	na	100	[118]
	International	1997-2007	117 pub	na	na	2º,3º	na	100	[90]
	Spain	2003-2004	1	20	t.p.	2º	na	99.8	[71]
	United Kingdom	2004	1	6	t.p.	na	na	100	[102]
	Korea	2005	4	24	g.s.	na	100	100	[137]
	Korea	2008	12	64	g.s.	na	na	95.1	[122]
	Spain	2008-2009	3	84/na	t.p.	2º	na	100	[113]
	Spain	2009	3	28/na	t.p.	2º	na	100	[113]
	Portugal	2009	2	4	g.s.	na	100	100	[20]
	Korea	2010	5	10	t.p.	2º	na	99.9	[98]
	Italy	2010	1	8	t.p.	3º	na	98	[114]
	Greece	2010-2011	8	64	t.p.	2º	70-100	91	[123]
	Portugal	2011	1	14/na	t.p.	2º	NE-99	94	[23]
	China	2012-2013	1	8	g.s.	3º	na	100	[126]
	USA	2013	1	66	g.s.	3º	na	97.1	[91]
	Greece	2013-2014	1	48	t.p.	2º	75-100	90	[24]
	Portugal	2013-2014	15	60	t.p.	2º,3º	71-100	97.5	[115,116]
							NE-100	96.0	

*Calculated by the mean removal efficiencies from the pharmaceuticals from the same therapeutic group (ALP and LOR)

NE – Not eliminated

na – not available

t.p. – time proportional sample

f.p. – flow proportional sample

g.s. – grab sample

pub - publications

Table 30. Absolute standard deviation between the predicted environmental concentrations and the measured environmental concentrations.

		Equation 4	**Equation 5**	**Equation 6**	**Equation 7**	**Equation 8**
Anx	ALP	42	134	1	1	17
	LOR	100	103	185	185	76
	ZOL	499	398	2	2	4
Antib	AZI	49,164	1468	560	560	420
	CIP	64,800	3880	4911	4911	2246
Lip Reg	BEZ	9600	19,037	19,419	19,419	18,924
	GEM	58,280	110	805	805	344
	SIM	500	4416	761	761	388
SSRIs	CIT/ESC	1904	490	95	95	94
	FLU	2979	1473	427	427	653
	PAR	11,633	2611	3344	3344	3333
Anti-inf	DIC	4300	4069	365	365	1063
	IBU	76,347	65,114	27,338	27,338	19,119
	PARA	168,000	246,611	54,704	54,704	98,382
Average		32,011	24,994	8061	8061	10,362

Chapter VI – Human pharmaceuticals in Portuguese rivers: the impact of water scarcity in the environmental risk

This publication focused the occurrence of pharmaceuticals in surface waters, evaluating the real impact instigated by wastewater effluents (WWEs). Since there was the need to validate a new analytical methodology, because a new environmental matrix was used, we took the opportunity to add new pharmaceuticals, that meanwhile entered the watch list (CLA, ERY, E1, E2 and EE2) and others that had continuously high frequencies and concentrations in other studies (CAR and NAP). Based also on other works performed, we also decided to include metabolites (N-CIT, Nor-FLU, Nor-SER and 4-OH-DIC) and one transformation product (4-PARA). Some pharmaceuticals that presented low frequencies and concentrations in wastewaters (ALP, LOR, ZOL and PAR) were removed from this analytical methodology.

The work presented and discussed in this chapter resulted in the following publication:
PEREIRA A.M.P.T., SILVA L.J.G., LARANJEIRO C.S.M., MEISEL L.M., LINO C.M., PENA A.. Human pharmaceuticals in Portuguese rivers: the impact of water scarcity in the environmental risk. Submitted to Science of the Total Environment.

VI1. Abstract

Pharmaceutical concentrations were assessed in surface waters, evaluating the impact of wastewater treatment plants (WWTPs) and of river flow rates in pharmaceutical concentrations, performing also the respective environmental risk. This was performed by analysing the presence of 23 pharmaceuticals of several therapeutic groups, including metabolites and transformation products, in 72 samples collected from 20 different sites, upstream and downstream WWTPs, in two different seasons, through solid phase extraction (SPE) and liquid chromatography coupled to tandem mass detection (LC-MS*n*).

The global frequency of contamination was of 27.8%, with the selective serotonin reuptake inhibitors (SSRIs), anti-inflammatories and antibiotics presenting the highest frequencies (27.8, 23.6 and 23.6%, respectively) as well as average concentrations (37.9, 36.1 and 33.5 ng L^{-1}, respectively). When assessing the influence of WWTPs, an increase of 21.4% in the total average concentration was observed in the samples located downstream these facilities.

Regarding the impact of the rivers flow rate, a trend was observed with increased frequencies and concentrations with lower flow rates, both by comparing summer with winter campaigns and by evaluating the different rivers.

Performing the environmental risk assessment (ERA), risk quotients (RQs) higher than one were found for two pharmaceuticals, concerning two trophic levels. However, and since Iberian rivers are highly influenced by water scarcity, the flow rates in these rivers, in drought periods, can decrease at least ten times from the lowest value observed in the sampling campaigns. In these conditions, RQs higher than 0.1 would be found for all of the eleven detected pharmaceuticals, including five that would present RQ higher than 1.

These results emphasize that the river flow rates represent an important parameter influencing pharmaceutical concentrations, highlighting the ecotoxicological pressure, especially due to water scarcity in drought periods. This should be a priority issue in the environmental policies for minimizing its impact in the aquatic environment.

Keywords

Environmental contaminants; pharmaceuticals; surface waters; occurrence and fate; water scarcity; environmental risk assessment.

VI2. Introduction

Human pharmaceuticals represent a group of widely used chemicals that contaminate the aquatic environment. Albeit in trace amounts, their continuous introduction into the environment is of concern since they are designed to perform a biological effect [1,115]. Worldwide has been recognized the environmental impact of medicinal products, and the potential for negative ecotoxicological effects in the aquatic environment, even at sublethal concentrations [3]. Nonetheless, all the ecotoxicological risks associated to the ubiquitous occurrence of pharmaceuticals in aquatic ecosystems are far from known [4].

The main source of pharmaceutical residues in the aquatic environment is from human excretion, and since wastewaters treatment plants (WWTPs) are not able to completely remove the pharmaceuticals, these, along with its metabolites and as transformation products are disseminated mainly through wastewater effluents (WWEs) into surface waters [5,8,11,12,161]. Here the fate and concentration of pharmaceuticals can be mainly reliant on the receiving water body flow rate, partitioning to sediments and photodegradation [34,85,143,150]. Although no legal limits have been established in surface water, 6 pharmaceuticals and one metabolite became part of the Water Framework Directive (WFD) watch list, established by the Directive 2013/39/EU and the recent Commission Implementing Decision from the European Union (EU) 2015/495. This list is dynamic, therefore, identifying and prioritizing new pharmaceuticals are important goals to support future updates [14]. In this way, high-quality monitoring data, along with environmental risk assessment (ERA), are essential for pharmaceuticals prioritization [1,16,17,353]. In addition, a good ecological status is currently achieved in only 43% of the reported freshwater bodies, and despite the enormous efforts, the picture that emerges for European rivers and lakes regarding ecological and chemical status is still incomplete, fragmented, and with contradictory assessments of the situation [29].

Heavy contamination pressures from extensive urban activities characterize the Portuguese main rivers, which might lead to high aquatic contamination levels. However, no national survey on surface waters has been conducted and a knowledge gap is observed [115]. When WWEs reach surface waters, the dilution effect varies significantly due to different flows in the receiving rivers. Nonetheless, this effect can be relatively low, especially in arid or semi-arid regions, like some Iberian rivers [151,152]. Additionally, in a larger vision of future water resource management sustainability, with the escalating population growth and intensified agricultural and industrial activity, water scarcity will tend to increase [27,28].

Therefore, this study aims to provide a clear insight on pharmaceuticals contamination on surface waters, observing the influence of river flow rates, embracing, not only several parent compounds (17), but also metabolites (5) and transformation products (1), belonging to different therapeutic groups including antibiotics, lipid regulators, antiepileptics, selective serotonin reuptake inhibitors (SSRIs), anti-inflammatories and analgesics (further referred only as anti-inflammatories) and hormones. The pharmaceuticals in study are key representatives of the major therapeutic groups, and were selected based on their high consumption in Portugal, legislation, previous data on the their occurrence in WWTPs and surface waters, their attenuation/persistence in surface waters and given the relative concern about their potential ecotoxicological impact [14,24–26,116]. A better understanding of the regional and global context, concerning the environmental risk posed by pharmaceuticals in different scenarios of the aquatic environment is provided.

VI3. Materials and methods

VI3.1. Sampling site and collection

The 72 surface water samples were collected from 20 different sites, across mainland Portugal, by national authorities, to ensure correct sampling procedures (Figure 30). Grab samples (1.5 L) were obtained at 1 m deep, 500 m upstream and downstream the effluent discharges of the selected WWTPs, during two sampling periods: between 9 September/11 November 2014 – summer/autumn (summer), and 19 February/15 March 2015 – winter. Sampling in Mondego (Figueira da Foz), Tagus and Guadiana rivers were performed in estuarine areas, in the last quarter of lower tide to prevent abnormal dilution effects, high salinity rates and to ensure that the natural flow, towards the river mouth, was observed.

Two samples, obtained in consecutive days, were grabbed upstream and downstream each WWTP, for each sampling season (Table 32, Supporting information).

After collection into high-density polyethylene containers, previously rinsed with bi-distilled water, samples were acidified to pH 3 with formic acid and refrigerated during transportation; on arrival at the lab they were stored at -20 ºC until analysis.

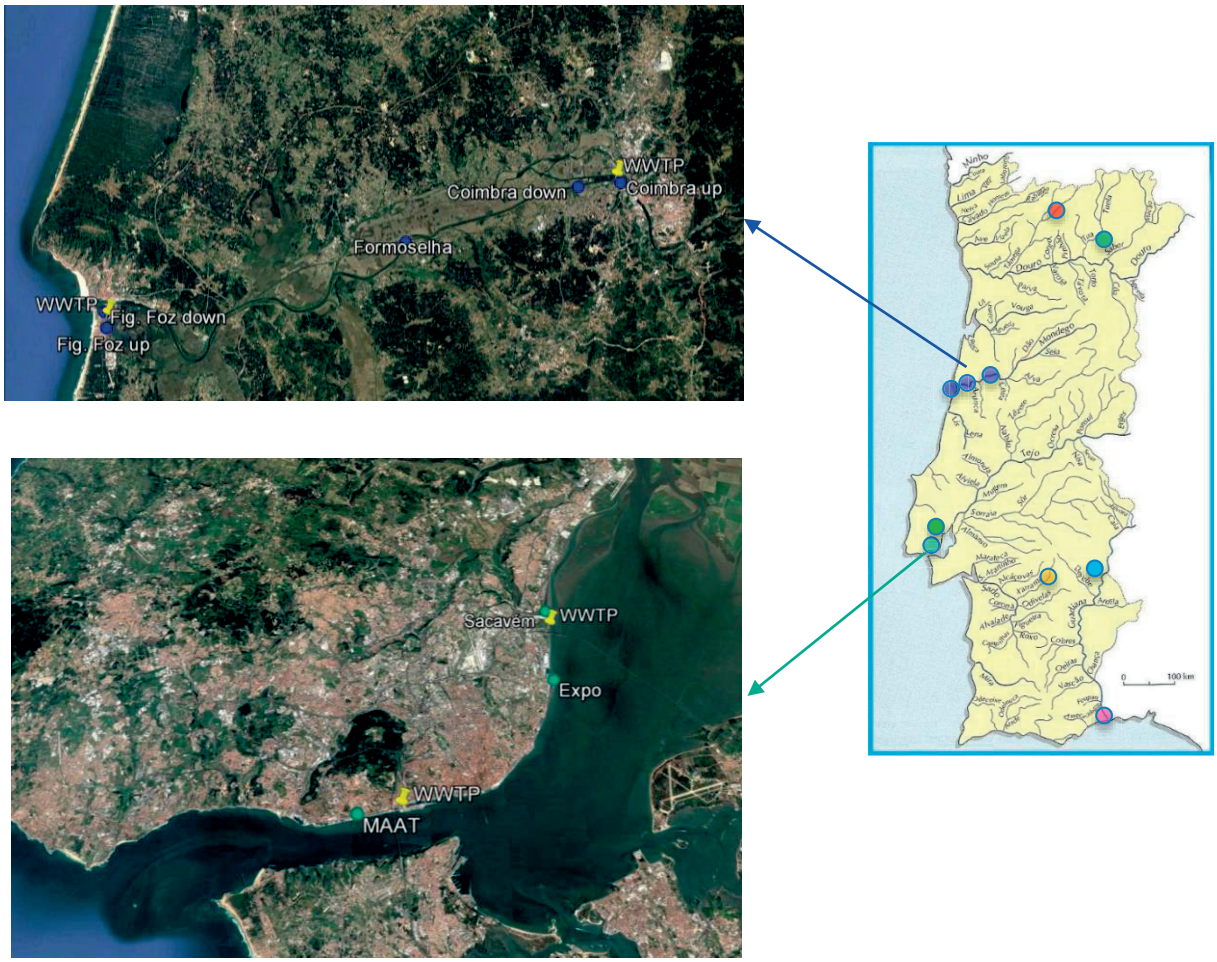


Figure 30. Sampling sites location.

VI3.2. Standards and chemicals

All pharmaceutical standards, with purity degree ≥ 98% or certified reference material, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock and intermediate solutions were prepared in acetonitrile at 500 µg mL^{-1} and 100 µg mL^{-1}, respectively, and stored at -20 °C for a maximum of 6 months. Mixed standard working solutions, renewed before each analytical run, and prepared at concentrations ranging between 25 and 250 ng mL^{-1}, in a mixture of water-methanol (90:10 v/v), were used for linearity, accuracy and repeatability assays. For the labelled surrogates, a concentration of 250 ng mL^{-1}, also in water-methanol (90:10 v/v), was used.
Sigma-Aldrich (St. Louis, MO, USA) supplied methanol and acetonitrile and Ultrapure Milli-Q water was obtained from a Millipore Milli Q system (Bedford, MA, USA). Formic acid (98%) was obtained from Merck (Darmstadt, Germany).

VI3.3. Experimental procedure

Analysis of pharmaceuticals, metabolites and transformation products were carried out using 500 mL of surface water, spiked with surrogate standards. Samples were subsequently vacuum filtered through glass microfiber filters (1.0 μm, 934-AH) and 0.45 and 0.2 μm polyamide membrane filters from Whatman Schleicher and Schuell (USA) and from Whatman (Dassel, Germany). Afterwards, the samples were loaded into solid phase extraction (SPE) cartridges Oasis HLB (200 mg, 6 mL), from Waters Corporation (Milford, Massachusetts, USA), previously conditioned with 2 mL methanol and 2 mL Milli-Q water. After rinsing with 5 mL of methanol/Milli-Q water (10:90 v/v) and left to dry for 15 min, elution was performed with 6 mL methanol. Finally, the eluate was evaporated to dryness under a gentle stream of nitrogen, at 40 ºC, and the dried extracts were stored at −20 ºC until analysis, that took place in a maximum of 48 h.

For liquid chromatography with mass spectrometry (LC-MS*n*) analysis, the dried eluate was reconstituted into 0.5 mL of water-methanol (90:10 v/v) and microfiltered. A 20 μL injection volume was used and a gradient of (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid at 200 μL min^{-1} were used (Table 33, Supporting information).

Chromatographic separation was achieved with a column Waters Spherisorb ODS2 (150 x 2.1 mm, 3 μm) (Waters Corporation, Milford, U.S.A.) preceded by a guard cartridge of the same packing material (10 x 4.6 mm, 5 μm) (Waters Corporation, Milford, U.S.A.). A hybrid Quadrupole Ion Trap Mass Spectrometer (LCQ Advantage MAX, Thermo Finnigan, San Jose, California, USA) was operated in the positive and negative electrospray ionization (ESI) modes using multiple reaction monitoring (MRM) acquisition. Source and capillary temperatures and voltages were set at 0 and 270 ºC and at 4.5 and 10 V, respectively. Nitrogen was used as nebulizing gas, with a sheath gas flow of 80 arb (arbitrary unit) and the auxiliary sweep gas flow of 20 arb. Two precursor-to-fragment transitions were acquired (MS1 and MS2), one for quantification purpose and the other for confirmation purpose. The collision gas was helium with normalized collision energy ranging between 21 and 39%. Retention time, product ions, and collision energy are also presented in Table 34 (Supporting information).

VI3.4. Statistical analysis

Complete statistical analysis was performed using GraphPad Prism (6.01, GraphPad Software, Inc., San Diego, USA). To test whether the dataset was of Gaussian distribution, D'Agostino–

Pearson normality test was used. Since most of the data set was not normally distributed, with non-homogeneous variances, nonparametric tests were applied. Kruskal–Wallis test with Dunns post-test were used to assess statistical differences between therapeutic groups and sampling locations. For the comparison between the two different sampling periods and concerning upstream and downstream samples, Mann-Whitney test was used. Pharmaceuticals not detected in all samples were excluded from this evaluation. The statistical significance level was set to $p<0.05$.

VI3.5. Environmental risk assessment (ERA)

The evaluation of the environmental risk of the aquatic compartment was based on the guideline on the ERA of medicinal products for human use [353]. Following this guideline, the risk quotients (RQs) associated to the selected pharmaceuticals were calculated by the ratio of measured environmental concentration (MEC) and predicted no-effect concentration (PNEC) on non-target organisms using three different trophic levels representatives of the aquatic ecosystem (algae, daphnids and fish) [23,115,372].

It should be taken into account that the choice of data can obviously affect the outcome and that maximum individual concentrations of pharmaceuticals found in surface waters were used as MECs, to set a worst-case scenario approach [23,372]

PNEC values were calculated by applying an uncertainty factor (UF) of 10 to the long-term no-observed-effect-concentration (NOEC) values or of 50 and 1000, to the short-term lowest-observed-effect-concentration (LOEC) and to the lethal (effective) concentration (L(E)C50) values, respectively, available in the literature. The UF is an expression of the degree of uncertainty in the extrapolation from the test data on a limited number of species to the actual environment [353]. When no experimental data were available, L(E)C50 values were estimated with ECOSAR 1.11. If RQ equals or is above 1 there is a potential environmental risk situation, whereas when values are lower than 1, no risk is expected.

VI4. Results and Discussion

VI4.1. Analytical quality control

Analytical quality control was performed encompassing different performance criteria such as sensitivity, linear range, matrix effects (ME), accuracy, and precision (Table 35, Supporting information). Linearity was studied analysing in triplicate at six concentration levels, using standard solutions between 25 and 250 ng mL^{-1}, that correspond, according to the analytical methodology, to the range of 25 to 250 ng L^{-1}, and also in matrix-matched calibrations, at the same concentrations. Linearity, achieved for every compound in the working standard solutions, was reliable as shown by the fact that the correlation coefficients (r^2) ranged from 0.9997, for SIM, 4-OH-DIC, IBU and 4-PARA and 1, for CLA, ERY, CIP, BEZ, GEM, CIT, N-CIT, Nor-FLU and SER. In matrix-matched solutions, r^2 values ranged between 0.9995, for DIC, NAP, PARA and 4-PARA and 1 for GEM.

MEs equalled the percentage of the matrix-matched calibration slope (B) divided by the slope of the standard calibration in solvent (A). Thus, the ratio (B/A x 100) was defined as the absolute matrix effect (ME%). The obtained value was interpreted as follows: a value of 100% denoted an absence of MEs, above 100% signal enhancement and below 100% signal suppression. MEs were considered negligible, since the values varied from 98.81 to 102.08%, for SIM and 4-OH-DIC, respectively.

The method detection (MDL) and quantification limits (MQL) were estimated through the matrix-matched calibration curve as |3.3Sy/x|/b and |10Sy/x|/b, respectively, where b is the slope and Sy/x the residual standard deviation of the linear function. MDL and MQL values ranged from 2.01 to 8.24 ng L^{-1}, and from 6.10 to 24.96 ng L^{-1}, for GEM and 4-OH-DIC, respectively.

Recovery tests were performed to determine the accuracy and precision of the method by spiking a surface water at three levels, 50, 150 and 250 ng L^{-1} (n=3), in three different days, and each sample was analysed in triplicate. Accuracy varied between 58.88 and 99.51%, for DIC and FLU, respectively, as for precision, evaluated through the relative standard deviation (RSD) of intra-day and inter-day repeatability, was below 8.88 and 8.54%, respectively.

These values are considered reliable and similar to other methods developed for the same purpose [26,163,166,419].

VI4.2. Occurrence

Table 31, Figure 31, Figure 32 and Table 36 (Supporting information) outline a summary on the occurrence data of the selected pharmaceuticals in surface water samples, their frequency, mean concentration, range and standard deviation observed.

From the 23 targeted pharmaceuticals, metabolites and transformation products, 11 were detected, and there were samples contaminated with up to 8 pharmaceuticals, being the hormones the only therapeutic group that did not present any positive result. Regarding the frequencies of detection, SSRIs was the most recurring therapeutic group, present in every contaminated samples (27.8%), followed by anti-inflammatories and antibiotics (23.6%), lipid regulators (8.3%) and antiepileptics (1.4%). As for each pharmaceutical, the highest frequency was observed for CIT (27.8%), followed by CLA (20.1%), DIC and PARA (19.4%), with the remaining pharmaceuticals with values under 7%.

The pattern for the average concentrations for each therapeutic group was similar to that of frequency, being in decreasing order SSRIs (37.9 ng L^{-1}), anti-inflammatories (36.1 ng L^{-1}), antibiotics (33.5 ng L^{-1}), antiepileptics (11.5 ng L^{-1}) and lipid regulators (9.4 ng L^{-1}). The highest individual concentrations were found for the anti-inflammatories PARA and DIC (69.2 and 51.2 ng L^{-1}, respectively), followed by the SSRIs CIT and FLU (53.0 and 25.4 ng L^{-1}, respectively) and the antibiotics CLA, ERY and AZI (39.1, 38.8 and 35.7 ng L^{-1}, respectively). These results are consistent, in some cases slightly lower, with those previously reported by other authors in Portuguese surface waters [81,191,204,206,213,420] and in other European countries [141,163,164,195,202].

Table 31. Occurrence of the selected pharmaceuticals.

Therapeutic group/Compound		Frequency	Minimum	Maximum	Mean	Standard deviation
Antib		23.61	24.80	39.10	33.53	4.84
Azithromycin (AZI)	PC	2.78	32.15	35.66	33.91	2.48
Ciprofloxacin (CIP)	PC	nd	nd	nd	nd	nd
Clarithromycin (CLA)	PC	20.83	24.80	39.10	33.08	5.39
Erythromycin (ERY)	PC	4.17	32.89	38.80	35.51	3.01
Lip reg		8.33	6.69	15.52	9.47	3.31
Bezafibrate (BEZ)	PC	2.78	11.86	15.52	13.69	2.59
Gemfibrozil (GEM)	PC	6.94	6.69	10.34	7.78	1.50
Simvastatin (SIM)	PC	nd	nd	nd	nd	nd
Antiepi		1.39	11.45	11.45	11.45	nd
Carbamazepine (CAR)	PC	1.39	11.45	11.45	11.45	nd
SSRIs		27.78	20.70	52.97	37.86	8.73
Citalopram (CIT)	PC	27.78	20.70	52.97	39.21	7.93
Desmethylcitalopram (N-Cit)	M	nd	nd	nd	nd	nd
Fluoxetine (FLU)	PC	1.39	25.37	25.37	25.37	nd
Norfluoxetine (Nor-FLU)	M	nd	nd	nd	nd	nd
Sertraline (SER)	PC	1.39	23.30	23.30	23.30	nd
Desmethylsertraline (Nor-SER)	M	nd	nd	nd	nd	nd
Anti-inf		23.61	18.91	69.15	36.13	12.04
Diclofenac (DIC)	PC	19.44	25.13	51.24	33.56	8.43
4-hydroxydiclofenac (4-OH-DIC)	M	nd	nd	nd	nd	nd
Ibuprofen (IBU)	PC	nd	nd	nd	nd	nd
Naproxen (NAP)	PC	nd	nd	nd	nd	nd
Paracetamol (PARA)	PC	19.44	18.91	69.15	38.69	14.69
4-aminophenol (4-PARA)	TP	nd	nd	nd	nd	nd
Horm		nd	nd	nd	nd	nd
17β-estradiol (E2)	PC	nd	nd	nd	nd	nd
Estrone (E1)	M	nd	nd	nd	nd	nd
17α-ethinylestradiol (EE2)	PC	nd	nd	nd	nd	nd

Antib - antibiotics; Antiepi - antiepileptics; Anti-inf - anti-inflammatories; Horm - hormones; Lip reg - lipid regulators; M - metabolite; PC – parent compound; nd – not detected; SSRIs - serotonin reuptake inhibitors; TP - transformation product.

VI4.2.1. Upstream and downstream comparison

When comparing the results regarding the sampling location position to the respective WWTPs, the results showed, as expected, and for all therapeutic groups, higher frequencies (30.8%) in the samples collected downstream the WWTPs than those in the upstream samples (24.2%) (Figure 31). As for the average concentrations, the same patterns were observed for all

therapeutic groups with a cumulative average concentration increase of 21.4%. Even though the observed trend, no significant statistical difference was observed with a *p*-value of 0.1924.

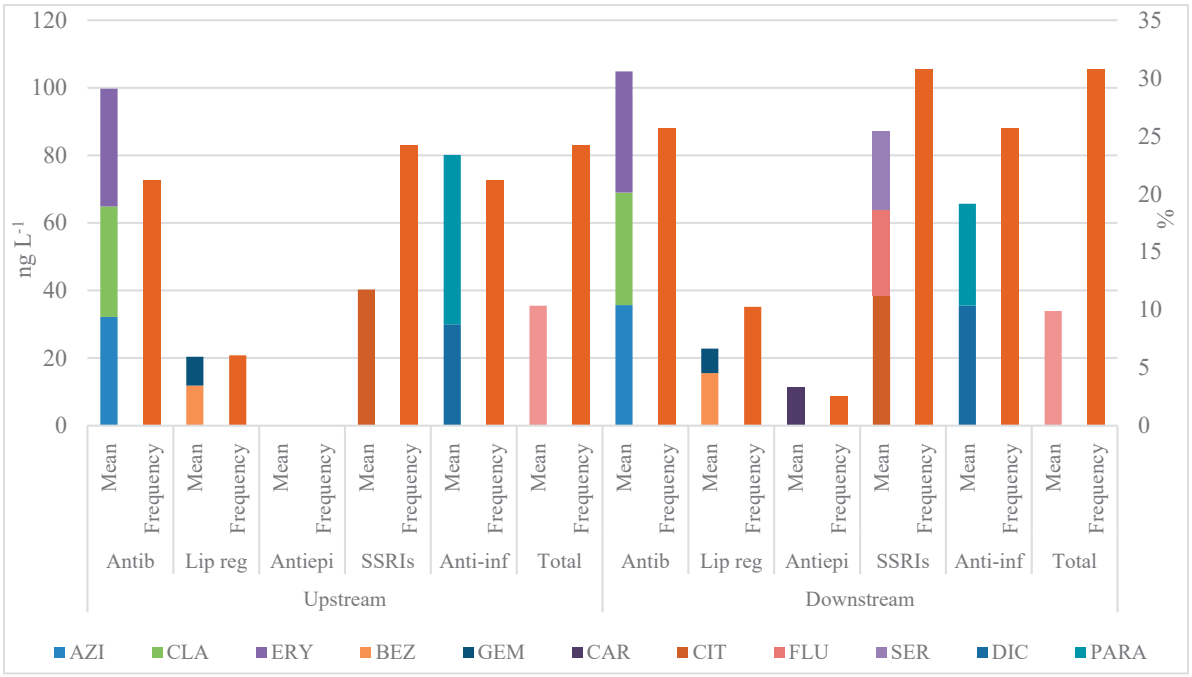


Figure 31. Frequency and concentrations of the selected pharmaceuticals, upstream and downstream the WWTPs comparison.
Antib - antibiotics; Lip reg - lipid regulators; Antiepi - antiepileptics; SSRIs – selective serotonin reuptake inhibitors; Anti-inf - anti-inflammatories.

This general increased concentration was already reported by other authors, since WWTPs are the major source of pharmaceuticals contamination [8,63,132]. However, some pharmaceuticals were only present in upstream samples or in higher concentrations than in the downstream samples of the same river. Although this was observed, there were no great discrepancies in the pharmaceutical concentrations, and this was also already reported in other studies [8,63]. These abnormal results could be mainly related to the fact that we used grab samples, which reflect the concentrations at a specific time. For practical reasons, sampling downstream and upstream were performed in the same day but not at the same time, therefore, some variations were expected [411]. Moreover, there are also additional contamination points like other WWTPs, illegal raw discharges (not yet connected to the sewage network) from buildings, commercial activities, agriculture and animal production farms present along the river banks that can also mislead in the results interpretation [8].

However, the upstream sample that presented the highest difference for the downstream samples was one from Sacavém (Tagus River), with 7 pharmaceuticals present. This sampling point is very close to Trancão River mouth, therefore, strongly influenced by its contamination. Moreover, we can observe a similar pattern of contamination in the referred Sacavém sample and the ones in Trancão River, supporting this correlation (Figure 30 and Table 36, Supporting information). Another unusual result is the one observed for Formoselha (Mondego River), since it has 4 pharmaceuticals that were not detected in the other samples located upstream (Coimbra downstream) (Figure 30 and Table 36, Supporting information). This could be explained by another WWTPs that is located between this two sampling points.

VI4.2.2. Influence of flow rates

Surface water was collected over a one year period, during two sampling seasons (summer and winter). It was impossible to collect the summer samples in drought periods and the ones in winter during heavy rain, that would allow to better access the influence of river flow variations, since the sampling process involved the coordination of several teams. Nevertheless, the flow rates in summer campaigns were clearly lower than in winter campaigns [421].

In general, the pharmaceuticals frequencies were found to be considerably higher during summer than in winter, with 38.2% and 18.4%, respectively (Figure 32). Regarding the concentrations, the same pattern was observed, with summer and winter results presenting average concentrations of 34.7 and 31.2 ng L^{-1}, respectively. This trend was observed for all therapeutic groups with the exception of lipid regulators. While no statistical significance was observed, the *p*-value obtained, 0.054, was very close to 0.05.

These results were also described by other authors, who referred that the rivers mass loads might be similar in different seasons, but concentrations are clearly lower in winter [132,420].

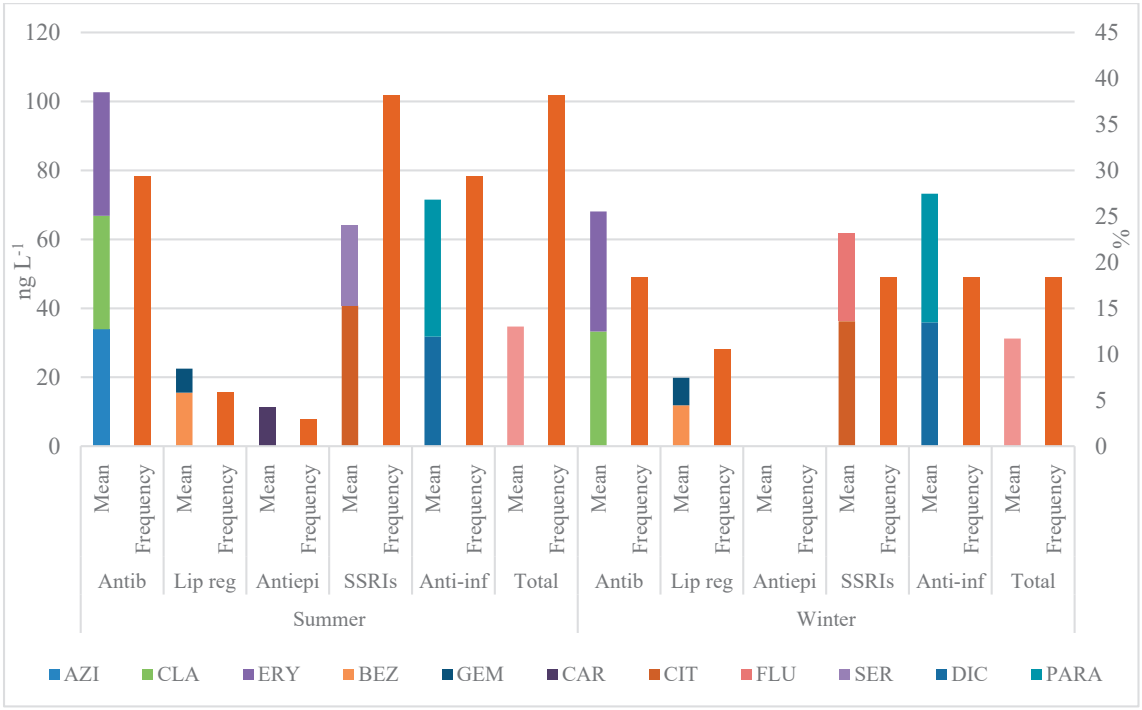


Figure 32. Frequency and concentrations of the selected pharmaceuticals, summer and winter comparison.

Antib - antibiotics; Lip reg - lipid regulators; Antiepi - antiepileptics; SSRIs – selective serotonin reuptake inhibitors; Anti-inf - anti-inflammatories.

When observing the presence of pharmaceuticals in different rivers, the total frequencies and total average concentrations in Trancão, Xarrama and Álamo (66.7, 75.9 and 77.5% and 31.0, 35.0 and 36.6 ng L^{-1}, respectively) were higher than in the other rivers, which presented total frequencies under 16.7% (Figure 33). The pharmaceuticals occurrence in the above mentioned rivers, presenting clearly lower flow rates, was statistical different from all the other rivers with the exception of Tagus. However, Tagus did not present statistical difference from any other river.

In estuarine areas, an additional dilution occurs due to influence of seawater. Therefore, with the exception of the above mentioned Sacavém sampling point, no positive samples were observed in estuarine regions like Tagus, Guadiana and Mondego rivers (Figueira da Foz).

Moreover, when using the average river flow rates and average WWEs discharge, we can observe that the downstream samples with greater percentage of WWEs were also the ones with higher concentrations, with Trancão, Xarrama and Álamo presenting 92.6, 15.9 and 1.9%, respectively [116].

Additionally, in some Iberian rivers, significant variations in water flow rates can occur due to water scarcity, as observed in the sampling sites with higher concentrations, like Xarrama,

Trancão and Álamo, namely in summer. In these rivers, higher percentages of WWE in the surface water would be observed, with the river flow composed almost exclusively by the WWE [151,152,422]. Also, in other European countries, high percentages of WWEs in surface waters were calculated for small rivers, up to 80% [107].

As referred, significant variations are observed in Portuguese river flow rates. For instance, in Tagus River, the monthly averages presented a minimum of 13 and a maximum of 1424 $m^3 s^{-1}$, with a variation up to 100 times [423]. However, when using Tagus River daily averages, the flow can be as low as 9 $m^3 s^{-1}$, 35 times lower than the reported average [423]. This was also observed in Guadiana daily averages, where the lowest flow rate reported was of 2 $m^3 s^{-1}$, 45 times lower than the average, and in Mondego River, where the annual average variations were observed between 27 and 140 $m^3 s^{-1}$ [424,425].

From the selected sampling points, only for Mondego River (Coimbra) data regarding daily flow rates was available and, in drought periods, the flow rate was more than ten times lower (2.4 $m^3 s^{-1}$) than the lowest observed in the sampling days, 27.9 $m^3 s^{-1}$ in summer [421]. In other hydrometric stations, like in Tâmega (near the river mouth) and Zêzere Rivers, the flow rate ratio between the summer sampling days and the lowest observed values were 53.4 and 24.1, respectively. These variations in the flow rates can promote much higher concentrations of pharmaceuticals in surface waters in drought periods.

Since we observed higher frequencies and concentrations in rivers with lower flow rates, either comparing different rivers or different sampling campaigns, we can access that the dilution factor represents the most important parameter influencing pharmaceuticals concentration in surface waters. In the rivers with lower flow rates, the increased partitioning to sediments and increased photodegradation and biodegradation, in summer, did not overcome the concentration effect due to the lower flow rates [34,85,143,150].

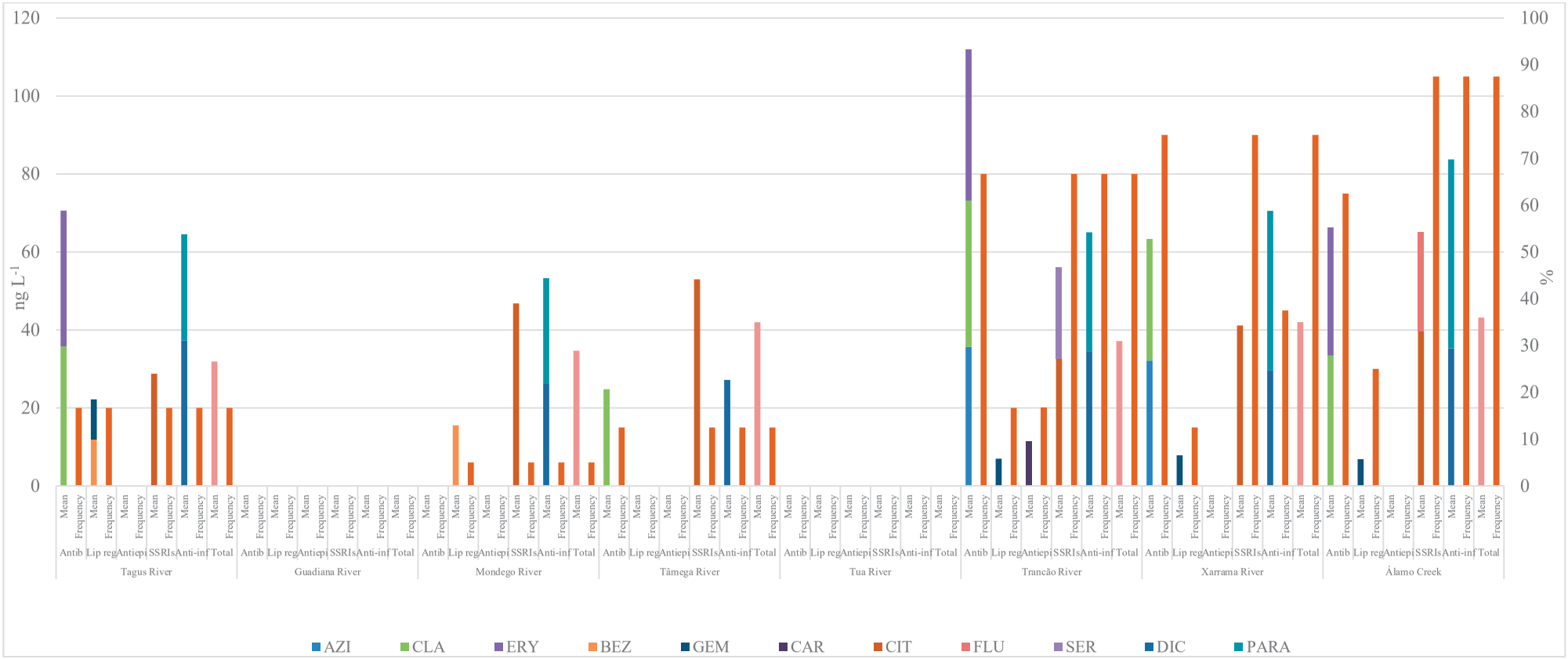


Figure 33. Frequency and average concentrations of the selected pharmaceuticals in the different rivers.
Antib - antibiotics; Lip reg - lipid regulators; Antiepi - antiepileptics; SSRIs – selective serotonin reuptake inhibitors; Anti-inf - anti-inflammatories.

VI4.3. Comparison with WWE concentrations

The occurrence results in surface waters showed a significant trend towards higher concentrations in summer, where flow rates are clearly lower. On the contrary, in another study performed in the WWTPs impacting the selected rivers and sampling locations, in 2013 and 2014, the concentrations found in winter for the WWEs were four times higher than the ones in summer, with even higher discrepancies in anti-inflammatories and lipid regulators [115,116,131]. Therefore, since concentrations in surface waters were higher in summer, the dilution factor overlapped the higher impact of WWEs in winter and other above mentioned factors, that could also decrease pharmaceuticals concentration in summer [34,85,143,150].

Even though the sampling period for the study on wastewaters did not exactly match the present study on surface waters, it is clear that the WWEs impacting the selected rivers which presented positive samples were neither the ones with higher concentrations, nor with high amount of pharmaceuticals released in the respective rivers [115,116,131]. The only correlation observed was the higher concentrations for PARA in the WWEs of Álamo Creek which was also witnessed in the respective surface water [115,116,131]. However, the concentrations in rivers appear to be primarily related to the river flow rates, suggesting, once again, that dilution factor is the main accountable for pharmaceutical river concentrations.

The range of pharmaceutical concentrations found in surface waters were in agreement with the ones predicted from the WWEs concentrations, up to 43 ng L^{-1} [116]. Nevertheless, the rivers that were expected to present higher concentrations, like Mondego and Tagus, had lower concentrations, probably due to most of the sampling being performed in estuarine areas.

VI4.4. Environmental risk assessment (ERA)

The above-mentioned data regarding occurrence is crucial in order to perform the ERA, which can be used to prioritize pharmaceuticals [202]. For that, RQ might be a useful tool, as previously found [202,413,414]. Therefore, the RQs deemed for each pharmaceutical are shown in Figure 34.

According to our results, only Álamo Creek had RQs higher than 1, with FLU and DIC presenting RQs for fish of 9.06 and 1.02, respectively; regarding daphnids only the RQ of FLU exceeded one (1.06). Therefore, risk might be expected for these trophic levels in this aquatic body. In addition, a certain risk might be expected in five rivers for CIT and SER in daphnids, for CLA and FLU in algae, and for DIC in fish with RQs calculated between 0.1 and 1.

Generally, algae appeared to be the most sensitive throphic level, with 7 pharmaceuticals with RQs above 0.01, however, some therapeutic groups presented higher sensibility for specific throphic levels, for example, SSRIs and daphnids.

Although there is known information on individual ecotoxicity of these compounds, it should be noted that, given their environmental presence in mixtures, and given their similar pharmacological mechanisms, namely in the same therapeutic group, additive or even synergistic effects may occur and thus, the real hazard may be greater than that calculated [23,133,230,232,405,426].

As observed for the frequencies and concentrations of pharmaceuticals in surface waters, the rivers with lower flow rates presented higher environmental risk, being these also the ones more susceptible to drought periods. Assuming that, as previously referred, since in drought periods the flow rate can decrease at least 10 times, the concentrations will also increase 10 times, since the pharmaceuticals mass load will remain the same. This would promote different RQs, with values higher than one for 5 pharmaceuticals (CLA, CIT, FLU, SER and DIC). Additionally, observing all rivers and all throphic levels, all the 11 pharmaceuticals would have a RQ higher than 0.1.

These results highlight that in drought periods the surface water concentrations can be unbalanced, additionally, due to climate changes, these events can occur more often and could promote severe consequences in aquatic biota [152].

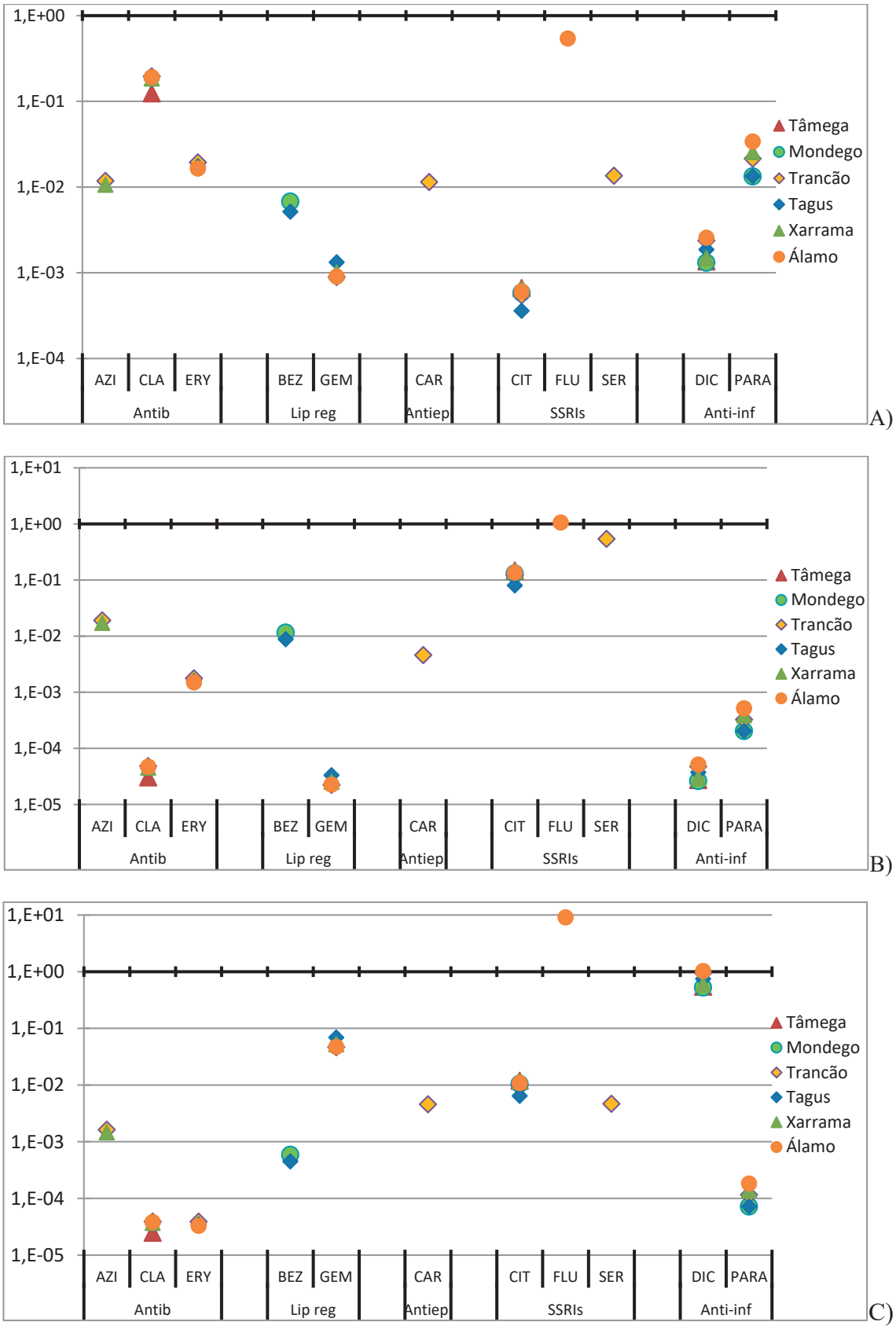


Figure 34. Environmental risk assessment of the selected pharmaceuticals in the different rivers for the three trophic levels. A) Algae; B) Daphnids; C) Fish.

Antib - antibiotics; Lip reg - lipid regulators; Antiepi - antiepileptics; SSRIs – selective serotonin reuptake inhibitors; Anti-inf - anti-inflammatories.

VI5. Conclusions

The work performed allowed to confirm the presence of 11 pharmaceuticals in Portuguese surface waters, presenting total frequencies of 27.8%, being each sample contaminated up to 8 pharmaceuticals, and with concentrations as high as 69.2 ng L^{-1} (PARA). Regarding each therapeutic group, the concentrations were, in decreasing order: SSRIs (37.9 ng L^{-1}), anti-inflammatories (36.1 ng L^{-1}), antibiotics (33.5 ng L^{-1}), antiepileptics (11.5 ng L^{-1}) and lipid regulators (9.4 ng L^{-1}).

Despite no statistical significance was observed, clearly the frequencies and concentrations were higher in downstream samples, with a 21.4% increase in concentrations, confirming the strong impact of WWTPs in surface waters.

Observing the influence of the flow rates in the pharmaceutical concentrations, it became clear, when observing the results in both sampling campaigns, in the different rivers and the correlation with the percentage of WWE in surface waters, that this parameter was closely related to the concentration of pharmaceuticals in the surface water. Additionally, in drought periods, which are increasing with climate changes, the flow rates can decrease up to a minimum of 10 times, when compared with the flow rates observed, which can lead to an increased concentration of the same ratio.

The ERA performed indicated RQs higher than one for FLU and DIC (9.06 and 1.02, respectively), regarding fish, and also a RQ of 1.06 for FLU, concerning daphnids. However, when using the predicted concentrations in drought periods, 5 pharmaceuticals (CLA, CIT, FLU, SER and DIC) presented RQs above 1, and all the remaining 11 pharmaceuticals detected had RQs higher than 0.1. These results underline the ecotoxicological pressure to which the aquatic biota are exposed in surface waters, namely during drought periods, with expected negative outcomes.

These results highlight the importance of pharmaceuticals contamination in surface waters, recognizing this issue as a priority for environmental policies and the importance of setting prioritizing measures and sustainable strategies, viewing the minimization of its impact in the aquatic environment. Since the Directive 2013/39/EU watch list is dynamic, it would be imperative to include the SSRIs CIT, FLU and SER in this list to better evaluate their environmental risk.

Moreover, to assess the risk to humans, groundwaters and drinking waters impacted from the most contaminated surface waters should also be evaluated, considering also the possible variations in drought seasons.

VI6. Supporting information

Table 32. Characterization and geographical location of the surface waters.

River (region)	Length (km) Basin area (km^2) Flow rate ($m^3 s^{-1}$)	Location	Position regarding WWTPs	Sample	Sampling date	Geographical coordinates
Tâmega River	164.5/3231/ 39.67	Chaves	upstream	U1	11-11-2014	41°43'06.3"N
				U2	12-11-2014	7°29'27.2"W
				U3	23-02-2015	
				U4	24-02-2015	
			downstream	D1	11-11-2014	41°42'57.6"N
				D2	12-11-2014	7°30'12.8"W
				D3	23-02-2015	
				D4	24-02-2015	
Tua River	40/560/39	Cachão (Mirandela)	upstream	U5	10-11-2014	41°23'30.6"N
				U6	11-11-2014	7°09'58.3"W
				U7	23-02-2015	
				U8	24-02-2015	
			downstream	D5	10-11-2014	41°23'02.2"N
				D6	11-11-2014	7°10'19.9"W
				D7	23-02-2015	
				D8	24-02-2015	
Mondego River	229/6 653/80	Figueira da Foz	upstream	U9	09-09-2014	40°07'49.3"N
				U10	10-09-2014	
				U11	11-03-2015	8°51'09.3"W
				U12	12-03-2015	
			downstream	D9	09-09-2014	40°08'24.3"N
				D10	10-09-2014	8°51'14.5"W
				D11	11-03-2015	
				D12	12-03-2015	
		Formoselha	downstream	D13	09-09-2014	40°10'58.8"N
				D14	10-09-2014	8°36'59.6"W
				D15	11-03-2015	
				D16	12-03-2015	
		Coimbra	upstream	U13	09-09-2014	40°13'09.1"N
				U14	10-09-2014	8°26'48.9"W
				U15	11-03-2015	
				U16	12-03-2015	
			downstream	D17	09-09-2014	40°13'00.0"N
				D18	10-09-2014	8°28'48.8"W
				D19	11-03-2015	
				D20	12-03-2015	
Trancão River	29/293/0.75	Frielas (Lisbon)	upstream	U17	18-09-2014	38°49'45.3"N
				U18	19-09-2014	9°08'36.0"W
				U19	09-03-2015	
			downstream	D21	18-09-2014	38°48'55.9"N
				D22	19-09-2014	9°09'15.7"W
				D23	09-03-2015	
Tagus River	891/80 906/315	Sacavém (Lisbon)	upstream	U20	19-02-2015	38°47'45.1"N
				U21	20-02-2015	9°05'28.2"W

Table 32. Characterization and geographical location of the surface waters. *(continued)*

River (region)	Length (km) Basin area (km^2) Flow rate ($m^3\ s^{-1}$)	Location	Position regarding WWTPs	Sample	Sampling date	Geographical coordinates
		Expo (Lisbon)	downstream	D24	19-02-2015	38°45'29.2"N
				D25	20-02-2015	9°05'27.1"W
		MAAT (Lisbon)	downstream	D26	19-02-2015	38°41'41.4"N
				D27	20-02-2015	9°11'47.5"W
Xarrama River	76/538/1	Évora	upstream	U22	13-09-2014	38°31'48.0"N
				U23	14-09-2014	7°55'19.4"W
				U24	14-03-2015	
				U25	15-03-2015	
			downstream	D28	13-09-2014	38°32'10.3"N
				D29	14-09-2014	7°54'26.0"W
				D30	14-03-2015	
				D31	15-03-2015	
Álamo Creek	na/na/0.75	Reguengos de Monsaraz	upstream	U26	13-09-2014	38°25'27.9"N
				U27	14-09-2014	7°30'02.9"W
				U28	14-03-2015	
				U29	15-03-2015	
			downstream	D32	13-09-2014	38°25'10.7"N
				D33	14-09-2014	7°28'59.0"W
				D34	14-03-2015	
				D35	15-03-2015	
Guadiana River	720/67 254/90	Vila Real de Santo António	upstream	U30	13-09-2014	37°12'49.5"N
				U31	14-09-2014	7°24'36.7"W
				U32	14-03-2015	
				U33	15-03-2015	
			downstream	D36	13-09-2014	37°12'08.3"N
				D37	14-09-2014	7°24'52.1"W
				D38	14-03-2015	
				D39	15-03-2015	

na – not available

Length and basin area in Portugal and Spain, since there are rivers that cross also Spain (Guadiana, Tagus and Tâmega rivers) [421]

Table 33. Gradient elution scheme.

TIME	% A	% B
0.00	90	10
3.00	90	10
3.10	55	45
5.00	55	45
8.00	15	85
9.00	15	85
9.10	5	95
14.00	5	95
14.10	90	10
30.00	90	10

Table 34. Retention time, product ions, ionization mode and collision energy.

Therapeutic group/Compound		Molecular Weight (g mol^{-1})	Retention time range (min)	Product ions	Ionization mode	Collision energy (%)
Antib		331.35 - 752.01	14.00 - 17.75			
AZI-D_3	D	752.01	17.69 - 17.75	594 (quantification) 576 (confirmation)	PI	27.0
AZI	PC	749.00	17.31 - 17.53	591 (quantification) 573 (confirmation)	PI	27.0
CLA	PC	747.97	17.39 - 17.57	590 (quantification) 558 (confirmation)	PI	27.0
ERY	PC	733.95	17.02 - 17.13	576 (quantification) 716 (confirmation)	PI	27.0
CIP-D_8	D	339.39	14.21 - 14.33	322 (quantification) 296 (confirmation)	PI	32.0
CIP	PC	331.35	14.00 - 14.15	314 (quantification) 288 (confirmation)	PI	32.0
Lip reg		250.34 - 424.60	15.50 - 16.81			
BEZ-D_4	D	365.84	16.20 - 16.29	320 (quantification) 280 (confirmation)	PI	35.0
BEZ	PC	361.83	15.99 - 16.21	316 (quantification) 276 (confirmation)	PI	35.0
GEM-D_6	D	256.37	15.63 - 15.77	121 (quantification) 239 (confirmation)	PI	33.0
GEM	PC	250.34	15.50 - 15.58	121 (quantification) 233 (confirmation)	PI	33.0
SIM-D_6	D	424.60	16.74 - 16.81	285 (quantification) 199 (confirmation)	PI	37.0
SIM	PC	418.58	16.41 - 16.54	285 (quantification) 199 (confirmation)	PI	37.0
Antiepi		236.28 – 246.33	15.18 – 15.48			
CAR-D_{10}	D	246.33	15.32 – 15.48	204 (quantification) 230 (confirmation)	PI	30.0
CAR	PC	236.28	15.18 – 15.27	194 (quantification) 220 (confirmation)	PI	30.0
SSRIs		291.06 - 330.43	10.55 - 13.94			
CIT-D_6	D	330.43	10.87 - 10.95	262 (quantification) 234 (confirmation)	PI	33.0
CIT	PC	324.16	10.77 - 10.85	262 (quantification) 234 (confirmation)	PI	33.0
N-CIT-D_3	D	313.38	10.65 - 10.72	262 (quantification) 109 (confirmation)	PI	37.0
N-CIT	M	310.15	10.55 - 10.62	262 (quantification) 109 (confirmation)	PI	37.0
FLU-D_5	D	314.36	11.54 - 11.59	153 (quantification) 122 (confirmation)	PI	30.0
FLU	PC	309.13	11.41 - 11.50	148 (quantification) 117 (confirmation)	PI	30.0
Nor-FLU-D_6	D	301.33	11.17 - 11.25	140 (quantification) 123 (confirmation)	PI	35.0
Nor-FLU	M	295.12	11.01 - 11.09	134 (quantification) 117 (confirmation)	PI	35.0
SER-D_3	D	309.25	13.88 - 13.94	275 (quantification) 159 (confirmation)	PI	35.0
SER	PC	305.07	13.78 - 13.85	275 (quantification) 159 (confirmation)	PI	35.0
Nor-SER-$^{13}C_6$	D	298.16	13.65 - 13.76	281 (quantification) 135 (confirmation)	PI	35.0

Table 34. Retention time, product ions, ionization mode and collision energy. *(continued)*

Therapeutic group/Compound		Molecular Weight (g mol^{-1})	Retention time range (min)	Product ions	Ionization mode	Collision energy (%)
Nor-SER	M	291.06	13.53 - 13.60	275 (quantification) 129 (confirmation)	PI	35.0
Anti-inf		109.13 - 318.10	5.29 - 8.96			
DIC-$^{13}C_6$	D	302.15	8.61 - 8.75	284 (quantification) 256 (confirmation)	PI	25.0
DIC	PC	296.15	8.47 - 8.59	278 (quantification) 250 (confirmation)	PI	25.0
4-OH-DIC-$^{13}C_6$	D	318.10	8.89 - 8.96	300 (quantification) 256 (confirmation)	PI	30.0
4-OH-DIC	M	312.15	8.78 - 8.86	294 (quantification) 250 (confirmation)	PI	30.0
IBU-D_3	D	209.30	6.45 - 6.51	164 (quantification) 192 (confirmation)	PI	31.0
IBU	PC	206.29	6.28 - 6.39	161 (quantification) 189 (confirmation)	PI	31.0
NAP	PC	230.26	7.11 - 7.26	185 (quantification) 213 (confirmation)	PI	31.0
PARA-D_4	D	155.19	6.13 - 6.19	138 (quantification) 114 (confirmation)	PI	21.0
PARA	PC	151.17	6.01 - 6.10	134 (quantification) 110 (confirmation)	PI	21.0
4-PARA	TP	109.13	5.29 - 5.33	92 (quantification) 93 (confirmation)	PI	21.0
Horm		270.37 – 296.41	9.01 - 9.83			
E2-D_5	D	277.41	9.31 - 9.39	185 (quantification) 147 (confirmation)	NI	39.0
E1	M	270.37	9.01 - 9.17	145 (quantification) 159 (confirmation)	NI	39.0
E2	PC	272.39	9.20 - 9.29	183 (quantification) 145 (confirmation)	NI	39.0
EE2	PC	296.41	9.75 - 9.83	185 (quantification) 159 (confirmation)	NI	39.0

Antib - antibiotics; Antiepi - antiepileptics; Anti-inf - anti-inflammatories; Horm - hormones; Lip reg - lipid regulators; M - metabolite; NI - negative ionization; PC – parent compound; PI - positive ionization; SSRIs - serotonin reuptake inhibitors; TP - transformation product.

Table 35. Analytical quality control for the quantification of each pharmaceutical, metabolite and transformation product in water spiked samples.

Therapeutic group/Compound	Matrix matched linearity (r^2)	MDL (ng L^{-1})	MQL (ng L^{-1})	ME (%)	Recovery (%)			RSD within-day (%)			RSD between-day (%)		
					50 ng L^{-1}	150 ng L^{-1}	250 ng L^{-1}	50 ng L^{-1}	150 ng L^{-1}	250 ng L^{-1}	50 ng L^{-1}	150 ng L^{-1}	250 ng L^{-1}
Antib													
AZI	0.9997	6.83	20.70	101.25	74.81	77.25	72.40	8.38	7.33	5.94	5.24	1.02	2.01
CLA	0.9996	6.43	19.49	100.00	80.02	77.61	82.75	6.81	4.08	3.82	2.49	1.52	0.71
ERY	0.9996	6.56	19.87	100.00	76.84	74.27	76.05	8.88	4.55	2.70	4.38	1.39	0.65
CIP	0.9996	7.36	22.29	100.00	85.26	85.58	87.37	4.20	4.13	0.92	3.26	4.03	0.59
Lip reg													
BEZ	0.9999	3.64	11.03	100.00	81.66	82.27	85.06	8.50	5.00	3.52	0.62	1.16	1.06
GEM	1	2.01	6.10	100.00	81.56	80.12	81.13	3.18	2.22	4.69	0.94	0.35	0.21
SIM	0.9999	3.49	10.58	98.81	81.55	81.09	76.61	2.71	3.87	4.03	0.93	0.72	0.36
Antiepi													
CAR	0.9996	7.19	21.80	100.00	77.33	78.79	77.59	3.06	3.64	4.16	1.23	0.82	0.67
SSRIs													
CIT	0.9997	5.81	17.60	100.00	96.45	98.84	97.42	2.30	0.45	2.50	0.42	0.25	1.69
N-CIT	0.9998	5.15	15.61	100.00	97.82	98.77	98.41	1.67	0.89	0.59	0.80	0.48	0.64
FLU	0.9998	5.08	15.39	100.00	97.72	99.01	99.51	1.57	0.69	2.34	0.65	0.31	0.97
Nor-FLU	0.9997	5.74	17.41	100.00	97.52	98.64	95.50	1.18	0.68	1.17	0.47	0.35	0.97
SER	0.9997	5.98	18.11	100.00	96.27	98.48	98.28	4.65	0.63	2.63	2.78	0.20	0.06
Nor-SER	0.9997	5.52	16.74	100.00	97.54	98.62	97.03	2.74	1.20	1.58	1.51	0.19	1.94

Table 35. Analytical quality control for the quantification of each pharmaceutical, metabolite and transformation product in water spiked samples. *(continued)*

Therapeutic group/Compound	Matrix matched linearity (r^2)	MDL (ng L^{-1})	MQL (ng L^{-1})	ME (%)	Recovery (%)			RSD within-day (%)			RSD between-day (%)		
					50 ng L^{-1}	150 ng L^{-1}	250 ng L^{-1}	50 ng L^{-1}	150 ng L^{-1}	250 ng L^{-1}	50 ng L^{-1}	150 ng L^{-1}	250 ng L^{-1}
Anti-inf													
DIC	0.9995	7.81	23.66	100.00	66.93	58.88	59.24	8.04	7.14	3.31	2.86	2.25	1.30
4-OH-DIC	0.9997	8.24	24.96	102.08	71.25	72.41	69.61	4.79	3.65	5.12	5.01	2.79	0.87
IBU	0.9997	7.07	21.41	101.89	71.60	67.10	66.98	5.47	2.70	2.64	7.15	0.94	0.48
NAP	0.9995	7.78	23.56	100.00	67.46	73.52	70.37	7.18	3.33	3.08	8.54	1.63	2.91
PARA	0.9995	7.74	23.44	100.00	67.98	68.86	69.78	6.82	5.57	4.83	7.87	1.39	5.56
4-PARA	0.9995	7.39	22.40	100.00	69.43	62.67	69.16	5.97	6.77	4.45	3.50	8.67	5.13
Horm													
E1	0.9997	6.35	19.23	100.00	64.78	69.86	67.77	5.71	5.09	2.58	3.91	2.75	0.83
E2	0.9996	7.24	21.94	100.00	69.97	67.69	67.02	4.68	5.19	4.44	2.37	1.29	2.31
EE2	0.9998	6.82	20.65	100.00	63.94	66.09	64.39	5.79	6.34	4.30	0.83	1.40	1.54

Antib - antibiotics; Antiepi - antiepileptics; Anti-inf - anti-inflammatories; Horm - hormones; Lip reg - lipid Regulators; MDL - method detection limit; ME - matrix effect; MQL - method quantification level; RSD - relative standard deviation; SSRIs - serotonin reuptake inhibitors.

Table 36. Occurrence of the selected pharmaceuticals in the different rivers, frequency, mean and standard deviation.

Therapeutic class				Antib				Lip reg			Antiepi	SSRIs						Anti-inf					Horm		
Pharmaceutical				AZI	CIP	CLA	ERY	BEZ	GEM	SIM	CAR	CIT	N-CIT	FLU	Nor-FLU	SER	Nor-SER	DIC	4-OH DIC	IBU	NAP	PARA	4-PARA	E1	E2	EE2
Tâmega River Chaves	up	11/11/2014	U1	nd	nd	24.80	nd	nd	nd	nd	nd	52.97	nd	nd	nd	nd	nd	27.17	nd	nd	nd	nd	nd	nd	nd	nd
		12/11/2014	U2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		23/02/2015	U3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		24/02/2015	U4	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	down	11/11/2014	D1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		12/11/2014	D2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		23/02/2015	D3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		24/02/2015	D4	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Total		Frequency		nd	nd	12.50	nd	nd	nd	nd	nd	12.50	nd	nd	nd	nd	nd	12.50	nd	nd	nd	nd	nd	nd	nd	nd
		Mean		nd	nd	24.80	nd	nd	nd	nd	nd	52.97	nd	nd	nd	nd	nd	27.17	nd	nd	nd	nd	nd	nd	nd	nd
Therapeutic group		Frequency		12.50				nd			nd	12.50						12.50					nd		
		Mean		24.80				nd			nd	52.97						nd					nd		
Tua River Cachão (Mirandela)	up	10/11/2014	U5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		11/11/2014	U6	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		23/02/2015	U7	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		24/02/2015	U8	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	down	10/11/2014	D5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		11/11/2014	D6	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		23/02/2015	D7	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		24/02/2015	D8	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Total		Frequency		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		Mean		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Therapeutic group		Frequency		nd				nd			nd	nd						nd					nd		
		Mean		nd				nd			nd	nd						nd					nd		
	up	09/09/2014	U9	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		10/09/2014	U10	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table 36. Occurrence of the selected pharmaceuticals in the different rivers, frequency, mean and standard deviation. *(continued)*

Therapeutic class				Antib				Lip reg			Antiepi	SSRIs						Anti-inf						Horm		
Pharmaceutical				AZI	CIP	CLA	ERY	BEZ	GEM	SIM	CAR	CIT	N-CIT	FLU	Nor-FLU	SER	Nor-SER	DIC	4-OH DIC	IBU	NAP	PARA	4-PARA	E1	E2	EE2
Mondego River		11/03/2015	U11	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		12/03/2015	U12	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Figueira da Foz	down	09/09/2014	D9	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		10/09/2014	D10	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		11/03/2015	D11	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		12/03/2015	D12	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Formoselha	down	09/09/2014	D13	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		10/09/2014	D14	nd	nd	nd	nd	15.52	nd	nd	nd	46.82	nd	nd	nd	nd	nd	26.15	nd	nd	nd	27.11	nd	nd	nd	nd
		11/03/2015	D15	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		12/03/2015	D16	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Coimbra	up	09/09/2014	U13	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		10/09/2014	U14	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		11/03/2015	U15	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		12/03/2015	U16	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	down	09/09/2014	D17	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		10/09/2014	D18	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		11/03/2015	D19	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		12/03/2015	D20	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Total		Frequency		nd	nd	nd	nd	5.00	nd	nd	nd	5.00	nd	nd	nd	nd	nd	5.00	nd	nd	nd	5.00	nd	nd	nd	nd
		Mean		nd	nd	nd	nd	15.52	nd	nd	nd	46.82	nd	nd	nd	nd	nd	26.15	nd	nd	nd	27.11	nd	nd	nd	nd
Therapeutic group		Frequency		nd				5.00			nd	5.00								5.00				nd		
		Mean		nd				15.52			nd	46.82								26.63				nd		
Trancão River	up	18/09/2014	U17	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		19/09/2014	U18	nd	nd	39.10	nd	nd	nd	nd	nd	43.71	nd	nd	nd	nd	nd	31.40	nd	nd	nd	43.76	nd	nd	nd	nd
		09/03/2015	U19	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table 36. Occurrence of the selected pharmaceuticals in the different rivers, frequency, mean and standard deviation. *(continued)*

Therapeutic class				Antib				Lip reg		Antiepi	SSRIs						Anti-inf						Horm		
Pharmaceutical				AZI	CIP	CLA	ERY	BEZ	GEM	SIM	CAR	CIT	N-CIT	FLU	Nor-FLU	SER	Nor-SER	DIC	4-OH DIC	IBU	NAP	PARA	4-PARA	E1	E2	EE2
Frielas (Lisbon)	down	18/09/2014	D21	35.66	nd	nd	38.80	nd	6.97	nd	11.45	20.70	nd	nd	nd	23.30	nd	47.42	nd	nd	nd	18.91	nd	nd	nd	nd
		19/09/2014	D22	nd	nd	39.05	nd	nd	nd	nd	nd	37.10	nd	nd	nd	nd	nd	31.93	nd	nd	nd	31.47	nd	nd	nd	nd
		09/03/2015	D23	nd	nd	34.45	nd	nd	nd	nd	nd	28.87	nd	nd	nd	nd	nd	27.46	nd	nd	nd	27.79	nd	nd	nd	nd
Total		Frequency		16.67	nd	50.00	16.67	nd	16.67	nd	16.67	66.67	nd	nd	nd	16.67	nd	66.67	nd	nd	nd	66.67	nd	nd	nd	nd
		Mean		35.66	nd	37.53	38.80	nd	6.97	nd	11.45	32.60	nd	nd	nd	23.30	nd	34.55	nd	nd	nd	30.48	nd	nd	nd	nd
Therapeutic group		Frequency				66.67			16.67		16.67			66.67						66.67					nd	
		Mean				37.41			6.97		11.45			30.74						nd					nd	
Tagus River Sacavém (Lisbon)	up	19/02/2015	U20	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		20/02/2015	U21	nd	nd	35.77	34.85	11.86	10.34	nd	nd	28.80	nd	nd	nd	nd	nd	37.25	nd	nd	nd	27.27	nd	nd	nd	nd
Expo (Lisbon)	down	19/02/2015	D24	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		20/02/2015	D25	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
MAAT (Lisbon)	down	19/02/2015	D26	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		20/02/2015	D27	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Total		Frequency		nd	nd	16.67	16.67	16.67	16.67	nd	nd	16.67	nd	nd	nd	nd	nd	16.67	nd	nd	nd	16.67	nd	nd	nd	nd
		Mean		nd	nd	35.77	34.85	11.86	10.34	nd	nd	28.80	nd	nd	nd	nd	nd	37.25	nd	nd	nd	27.27	nd	nd	nd	nd
Therapeutic group		Frequency				16.67			16.67		nd			16.67						16.67					nd	
		Mean				35.31			nd		nd			28.80						32.26					nd	
Xarrama River	up	13/09/2014	U22	32.15	nd	nd	nd	nd	nd	nd	nd	39.96	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		14/09/2014	U23	nd	nd	35.46	nd	nd	nd	nd	nd	41.08	nd	nd	nd	nd	nd	nd	nd	nd	nd	52.05	nd	nd	nd	nd
		14/03/2015	U24	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		15/03/2015	U25	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Évora	down	13/09/2014	D28	nd	nd	25.21	nd	nd	nd	nd	nd	42.42	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		14/09/2014	D29	nd	nd	25.72	nd	nd	nd	nd	nd	51.82	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		14/03/2015	D30	nd	nd	32.64	nd	nd	nd	nd	nd	31.84	nd	nd	nd	nd	nd	nd	nd	nd	nd	27.05	nd	nd	nd	nd
		15/03/2015	D31	nd	nd	36.87	nd	nd	7.86	nd	nd	39.82	nd	nd	nd	nd	nd	29.61	nd	nd	nd	43.75	nd	nd	nd	nd

Table 36. Occurrence of the selected pharmaceuticals in the different rivers, frequency, mean and standard deviation. *(continued)*

Therapeutic class				Antib				Lip reg		Antiepi	SSRIs						Anti-inf					Horm		
Pharmaceutical				AZI	CIP	CLA	ERY	BEZ	GEM	SIM	CAR	CIT	N-CIT	FLU	Nor-FLU	SER	Nor-SER	DIC	4-OH DIC	IBU	NAP	PARA	4-PARA	E1	E2	EE2
Total		Frequency		12.50	nd	62.50	nd	nd	12.50	nd	nd	75.00	nd	nd	nd	nd	nd	12.50	nd	nd	nd	37.50	nd	nd	nd	nd
		Mean		32.15	nd	31.18	nd	nd	7.86	nd	nd	41.16	nd	nd	nd	nd	nd	29.61	nd	nd	nd	40.95	nd	nd	nd	nd
Therapeutic group		Frequency				75.00			12.50		nd			75.00							37.50				nd	
		Mean				31.34			7.86		nd			41.16							38.12				nd	
Álamo Creek	up	13/09/2014	U26	nd	nd	nd	nd	nd	nd	nd	nd	38.54	nd	nd	nd	nd	nd	29.07	nd	nd	nd	48.95	nd	nd	nd	nd
		14/09/2014	U27	nd	nd	35.80	nd	nd	nd	nd	nd	41.86	nd	nd	nd	nd	nd	nd	nd	nd	nd	69.15	nd	nd	nd	nd
		14/03/2015	U28	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		15/03/2015	U29	nd	nd	25.33	nd	nd	6.69	nd	nd	35.42	nd	nd	nd	nd	nd	25.13	nd	nd	nd	59.40	nd	nd	nd	nd
Reguengos de Monsaraz	down	13/09/2014	D32	nd	nd	38.32	32.89	nd	nd	nd	nd	34.00	nd	nd	nd	nd	nd	33.23	nd	nd	nd	26.34	nd	nd	nd	nd
		14/09/2014	D33	nd	nd	nd	nd	nd	nd	nd	nd	39.34	nd	nd	nd	nd	nd	27.99	nd	nd	nd	nd	nd	nd	nd	nd
		14/03/2015	D34	nd	nd	30.35	nd	nd	7.04	nd	nd	48.46	nd	nd	nd	nd	nd	51.24	nd	nd	nd	nd	nd	nd	nd	nd
		15/03/2015	D35	nd	nd	37.32	nd	nd	nd	nd	nd	40.63	nd	25.37	nd	nd	nd	44.82	nd	nd	nd	38.68	nd	nd	nd	nd
Total		Frequency		nd	nd	62.50	12.50	nd	25.00	nd	nd	87.50	nd	12.50	nd	nd	nd	75.00	nd	nd	nd	62.50	nd	nd	nd	nd
		Mean		nd	nd	33.42	32.89	nd	6.87	nd	nd	39.75	nd	25.37	nd	nd	nd	35.25	nd	nd	nd	48.50	nd	nd	nd	nd
Therapeutic group		Frequency				62.50			25.00		nd			87.50							87.50				nd	
		Mean				33.34			6.87		nd			37.95							41.27				nd	
Guadiana River	up	13/09/2014	U30	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		14/09/2014	U31	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		14/03/2015	U32	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Vila Real de Santo António		15/03/2015	U33	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	down	13/09/2014	D36	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		14/09/2014	D37	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		14/03/2015	D38	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		15/03/2015	D39	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Total		Frequency		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		Mean		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Therapeutic group		Frequency				nd			nd		nd			nd							nd				nd	

Table 36. Occurrence of the selected pharmaceuticals in the different rivers, frequency, mean and standard deviation. *(continued)*

Therapeutic class		**Antib**				**Lip reg**			**Antiepi**	**SSRIs**						**Anti-inf**						**Horm**		
Pharmaceutical		**AZI**	**CIP**	**CLA**	**ERY**	**BEZ**	**GEM**	**SIM**	**CAR**	**CIT**	**N-CIT**	**FLU**	**Nor-FLU**	**SER**	**Nor-SER**	**DIC**	**4-OH DIC**	**IBU**	**NAP**	**PARA**	**4-PARA**	**E1**	**E2**	**EE2**
	Mean	nd				nd			nd	nd								nd					nd	
Total pharmaceutical	Frequency	2.78	nd	20.83	4.17	2.78	6.94	nd	1.39	27.78	nd	1.39	nd	1.39	nd	19.44	nd	nd	nd	19.44	nd	nd	nd	nd
	Min	32.15	nd	24.80	32.89	11.86	6.69	nd	11.45	20.70	nd	25.37	nd	23.30	nd	25.13	nd	nd	nd	18.91	nd	nd	nd	nd
	Max	35.66	nd	39.10	38.80	15.52	10.34	nd	11.45	52.97	nd	25.37	nd	23.30	nd	51.24	nd	nd	nd	69.15	nd	nd	nd	nd
	Mean	33.91	nd	33.08	35.51	13.69	7.78	nd	11.45	39.21	nd	25.37	nd	23.30	nd	33.56	nd	nd	nd	38.69	nd	nd	nd	nd
	SD	2.48	nd	5.39	3.01	2.59	1.50	nd	nd	7.93	nd	nd	nd	nd	nd	8.43	nd	nd	nd	14.69	nd	nd	nd	nd
Total therapeutic group	Frequency	23.61				8.33			1.39	27.78								23.61					nd	
	Min	24.80				6.69			11.45	20.70								18.91					nd	
	Max	39.10				15.52			11.45	52.97								69.15					nd	
	Mean	33.53				9.47			11.45	37.86								36.13					nd	
	SD	4.84				3.31			nd	8.73								12.04					nd	
Total	Frequency	27.78																						
	Min	6.69																						
	Max	69.15																						
	Mean	33.24																						
	SD	12.04																						

Antib - antibiotics; Antiepi - antiepileptics; Anti-inf - anti-inflammatories; Horm - hormones; nd - not detected; Lip reg - lipid regulators; SSRIs - serotonin reuptake inhibitors.

Part C – Final remarks and future perspectives

Since global population and pharmaceutical consumption continues to rise, the issue of the presence in aquatic environment of pharmaceuticals is a pressing subject. Therefore, the work described in this thesis provides additional data regarding the presence of pharmaceuticals in the Portuguese aquatic environment, namely in wastewaters and surface waters.

A careful literature review was conducted in order to understand the sources and fate, occurrence, toxicity and environmental risk assessment (ERA) of pharmaceuticals in the above mentioned matrices. In this context, a broad and highly specialized background was obtained, enabling a complete overview of the state of the art in these subjects.

Three main topics regarding the pharmaceuticals presence in the aquatic environment were studied. Firstly, the occurrence, fate, geographical and seasonal influence and ERA of different pharmaceuticals were studied in wastewater treatment plants (WWTPs) across Portugal. This was performed through solid phase extraction (SPE) and liquid chromatography coupled with tandem mass detection (LC-MS*n*). The results clearly showed that wastewater influent (WWI) samples presented higher frequencies and contamination levels. Also, all samples were contaminated with concentrations up to 150 and 32 μg L^{-1} for WWIs and wastewater effluents (WWEs), respectively, evidencing that WWTPs are not capable to completely remove these pharmaceuticals. Higher mass loads in WWEs in the winter season, Center and Algarve regions were observed, supporting that geographical and seasonal variations do occur, influenced by variations in population and meteorological conditions. In line with the Directive 2013/39/EU, the most impacted rivers by WWEs (Mondego, Tagus, Ave, Trancão, Fervença and Xarrama) were also selected for future monitoring stations in surface waters. Risk quotients (RQs) higher than 1 were observed for 7 pharmaceuticals in WWEs, presenting an ecotoxicological pressure for the three aquatic trophic levels.

Secondly, based on the occurrence of pharmaceuticals in WWEs, and gathering additional information on consumption, excretion, WWTPs removal and wastewater produced by the Portuguese population, a critical evaluation on the European guideline on ERA was performed. Improvements to this legislation were suggested, such as, changes in the penetration factor value and inclusion of consumption and excretion data to provide more accurate predicted environmental concentrations (PECs). Also, since the majority of prioritization lists of pharmaceuticals are based on the ERA concept, these suggestions can be used to better assess which pharmaceuticals ought to be studied in the aquatic environment.

Finally, the work done in this doctoral project evaluated the presence of pharmaceuticals in the selected surface waters most impacted by WWEs. In this aquatic compartment, 11 pharmaceuticals were found with concentrations up to 69.2 ng L^{-1}. The impact of WWEs in

surface waters was observed, with a 21.4% increase in concentrations downstream WWTPs. On the contrary to wastewaters, due to lower flow rates, summer was the season with higher concentrations. This feature also influenced the concentrations in each river, with small rivers, with lower flow rates, presenting higher concentrations. Additionally, in drought periods, flow rates can decrease about ten times, with an expected increase in concentrations. Performing the ERA, using the expected concentrations in drought periods, RQs higher than one were found for five pharmaceuticals.

The results obtained in this thesis evidence that concentrations of pharmaceuticals are present, in decreasing order, in WWIs, WWEs and surface waters. Moreover, the results show that WWTPs are a source of pharmaceuticals contamination, and that surface water concentrations are strongly influenced by river flow rates, not only by their average flow or their normal seasonal variations but also by the escalating problem of water scarcity, that can promote levels of pharmaceuticals that can enable RQs higher than one in some Portuguese rivers.

The pressure of pharmaceuticals on aquatic bodies will continue to rise, and therefore, it is important to further assess other matrices to evaluate the complete extent of pharmaceuticals occurrence and risk in the aquatic environment. Since the contaminated wastewaters and surface waters drain into the sea, seawater should be evaluated. However, the expected low concentrations and the sensitivity of current analytical methodologies can make it difficult to assess this matrix and thus, the use of bioindicators, such as bivalves, ought to be considered, since they bioconcentrate these compounds allowing their easier detection.

Other possible outcome of the presence of pharmaceuticals in the aquatic environment is the possibility of reaching groundwaters and drinking waters. Therefore, groundwaters and drinking water treatment plant influents and effluents should also be evaluated, to realize the contribution of these facilities in the removal of pharmaceuticals. Finally, tap waters and mineral waters ought to be assessed, in order to evaluate the risk for humans. In this way, the complete scenario of the contamination of pharmaceuticals in the Portuguese aquatic environment and their risk could be acquired, contributing to future improvements in minimization measures and legislation.

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