Micropollutants in aquaculture: constructed wetlands and advanced oxidation technologies as prospective treatments

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Other specific acknowledgements are included in the published version of each Chapter (Appendix A).

Statement of Originality

I certify that this work does not contain any material that has been nor will be used for the award of any other degree or diploma in my name or anyone, in any university or institution. In addition, I certify that, to the best of my knowledge, this work does not contain any material previously published or written by another person, except where due reference has been made in the text. Accordingly, all the contents of the work herein presented are exclusively from my own with the collaborations mentioned in the thesis. The full agreement of the co-authors on the inclusion of the manuscripts in the present thesis can be found in Appendix B.

Ana Margarida Gorito Gonçalves

A dúvida é o princípio da sabedoria

- Aristóteles -

Aos meus,
Pai.
Mãe.
Duarte.

- Têm o meu ♡ -

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Thesis outline

The present manuscript describes the PhD project entitled "*Micropollutants in aquaculture: constructed wetlands and advanced oxidation technologies as prospective treatments*", which has been performed between 2017 and 2022 under the Doctoral Programme in Environmental Engineering (PDEA) at the Faculty of Engineering of the University of Porto (FEUP) and supported by FCT. This PhD project has been implemented at the Associate Laboratory of Separation and Reaction Engineering – Laboratory of Catalysis and Materials (LSRE-LCM/FEUP), under the supervision of Professor Adrián M.T. Silva and Doctor Ana Rita L. Ribeiro, and at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) under the supervision of Doctor Cristina Marisa R. Almeida.

The manuscript consists of 8 chapters: general introduction to the topic (*Chapter 1*); state-of-the-art, motivation and objectives of the work (*Chapter 2*); experimental studies developed on the investigated water treatment technologies (*Chapters 3, 4, 5, and 6*); final remarks (*Chapter 7*), and main conclusions and recommended future work (*Chapter 8*). The information contained in *Chapters 2* (*sub-chapters 2.1.1 and 2.1.3*) to *6* is based on manuscripts published [1].

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[1]

Chapter 2.1.1. Gorito, A.M., Ribeiro, A.R., Almeida, C.M.R, Silva, A.M.T. *A review on the application of constructed wetlands for the removal of priority substances and contaminants of emerging concern listed in recently launched EU legislation*, Environmental Pollution, 227 (2017) 428-443.

Chapter 2.1.3. Gorito, A.M., Ribeiro, A.R.L., Pereira, M.F.R., Almeida, C.M.R, Silva, A.M.T. *Advanced oxidation technologies and constructed wetlands in aquaculture farms: What do we know so far about micropollutant removal?* Environmental Research, 204 (2022) 111955.

Chapter 3. Gorito, A.M., Ribeiro, A.R., Gomes, Carlos Rocha., Almeida, C.M.R, Silva, A.M.T. *Constructed wetland microcosms for the removal of organic micropollutants from freshwater aquaculture effluents*, Science of the Total Environment, 644 (2018) 1171-1180.

Chapter 4. Gorito, A.M., Barbosa, M.O., Almeida, C.M.R, Pereira, M.F.R., Silva, A.M.T., Ribeiro, A.R.L. *Quenchers in advanced oxidation technologies for analysis of micropollutants by liquid chromatography coupled to mass spectrometry: Sodium sulphite or catalase?*, Science of the Total Environment, 692 (2019) 995-1004.

Chapter 5. Gorito, A.M., Pesqueira, J.F.J.R., Moreira, N.F.F, Ribeiro, A.R., Pereira, M.F.R., Nunes, O.C., Almeida, C.M.R., Silva, A.M.T. *Ozone-based water treatment (O_3, O_3/UV, O_3/H_2O_2) for removal of organic micropollutants, bacteria inactivation and regrowth prevention*, Journal of Environmental Chemical Engineering, 9 (2021) 105315.

Chapter 6. Gorito, A.M., Rodrigues, P., Ribeiro, A.R.L., Pereira, M.F.R., Guimarães, L., Almeida, C.M.R., Silva, A.M.T. *Antibiotics removal from aquaculture effluents by ozonation: chemical and toxicity descriptors*, Water Research, 218 (2022) 118497.

Abstract

The presence of organic micropollutants (MPs) in the aquatic environment (usually between ng L^{-1} and µg L^{-1}) and their potential adverse effects to wildlife and human health are worldwide issues of increasing concern. Most of these compounds are not effectively eliminated in conventional water and wastewater treatment plants (WWTPs), being discharged into receiving waters bodies and spread in the environment. For this reason, the European Union (EU) has listed several MPs (namely priority substances (PSs) in Directive 2013/39/EU and contaminants of emerging concern (CECs) in Decisions 2015/495, 2018/840 and 2020/1161), which must be monitored in surface waters (SW) within the EU. Moreover, these documents underline the need to develop innovative water/wastewater treatment technologies to remove them.

MPs encompass several classes of substances that can reach the environment from diverse sources, as is the case of aquaculture. The artificial food and medication used in this type of food industry can originate MPs in the resulting effluents. Moreover, the water supplying these activities can also contain MPs. In this scenario, these compounds can be present in both aquaculture influents and effluents and in the resulting food products, impacting the environment and posing risks for wildlife and human health. Appropriate treatment processes to remove MPs are thus needed in aquaculture farms. With this in mind, the main technological goal of this PhD project was to develop a treatment solution based on the application of constructed wetlands (CWs) and/or advanced oxidation technologies (AOTs) to remove organic MPs from aquaculture waters.

Initially, this PhD comprised an extensive literature survey to gain knowledge on the application of CWs and AOTs for MPs elimination. Both technologies have potentialities to remove MPs, but information on aquaculture waters was limited to 34 studies, and combined CWs-AOTs or AOTs-CWs systems were not investigated in these reports.

The acquired know-how on CW systems highlighted the need to extend studies for more PSs and CECs, mainly when occurring as mixtures and in realistic (*i.e.*, not simulated) matrices. Therefore, one of the first parts of this PhD project was dedicated to evaluate the performance of CWs for removing a mixture of multi-class PSs and CECs from aquaculture effluents. The CW microcosms

were assembled according to the information available in the literature, attempting to maximize the removal of a larger number of MPs. Thus, vertical subsurface flow systems (VSSF) planted with *Phragmites australis* operating automatically under batch mode for 7 days led to excellent results, with most target MPs (in spiked and non-spiked influents) being totally removed during 1-month of CWs functioning. Interestingly, 9 of the 35 studied MPs were detected in the collected aquaculture water samples, evidencing the concern regarding the occurrence of these compounds in this type of food industry.

While CWs require investigation for more PSs and CECs, studies on AOTs lack more information of operating conditions resembling those occurring in real situations (*i.e.,* natural aqueous matrices, environmentally relevant concentrations, suitable treatment times, reasonable quantities of reagents, among others). For this reason, the second stage of this PhD was focused on the assessment of AOTs for removing organic MPs in conditions as close as possible to those of on-field realistic scenarios. Unexpectedly, the first trials of AOTs with ultraviolet radiation and hydrogen peroxide (UV/H_2O_2) resulted in an analytical limitation that hampered the accurate quantification of MPs. In this context, additional experiments revealed that the use of sodium sulphite (Na_2SO_3) as quencher of residual H_2O_2 triggered a significant suppression on the signal of basic MPs ($pK_a > 5.9$) monitored under positive ionization (PI) mode in liquid chromatography tandem mass spectrometry (LC-MS/MS). Thus, the use of catalase as quencher of oxidizing agents in AOTs studies (*e.g.*, H_2O_2 and ozone (O_3)) should be preferred because it did not lead to interferences on MPs analysis. UV and ozonation are among the most studied AOTs for MPs removal, and thus UV/H_2O_2, O_3, O_3/UVA-light-emitting diodes (LEDs), and O_3/H_2O_2 were assessed in a flow-through reactor operating in continuous mode to remove organic MPs from spiked SW. It was perceptible that UV/H_2O_2 was the less effective process (< 21%) in opposition to all the O_3-based treatments that showed high removals of all MPs (> 85%), except perfluorooctanesulfonic acid (PFOS) (< 50%). Among the tested processes, single ozonation represented the more ecofriendly and economical solution because it did not require radiation or other oxidants, apart from O_3, to achieve removals of MPs similar to those obtained in the other O_3-based AOTs.

Nowadays, O_3 is often used in drinking water treatment plants (DWTPs) for disinfection, and thus, the subsequent O_3 experiments were conducted applying conditions described in the literature for this purpose (*i.e.*, 1.5 or 3 mg O_3 L^{-1}; hydraulic retention time (HRT) of 10 min). Based on this information, beyond MPs elimination, O_3 was also investigated to simultaneously inactivate microorganisms (*Escherichia coli*, enterococci, and total heterotrophs) in the treatment of a SW matrix. The bacteriologic component proved to be the decisive factor to establish the O_3 dose, since the lowest studied concentration (1.5 mg O_3 L^{-1}) did not allow to comply with the established limits for target bacteria, when considering the regrowth occurring in treated water stored for 3 days. In this sense, continuous ozonation at 3 mg O_3 L^{-1} during 10 min of HRT resulted in a safe inactivation of bacteria and in excellent removals of MPs (> 85%), except PFOS, whereas 1.5 mg O_3 L^{-1} was enough for the removal of MPs only.

Intensive aquaculture generally includes the use of antibiotics to prevent bacterial infections of fish and, for this reason, next studies on single ozonation were extended to a specific group of 5 antibiotics typically applied in these farms (florfenicol, oxytetracycline, sulfadimethoxine, sulfamethoxazole, and trimethoprim). Hence, O_3 experiments were conducted at continuous mode with the same HRT (10 min) and applying the lowest O_3 concentration tested (1.5 mg L^{-1}). All antibiotics were highly removed from aquaculture effluents (> 98%), except florfenicol (< 68%). Complementary studies showed that florfenicol was poorly receptive to direct oxidation by molecular O_3, being predominately eliminated by hydroxyl radicals ($HO^{\bullet}$). The water matrix under treatment had a great influence on florfenicol removal because it might control the generation and/or catching of $HO^{\bullet}$. Other chemical descriptors under analysis showed that although O_3 was efficient to remove the parent antibiotics, it was not as efficient to eliminate the by-products of ozonation. This emphasises the importance of evaluating ecotoxicity before and after AOTs. In the study carried out, toxicity tests with zebrafish embryos confirmed that the water toxicity did not increase with ozonation.

Concluding, both CWs and AOTs technologies were highly efficient to remove organic MPs from aquaculture effluents. However, depending on their specific characteristics, they can be useful for different types of aquaculture farms. CWs as a “green” and low-cost technology are recommended

for rural aquaculture farms, which generally have a simple process without water recirculation and do not include complex water treatments. Contrariwise, AOTs (specifically O_3) as a more expensive and developed alternative, are suggested for modernised facilities that generally involve water recirculation and equipment to control the quality of water. Interestingly, the combination of CWs and AOTs is not an expected option for the removal of MPs since they are both efficient in this regard, and because the advantages of the combined system might not be substantial enough considering the high investment that is expected to be required.

Summarizing, this PhD thesis contributed to expand the knowledge on the potential of CWs and AOTs application for removing MPs, having embraced critical gaps identified in this research field. Potential solutions were proposed, needing further investigation at full scale, which can contribute to a more sustainable aquaculture sector able to minimize the risks associated with the presence of MPs in their waters.

Resumo

A presença de micropoluentes orgânicos no ambiente aquático (geralmente entre ng e μg L^{-1}) e os seus potenciais efeitos adversos para a fauna, flora, e saúde humana são assuntos de crescente preocupação a nível mundial. A maioria destes compostos não são eficazmente eliminados nas estações de tratamento de águas e águas residuais convencionais, sendo descarregados no meio aquático recetor e disseminados no meio ambiente. Por este motivo, a União Europeia (UE) tem identificado vários micropoluentes (nomeadamente substâncias prioritárias na Diretiva 2013/39/EU e poluentes emergentes nas Decisões 2015/495, 2018/840 e 2020/1161) que devem ser monitorizados em águas de superfície da UE. Estes documentos também reforçam a necessidade de desenvolver novas tecnologias de tratamento de água/água residual capazes de remover estes contaminantes.

Os micropoluentes incluem várias classes de substâncias que chegam ao ambiente a partir de diversas origens, como é o caso das aquaculturas. A alimentação e medicação artificial fornecida nesta indústria pode conduzir à presença de micropoluentes nos seus efluentes. Além disso, a própria água que abastece estas unidades também pode conter micropoluentes. Neste cenário, estes compostos podem estar presentes tanto nos seus influentes e efluentes, como nos produtos alimentares resultantes, representando um risco para o meio ambiente e ecossistemas associados, assim como para a saúde humana. São assim necessários tratamentos adequados para a remoção de micropoluentes nas unidades aquícolas. Com este propósito, o principal objetivo deste projeto de doutoramento foi identificar uma solução de tratamento baseada na aplicação de leitos de macrófitas e/ou tecnologias avançadas de oxidação para remover micropoluentes orgânicos das águas de aquacultura.

Numa fase inicial, este projeto de doutoramento incluiu uma extensa pesquisa na literatura para adquirir conhecimento sobre a aplicação de leitos de macrófitas e tecnologias avançadas de oxidação para remover micropoluentes. Ambas as tecnologias demonstraram ter potencial para remover estes compostos, embora a informação para águas de aquacultura esteja limitada a 34 estudos, os quais ainda não contemplam a aplicação combinada das duas tecnologias.

O conhecimento adquirido em relação aos leitos de macrófitas destacou a necessidade de alargar os estudos a mais substâncias prioritárias e compostos de preocupação emergente, sobretudo em mistura e matrizes reais (*i.e.*, não sintéticas). Portanto, uma das primeiras fases deste doutoramento foi dedicada a avaliar o desempenho dos leitos de macrófitas para remover uma mistura de várias classes destes compostos de efluentes de aquacultura. Os leitos de macrófitas à escala laboratorial foram construídos de acordo com a informação disponível na literatura, para tentar potenciar a remoção dos micropoluentes. Assim, sistemas com fluxo vertical subterrâneo plantados com *Phragmites australis*, operando automaticamente em modo descontínuo durante sete dias, conduziram a excelentes resultados para a maioria dos compostos em análise (incluindo influentes com e sem dopagem), que foram totalmente removidos durante um mês de funcionamento dos sistemas. Foi interessante constatar que 9 dos 35 compostos alvo foram detetados nas amostras de água de aquacultura, destacando a preocupação relativa à presença destes compostos neste tipo de indústria alimentar.

Enquanto os leitos de macrófitas requerem investigação para um maior número de substâncias prioritárias e poluentes emergentes, os estudos focados nas tecnologias avançadas de oxidação necessitam de mais informação visando condições operacionais semelhantes àquelas que ocorrem em situações reais (nomeadamente matrizes aquosas naturais, concentrações relevantes a nível ambiental, tempos de tratamento adequados, quantidade de reagentes razoável, entre outras). Por esta razão, a segunda etapa deste doutoramento focou-se na avaliação das tecnologias avançadas de oxidação para remover micropoluentes em condições tanto mais próximas quanto possível de cenários reais. Inesperadamente, o primeiro conjunto de experiências com radiação ultravioleta e peróxido de hidrogénio (UV/H_2O_2) resultou numa limitação analítica que impediu a correta quantificação dos micropoluentes. Neste contexto, experiências posteriores revelaram que o uso de sulfito de sódio (Na_2SO_3) como inibidor do H_2O_2 remanescente causou uma supressão significativa no sinal dos micropoluentes mais básicos ($pK_a > 5.9$) e monitorizados sob ionização positiva por cromatografia líquida acoplada à espectrometria de massa. Deste modo, deve preferir-se a utilização de catalase como inibidor de possíveis oxidantes aplicados em

tecnologias avançadas de oxidação (p. ex. H_2O_2 e ozono (O_3)), uma vez que a mesma não interferiu na análise dos micropoluentes.

Processos com UV e O_3 estão entre as tecnologias avançadas de oxidação mais estudadas para a remoção de micropoluentes e, por isso, UV/H_2O_2, O_3, O_3/UVA-LEDs e O_3/H_2O_2 foram avaliados num reator de fluxo em modo contínuo para remover micropoluentes orgânicos de águas de superfície dopadas. Foi percetível que o UV/H_2O_2 foi o processo menos eficaz (< 21%), ao contrário de todos os outros baseados na aplicação de O_3 que apresentaram remoções de micropoluentes elevadas (> 85%), com exceção do ácido perfluoro-octanessulfônico (PFOS) (< 50%). De entre os processos estudados, a ozonização aplicada isoladamente foi a solução mais ecológica e económica, porque não exigiu radiação ou outros oxidantes para além do O_3, para registar remoções de micropoluentes similares às obtidas nos restantes tratamentos derivados do O_3.

Nos dias de hoje, o O_3 é frequentemente aplicado em estações de tratamento de água para desinfeção e, por isso, os estudos subsequentes com O_3 foram realizados aplicando condições descritas na literatura para este propósito (*i.e.*, 1,5 ou 3 mg O_3 L^{-1}; tempo de retenção hidráulico de 10 min). Com base nesta informação, para além da eliminação dos micropoluentes, o O_3 foi simultaneamente estudado para inativar microrganismos (*Escherichia coli*, enterococcus e heterotróficos totais) de uma matriz de água de superfície. A componente bacteriológica demonstrou ser o fator preponderante para estabelecer a dose de O_3, uma vez que a concentração mais baixa que foi testada (1,5 mg O_3 L^{-1}) não permitiu cumprir com os limites estabelecidos para as bactérias em análise, quando considerado um possível recrescimento dos microrganismos na água tratada que foi armazenada durante 3 dias. Neste sentido, a ozonização com 3 mg O_3 L^{-1} durante um tempo de retenção hidráulico de 10 minutos, resultou numa inativação segura das bactérias alvo e excelente remoção dos micropoluentes (> 85%), menos o PFOS, enquanto 1,5 mg O_3 L^{-1} foi suficiente apenas para remover os micropoluentes.

A aquacultura intensiva geralmente inclui o uso de antibióticos para prevenir infeções bacterianas nos peixes e, por isso, os estudos seguintes envolvendo ozonização foram alargados a um grupo específico de 5 antibióticos frequentemente aplicados nestas indústrias (florfenicol, oxitetraciclina, sulfadimetoxina, sulfametoxazol e trimetoprim). Assim, os estudos com O_3 foram conduzidos em

modo contínuo aplicando o mesmo tempo de retenção hidráulico de 10 minutos, e a concentração de O_3 mais baixa testada (1,5 mg L^{-1}). Todos os antibióticos foram significativamente removidos dos efluentes de aquacultura (> 98%), excetuando o florfenicol (< 68%). Estudos complementares mostraram que o florfenicol é pouco recetivo à oxidação direta pelo O_3 molecular, sendo predominantemente eliminado por radicais hidroxilo ($HO^{\bullet}$). A matriz aquosa em tratamento acabou por ter uma forte influência na remoção do florfenicol, uma vez que esta pode controlar a formação e/ou captura dos $HO^{\bullet}$. Outros descritores químicos em análise demostraram que apesar do O_3 ter sido eficiente a remover os antibióticos parentais, não foi tão eficiente na eliminação dos subprodutos de ozonização. Isto destaca a importância de avaliar a ecotoxicidade antes e depois dos tratamentos avançados de oxidação. No estudo realizado, os testes de toxicidade com embriões de peixe zebra confirmaram que a toxicidade da água não aumentou depois da ozonização.

Concluindo, os leitos de macrófitas e as tecnologias avançadas de oxidação foram altamente eficazes na remoção de micropoluentes orgânicos de efluentes de aquacultura. No entanto, dependendo das suas características específicas, estes processos podem ser úteis para diferentes tipos de aquacultura. Os leitos de macrófitas sendo uma tecnologia “verde” e de baixo custo são recomendados para aquaculturas rurais, que em geral têm um funcionamento simples, sem recirculação de água, e não incluem tratamentos complexos da mesma. Pelo contrário, as tecnologias avançadas de oxidação (neste caso o O_3), sendo alternativas mais caras e desenvolvidas, são sugeridas para aquaculturas modernas que normalmente envolvem recirculação de água e equipamentos para controlar a sua qualidade. Curiosamente, a combinação dos 2 processos referidos não é uma opção expectável para remover micropoluentes, porque ambos são individualmente eficientes na remoção destes compostos, e as vantagens do sistema combinado podem não ser tão substanciais considerando o elevado investimento expectável.

Em suma, esta tese de doutoramento contribuiu para expandir o conhecimento sobre o potencial dos leitos de macrófitas e tecnologias avançadas de oxidação para remover micropoluentes, tendo abordado lacunas que têm sido identificadas neste campo de investigação. Foram propostas

potenciais soluções para o problema em estudo, que carecem de investigação adicional à escala real, mas que podem contribuir para um sector aquícola mais sustentável capaz de minimizar os riscos associados à presença de micropoluentes nas suas águas.

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List of Abbreviations

AOPs	Advanced oxidation processes
AOTs	Advanced oxidation technologies
ARB	Antibiotic-resistant bacteria
ARGs	Antibiotic resistance genes
BEA	Bile Esculin Azide Agar
BOD	Biological oxygen demand
CCA	Chromogenic Coliform Agar
CECs	Contaminants of emerging concern
COD	Chemical oxygen demand
CWs	Constructed wetlands
DAD	Diode array detection
DEHP	Di(2-ethylhexyl)phthalate
DOC	Dissolved organic carbon
DW	Drinking water
DWTPs	Drinking water treatment plants
E1	Estrone
E2	Beta-oestradiol
E3	Oestriol
EE2	17-alpha-ethinyloestradiol
EHMC	2-ethylhexyl-4-methoxycinnamate
EQS	Environmental quality standard
ESI	Electrospray ionization
EU	European Union

FD	Fluorescence detection
HLB	Hydrophilic-lipophilic-balanced
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
HT_{50}	Median time to hatching
k_{O3}	Kinetic rate constant with ozone
K_{ow}	Octanol-water partition coefficient
LC	Liquid chromatography
LC–MS/MS	Liquid chromatography with tandem mass spectrometry
LEDs	Light-emitting diodes
MAC	Maximum allowable concentration
MPs	Micropollutants
MSFD	Marine Strategy Framework Directive
MDL	Method detection limit
MQL	Method quantification limit
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NI	Negative ionization
Non-RAS	Non-recirculated aquaculture systems
PAA	Peracetic acid
PAHs	Polycyclic aromatic hydrocarbons
PCA	Plate Count Agar
PFOS	Perfluorooctanesulfonic acid
PI	Positive ionization
pK_a	Acid dissociation constant (negative base-10 logarithm)
PMS	Peroxymonosulfate
PPCPs	Pharmaceuticals and personal care products
PS	Persulfate

PSs	Priority substances
RAS	Recirculating aquaculture systems
SB	Slanetz and Bartley
SF	Surface flow
SSF	Subsurface flow
HSSF	Horizontal subsurface flow
VSSF	Vertical subsurface flow
SPE	Solid-phase extraction
SW	Surface water
TDS	Total dissolved solids
T&O	Taste and odour compounds
TSS	Total suspended solids
UHPLC	Ultra-high performance liquid chromatography
UHPLC – MS/MS	Ultra-high performance liquid chromatography with tandem mass spectrometry
US	Ultrasound
UPW	Ultrapure water
UV	Ultraviolet
WFD	Water Framework Directive
WWTPs	Wastewater treatment plants

Chapter

1

General Introduction

1.1. Micropollutants in aquatic systems

Freshwater is one of the most important resources on Earth but its scarcity is an issue that many regions face today, with a tendency to expand to other areas around the world. The continuous world population growth and intensive urbanisation led to the increase of water usage for domestic, agricultural, and industrial purposes, resulting in a great production of wastewater (WW) that require proper management and control [1]. Ensuring water quality is intrinsically related to preservation of ecosystems, food safety, human health, poverty reduction, and other associated aspects, such as economic growth and social development. Therefore, maintaining global water quality is a requirement for sustainable progress, in order to satisfy ecosystem requirements and to guarantee the overall well-being of humans [2].

Water pollution is defined by the presence of particular pollutants at certain concentrations and during a period enough to cause undesirable effects [1]. Until very recently, the main focus on the impacts of water pollution has been related to conventional pollutants, such as metals and persistent organic pollutants. For this reason, they have been extensively studied, being developed measures to avoid and/or control their presence in water [3]. However, over the last few decades, the occurrence of micropollutants (MPs) in aquatic systems has become a worldwide issue of increasing concern [4]. MPs can be anthropogenic or natural substances, commonly present in water at trace concentrations, ranging from a few ng L^{-1} to several µg L^{-1}. These compounds include pharmaceuticals and personal care products (PPCPs), X-ray contrast media, steroid hormones, industrial compounds, pesticides, among others, which are integrated on the so-called priority and emerging contaminants [4]. Although some of these pollutants persist in the environment, others are considered “pseudo-persistent” due to their continuous introduction into the environment, which is not surpassed by their elimination rates [5]. The “low concentration” and diversity of MPs complicate the analytical procedures for their detection [6] and create problems for conventional water and WW treatment systems [4], which are not specifically designated to eliminate many of them.

1.1.1. Pollution sources

Industries are one of the possible sources of MPs since their effluents can contain various compounds, according to each specific producing sector, which can be released in the environment [7]. On the other hand, hospital effluents and domestic WW can also be significant MPs pathways [7, 8]. Pharmaceuticals can be completely or partially metabolised in the human body, and thus, the unchanged parent drugs and the produced metabolites are excreted via urine and faeces, and then discharged into the sewer system [9]. In addition, other products commonly used at home and hospitals (*e.g.*, soaps, shampoo, toothpaste, and disinfectants) contain various types of substances that end up also in the sewer system. Agriculture is another important source of MPs, mainly due to the pesticides used to improve the productivity (*e.g.*, controlling the pests and vectors), which can be found in waterways as a result of agricultural runoff [10]. Moreover, veterinary drugs and food additives used in livestock farming are excreted by animals and may reach the aquatic systems via runoff. A fraction of these pollutants can also infiltrate the soil after manure application and pollute the groundwater system. Leaching from dumping sites and sewage treatment facilities, as well as environmental disasters, are other possible sources of MPs [5, 8].

Despite all existing pollution routes of MPs, the most significant for the aquatic environment are the industrial, hospital and urban wastewater treatment plants (WWTPs) effluents [4, 8]. Although many substances are efficiently eliminated in WWTPs, the removal of MPs is often insufficient. Conventional WWTPs are not specifically designed to eliminate this type of pollutants which end up passing through WW treatment processes due to their persistence and very low concentration levels. Likewise, drinking water treatment plants (DWTPs) cannot completely remove most MPs from supplying water. Consequently, tap water containing MPs can be ingested by humans, exposing them to such substances and their possible long-term effects [7].

1.1.2. Environmental fate and effects

The environmental fate of MPs is basically determined by the properties of the compound (chemical structure, octanol partition coefficient, acidity constant, etc.) and by the conditions of the

surrounding environment. Depending on these aspects, processes such as sorption, volatilization, dispersion, hydrolysis, oxidation, isomerisation, photodegradation and biodegradation can occur [8]. As the environmental compartments are constantly interconnected, MPs are spread by surface water (SW), groundwater, soil, and air. Furthermore, these compounds may also bioaccumulate in plants and other organisms (Fig. 1) [11].

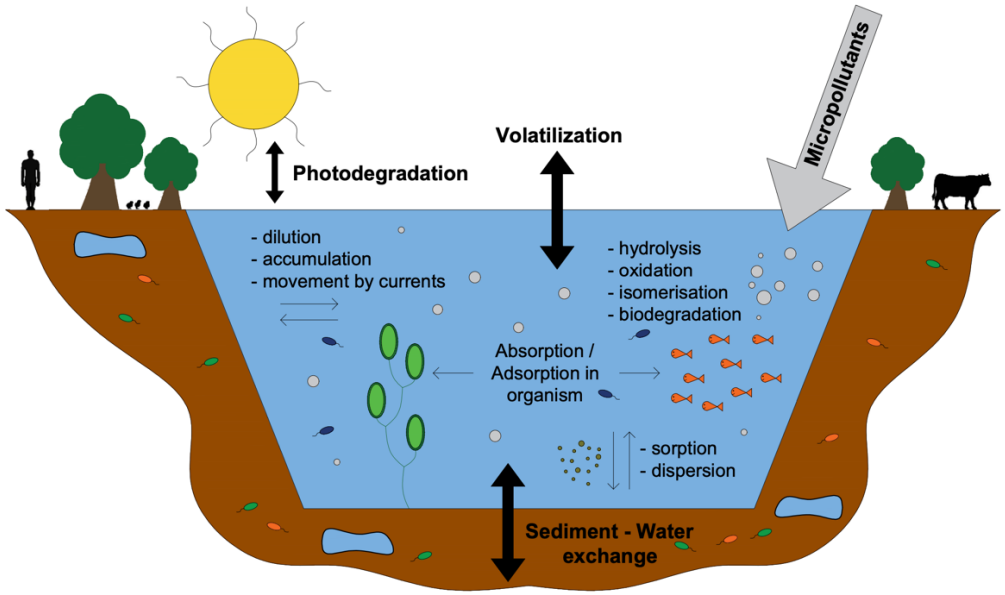

Figure 1. Fate of MPs in water systems and their spread by the different environmental compartments - adapted from [8].

The occurrence of pollutants in the aquatic environment has been frequently linked to ecotoxicological adverse effects, which are still not totally understood. Although the acute toxic effects are unlikely to occur due to the quite low concentrations of MPs in water, the precautionary principle should prevail due to the possible long-term exposure, which may originate associated effects that are difficult to predict [12]. Some studies have shown that the exposure to certain MPs can originate impacts on biological systems, mostly in fish and aquatic species [13]. Moreover, the accumulation of some pollutants, such as antibiotics, can potentially promote the development and accumulation of antibiotic-resistant bacteria and/or resistance genes (ARB&ARGs) in the

environment [14-16]. Some researchers found higher levels of ARGs downstream of WWTPs compared to upstream, suggesting that WWTPs effluents can be a significant source of ARB&ARGs spreading into the environment [13, 15]. Besides the development of resistance mechanisms by bacteria and other chronic toxic effects, another issue of concern is the endocrine disrupting effects caused by some MPs, which may interfere with organisms endocrine system and produce adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife [2, 13].

1.1.3. European Policy

MPs can be classified either as priority substances (PSs), "presenting a significant risk to or via the aquatic environment" as indicated in the Water Framework Directive (WFD) [17], or as contaminants of emerging concern (CECs), depending on the existence or absence of regulation, respectively. Most PSs are organic substances, but some toxic metals and organometallic compounds have also been identified as PSs [18]. In turn, CECs include new synthetized substances as well as substances that have been recently detected in natural streams (often due to improved analytical detection capacity) and/or which are suspect to pose a risk to ecosystems and/or human health [13, 19, 20]. CECs are commonly designated by "emerging compounds" and comprise PPCPs, steroids and hormones, pesticides, industrial and household chemicals, metals, surfactants, industrial additives, among others [13, 19].

Although there are no legal discharge limits for MPs, some regulations have been published. In 2008, 33 PSs and 8 "certain other pollutants" were ratified by the Directive 2008/107/CE, which listed the Environmental Quality Standards (EQS) for each of these compounds, indicating the concentration in water, sediment or biota that should not be exceeded for the protection of the environment and human health [21]. This document was subsequently updated by the Directive 2013/39/EU that revised EQS of some PSs already in the previous Directive, and included more PSs, and their respective EQS, totalling an inventory of 45 PSs (41 organic substances and 4 metals) and 8 "certain other pollutants" with EQS to be considered [18]. Directive 2013/39/EU also

calls the attention to the significance of monitoring some CECs for which legislation still does not exist. In this context, the first watch list of CECs for the European Union (EU)-wide monitoring was published by Decision 2015/495/EU [22], which was revised by Decision 2018/840/EU [23], and more recently by Decision 2020/1161/EU. Now the latter includes 19 CECs that should be monitored in order to gather information for further possible prioritisation exercises [24]. In addition to the monitoring of PSs and CECs, Directive 2013/39/EU highlights the importance of preventive actions, the identification of pollution causes, the need to deal with emissions of pollutants at the source and the need to develop innovative water/wastewater treatment technologies [18].

1.2. Concern on MPs in aquaculture

Aquaculture is the farming of aquatic organisms (finfish, shellfish and/or plants) in fresh, salty, or brackish water, using specific techniques to increase their production beyond the natural capacity of the environment [25]. It is classified as extensive, semi-intensive and intensive according to the production levels of the organisms and the resources needed to support them, respectively almost nil, media and high [26, 27]. Industrial-scale aquaculture farms often work under an intensive regime involving a large number of produced organisms and the control of several parameters through external intervention, namely the water quality and flow, fish breeding and growth, and food and medication supplementation [25].

Nitrogen and phosphorus are the main focus of most studies on the environmental impacts of aquaculture effluents, as they are excreted by aquatic species and contribute to eutrophication [28, 29], but MPs have also gained attention in this domain [25, 30-32]. MPs are mainly introduced in aquaculture waters due to the addition of certain substances during production [31]. Veterinary drugs, disinfectants, and antifoulants are frequently used to improve the survival rates of cultured organisms and to control pathogens [33]. Tetracyclines, trimethoprim, sulfonamides and phenicols are some of the most commonly used pharmaceuticals [34], whereas dichlorvos and diuron are examples of other chemicals that are used to disinfect water and prevent the biofouling of immersed surfaces and equipment [35, 36]. These substances and their respective by-products and

metabolites can be considered MPs when the outlet water is discharged into the environment without proper treatment. Moreover, water reservoirs that supply water to aquaculture farms are threatened by the presence of MPs. Many studies have reported the occurrence of MPs in surface and ground waters [4, 7, 37, 38], highlighting the high probability of the presence of MPs in these aquatic compartments and, consequently, in the aquaculture waters that they provide. Since aquaculture facilities are generally interconnected with surrounding water bodies, MPs not ingested/absorbed or excreted by farmed organisms and their related by-products and metabolites can spread to different environments [39]. Moreover, within farms, MPs can also be bioaccumulated by organisms, which are subsequently consumed by humans [36].

1.2.1. Environmental guidelines

Currently, aquaculture provides near half of the total fish consumed worldwide, and a production increase of more than 60% is expected during the next years [40]. Therefore, sustainability and good environmental practices are common goals for aquaculture production, but the means to achieve it depends on the traditions and values of each region [41]. In EU countries, the WFD [17] and the Marine Strategy Framework Directive (MSFD) [42] are the two main documents that guide the practices for good water quality, envisaging a healthy ecological and chemical status for both fresh and marine waters. With respect to MPs, the EU recognises concerns about their occurrence in aquaculture activities, namely cleaning agents and pharmaceuticals, which are often used to improve the survival, performance, and quality of fish [33]. In this sense, a recent European Commission guiding document has provided best practice guidelines and suggestions to national authorities and aquaculture producers, aiming to minimise the possible undesirable effects of these substances in the environment. Some recommendations include enforcing limits on the administration of veterinary medicines, prediction models to assess environmental impacts, monitoring results published on open access websites and applying alternative cleaning techniques over the use of antifoulants [33]. However, specific water treatment processes to remove MPs have not been specified yet.

1.2.2. Treatment options

Water treatment options suitable for MP removal must be considered in aquaculture farms due to the possible adverse effects of these substances on the aquaculture farmed organisms, surrounding ecosystems and human health. Since conventional water/wastewater treatment processes are not specifically designed to remove MPs, alternative technologies are required. Although concerns about MP removal are mainly centred on aquaculture-produced wastewaters, the treatment of influent waters should also not be disregarded.

Adsorption on activated carbon and membrane processes (including microfiltration, ultrafiltration, nanofiltration and reverse osmosis) are some examples of alternative technologies already applied to remove MPs at full scale, namely in WWTPs and DWTPs [43-46]. Powdered and granular activated carbon systems are highly effective at adsorbing MPs from water; however, their performance might be compromised by the competition between MPs and natural organic matter for binding sites [47]. Furthermore, activated carbon must be incinerated or regenerated when its adsorption capacity is achieved, which consumes high amounts of energy and generates a stream that itself must be treated as hazardous waste [48]. Among the membrane processes, high-pressure systems (including nanofiltration and reverse osmosis) are more suitable for the removal of MPs [49-51]. Nevertheless, such processes, which show better performance with low-molecular-weight and neutrally charged MPs, also originate a concentrate containing all retained compounds, which requires further treatment and disposal [48]. Due to this, advanced oxidation technologies (AOTs) such as ozonation and ultraviolet (UV)-based processes are gaining attention in this domain, as they can degrade organic MPs without either their transfer to a different phase or their retention in a concentrate, which both would require additional treatment [52-55]. Nonetheless, AOTs are typically costly options and could lead to the formation of intermediate products, which in some cases, can be more toxic than the original compounds [56]. Another interesting option is the application of nature-based solutions such as constructed wetlands (CWs). Although CWs were originally applied to remove conventional pollutants (*e.g.*, organic matter, nutrients, and metals) from WW in small communities or industries, they have demonstrated promising performance concerning the removal of organic MPs [57-59]. When compared with AOTs, CWs involve lower

costs of implementation and operation, but require longer treatment time and more space. Moreover, CWs might not be as efficient as AOTs in removing MPs.

1.3. Treatment of water containing organic MPs

1.3.1. Constructed Wetlands

CWs are engineered systems that recreate the conditions and processes occurring in natural wetlands, which are well recognised by their capacity for improving water quality [60]. Since CWs are constituted by water, substrate, plants (in most cases), and microorganisms, several biotic and abiotic processes may co-occur, contributing to the elimination of different types of compounds, namely MPs (Fig. 2) [57, 61]. Microbial degradation and uptake by plants are the main biotic mechanisms for the removal of contaminants from water passing through CWs, whereas abiotic processes include sedimentation, substrate adsorption, volatilization and photodegradation [61]. However, the balance between such processes is highly dependent on several factors, namely the specific characteristics of the compounds and the conditions of the surrounding environment [57].

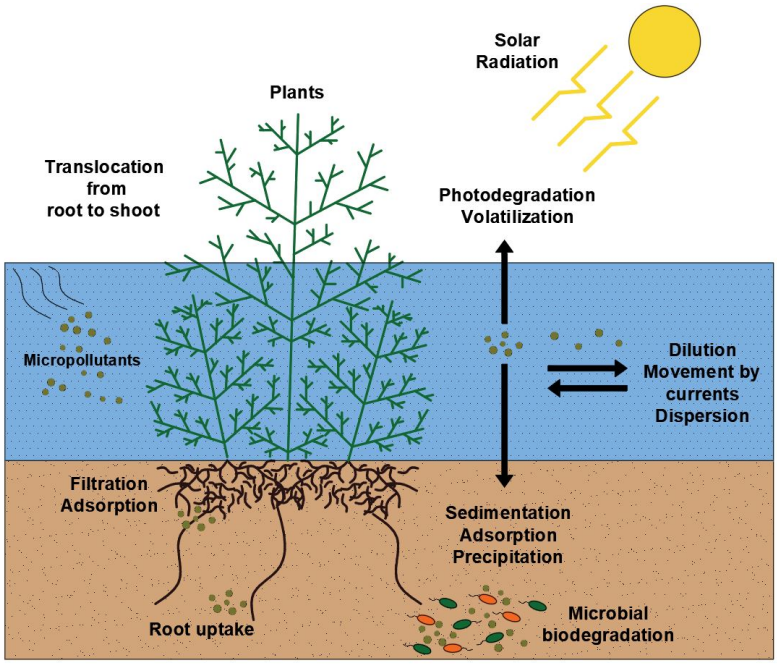

Figure 2. Biotic and abiotic processes removing MPs from CWs systems – taken from [62] with permission of Elsevier.

Regarding the properties of the pollutants, substances with higher vapour pressure values, for instance, are more likely to volatilise than others [63]. Furthermore, hydrophobic compounds (with a high octanol-water partition coefficient (K_{ow})) are easily adsorbed onto soil, mineral surfaces, and biofilms, whereas hydrophilic compounds (low K_{ow}) are mostly removed by other processes [59]. In addition, it was reported that compounds with a log K_{ow} between 0.5 and 3 are easily uptake by plants [64]. In turn, photodegradation is influenced by the specific absorbance spectrum of each compound and the seasonal variation of light intensity [59].

The surrounding environment also plays an important role in the removal of pollutants by CWs. For instance, parameters such as pH, temperature, ionic strength, and oxygen (O_2) availability can affect adsorption, uptake, and microbial degradation mechanisms [64]. The growth of plants as well as microbial activity in CWs require optimal pH values (near neutral), and high pH can affect the adsorption behaviour of pollutants due to their dissociation and subsequent attachment to

substrate/sediment by ion exchange. Moreover, the dissolved O_2 and temperature have a great impact on macrophytes and microorganisms, with the high availability of O_2 promoting aerobic degradation by microbial communities, and the warm temperature (15–25 °C) enhancing the action of some organisms such as nitrifying and proteolytic bacteria [65]. The performance of CWs is also influenced by climatic conditions, which can affect evapotranspiration, plants photosynthesis and microbial activity [66].

CWs design can also affect the removal of pollutants [57, 59]. Typically, CWs are differentiated according to: (i) their hydrology (water surface flow (SF) and sub-surface flow (SSF)), (ii) plant growth (free-floating, floating-leaved, submerged, or emergent), and (iii) the flow path (horizontal (H) or vertical (V)) (Fig. 3). SF-CWs can be a good solution for compounds susceptible to photodegradation because the water is directly exposed to sunlight, whereas SSF-CWs are a better alternative for biodegradable pollutants because the water passage in the substrate matrix triggers several biological and chemical interactions that promote their removal. Moreover, in VSSF-CWs, the flow path can improve microbial degradation due to the higher oxygenation of the different substrate layers when compared with HSSF ones [59]. In addition, various types of CWs may be combined (hybrid systems) to achieve higher removal efficiencies [67].

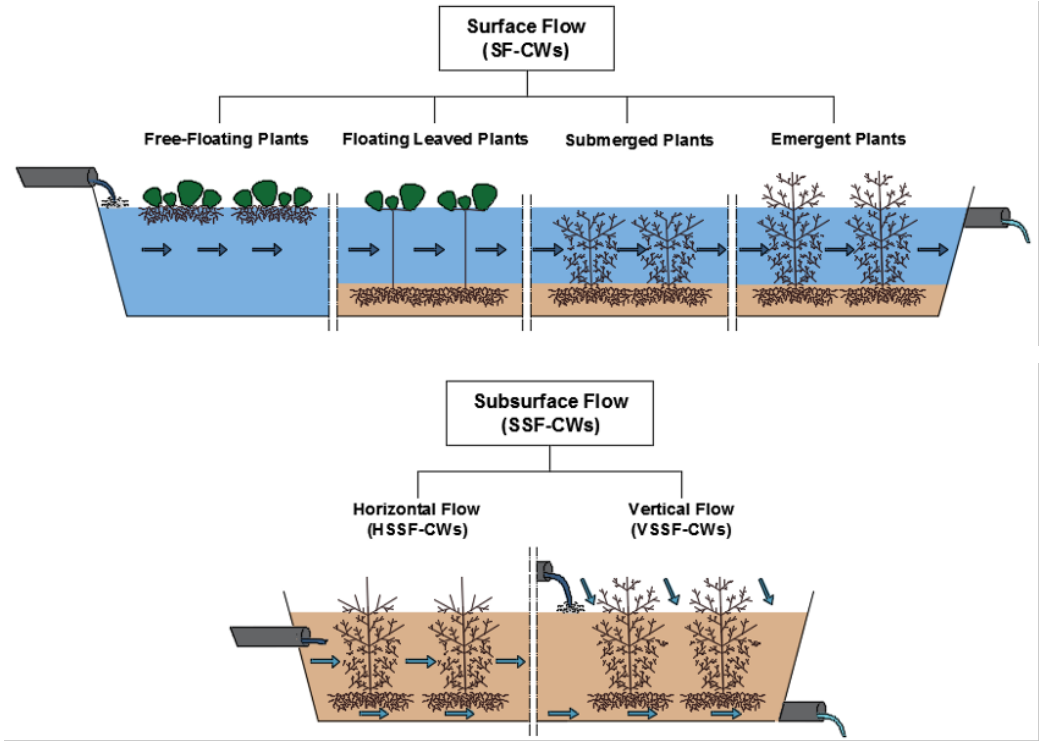

Figure 3. CWs configurations, according to hydrology (surface or subsurface flow, SF and SSF respectively), plants growth forms for SF-CWs (free-floating, floating leaved, submerged, or emergent) and flow path for SSF-CWs (horizontal or vertical, HSSF and VSSF respectively) – taken from [57] with permission of Elsevier.

Besides configuration, operational parameters such as operating mode (batch or continuous), hydraulic retention time (HRT), plant species, and substrate composition are also important for CWs performance since they can significantly affect their efficiency [57]. For instance, CWs operating in batch mode generally has better efficiency than those under continuous feed because an aerobic environment, promoted through draining and filling operations, enhances the microbial activity and consequently the degradation of pollutants [57]. As consequence of the surrounding conditions of the media (for instance, aerobic and anaerobic zones), organic MPs (used as a source of energy by microorganisms) can be removed by several mechanisms [68, 69]. For instance,

aerobic bacteria oxidize the pollutants utilizing O_2 as the final electron acceptor, while anaerobic bacteria degrade the pollutants through other final electron acceptors (such as carbon dioxide (CO_2), SO_4^{2-} and NO_3^-) or fermentation and methanogenesis [69]. Regarding the time of treatment, higher HRTs allow a longer interaction of contaminants with the wetland system; however, a period of 4–15 d has been reported to be suitable for achieving the good performance of CWs [59]. The selection of plant species and of the substrate matrix is also important to remove specific types of pollutants and to support the environment for plant and microbial activity [57]. *Typha* ssp. and *Phragmites* ssp. are the most popular species used in CWs [70], while the type of substrate matrix and their respective depths are very adaptable according to the implemented CWs [71-73]. Given all these factors, CWs can be adapted according to the characteristics of the target contaminants to be removed and the environmental conditions available in their locations or implementation sites. In general, the applications of CW systems have important advantages, such as moderate capital costs, low energy consumption, small maintenance requirements and benefits for wildlife habitats and biodiversity [74]. Considering their impressive performances in removing conventional pollutants from WW, such as nitrogen, phosphorus, organic matter and bacteria, their applicability for the removal of organic MPs has only recently begun to be investigated [57], including studies on aquaculture waters [74, 75].

A review on the application of CWs to remove MPs from water and WW is presented in *Chapter 2*, highlighting current gaps that still need research, including CWs possible use for aquaculture effluents, and influents, treatment.

1.3.2. Advanced oxidation technologies

AOTs are treatment technologies based on the production of highly reactive species that oxidize organic compounds [76]. In most cases, AOTs involve the generation of hydroxyl radicals ($HO^\bullet$), *i.e.,* conceptually known as advanced oxidation processes (AOPs) [77], but other reactive species can also be involved, such as the oxide anion ($O_2^{\bullet-}$), hydroperoxyl ($HO_2^\bullet$), ozonide anion ($O_3^{\bullet-}$), sulphate ($SO_4^{\bullet-}$) and chlorine ($Cl^\bullet$) radicals (Fig. 4) [78, 79]. As organic MPs are not efficiently

eliminated by conventional water/wastewater treatment processes, AOTs have attracted significant interest as a suitable alternative to remove these contaminants from aqueous matrices [56, 80, 81]. There are several types of AOTs (Fig. 4), which can be categorized according to: (i) the way as the highly reactive species are generated (chemical, photochemical, electrochemical, and sonochemical); and (ii) the number of phases (one or more) involved in the oxidative reactions (homogeneous or heterogeneous processes, respectively) [82]. Chemical technologies involve the use of oxidants such as ozone (O_3) and hydrogen peroxide (H_2O_2), or catalytic mixtures as the Fenton reagent, whereas photochemical processes require radiation (such as UV/O_3, UV/H_2O_2, UV/Fenton). Typically, these reactions are conducted at near-ambient temperature and pressure, but more severe conditions can also be applied to oxidize organic MPs (*e.g.,* in wet air oxidation and supercritical water oxidation, both using O_2 to produce free radicals, or peroxide wet oxidation that applies H_2O_2 to enhance the production of these radicals) [5, 83, 84]. Electrochemical and sonochemical processes involve more complex treatments, respectively using electrical currents (such as anodic oxidation, photoelectrocatalysis, electro-Fenton, photo-electro-Fenton, and solar photo-electro-Fenton processes) and ultrasonication (including sonolysis, sonocatalysis, sonophotolysis, and sonophotocatalysis) to produce highly reactive species [85, 86]. In electrochemical processes, the organic MPs can be oxidized either indirectly, via the reactive species produced from the water oxidation on the anode, or directly via electron transfer from the contaminant to the anode surface [87, 88]. In the case of photoelectrocatalysis, photocatalysis is an additional pathway for the generation of reactive species beyond the electrochemical oxidation [88]. Fenton-process is based on the generation of $HO^{\bullet}$ through the reaction between H_2O_2 and Fe^{2+} in acidic media, and thus, electro-Fenton based techniques can decrease (or avoid) the addition of H_2O_2 by its *in-situ* production from the reduction of dissolved O_2 in the cathode [88]. In sonochemical processes, the ultrasonic waves lead to the pyrolytic cleavage of water molecules (acoustic cavitation), being formed $HO^{\bullet}$ and other reactive species that can remove organic MPs [85, 89].

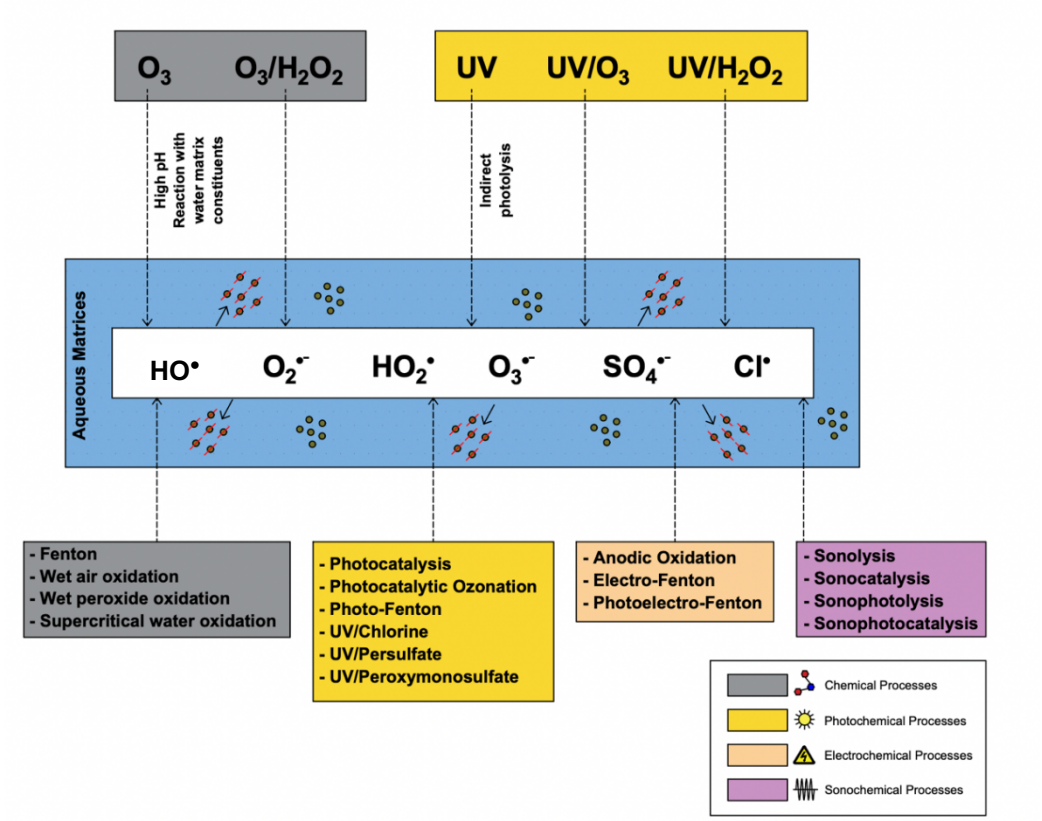

Figure 4. AOTs general processes for removing organic MPs from aqueous matrices (chemical, photochemical, electrochemical and sonochemical processes) – taken from [62] with permission of Elsevier.

Homogeneous processes occur in a single phase, whereas heterogeneous processes occur in two or more phases. Metal oxides (based on Ti, Cu, Zn, Mn, Fe, Co, and Bi), noble metals (such as Ru, Pt, Pd, Ir, and Rh) on different supports or even metal-free carbon materials, such as activated carbons, carbon xerogels, carbon nanotubes, carbon foams and fibres, and graphene derivatives are examples of catalysts that can be applied in heterogeneous AOTs [5]. In particular, heterogeneous photocatalysis is based on the activation of the catalyst by a radiation source with energy equal to or higher than its band gap, leading to the generation of electrons and holes, highly reactive species, and subsequent degradation of organic MPs [90, 91]. Titanium dioxide (TiO_2) is the most widely used photocatalyst because of its outstanding activity, stability, low cost, and

relatively low toxicity [92, 93]. To increase the treatment efficiency and removal of MPs, heterogeneous photocatalysts have been combined with O_3 in a process called photocatalytic ozonation [94-98].

Overall, O_3-based technologies have been extensively studied to remove organic MPs from water/wastewater [80, 99-102]. O_3 is an unstable and strong oxidant that can degrade organic MPs via direct, selective reactions or can undergo decomposition via a chain reaction mechanism [103-105], resulting in the production of secondary oxidants such as $HO^{\bullet}$ (indirect reaction) (Appendix C). Since hydroxyl and hydroperoxide ions are the driving force for O_3 decay, $HO^{\bullet}$ are mainly produced at high pH [106, 107]. During ozonation, molecular O_3 attacks MPs with electron-rich moieties (*e.g.*, olefins, activated aromatics, and sulphur groups), while $HO^{\bullet}$ reacts nonselectively with the O_3-resistant compounds [108]. There are a wide variety of parameters (such as pH, organic/inorganic carbon, temperature, and salts) that can promote or inhibit the decomposition of O_3, and thus, the degradation of organic MPs is highly dependent on the type of water matrix to be treated [109, 110]. Various alternatives can be applied to maximize the generation of $HO^{\bullet}$ and consequently the degradation of organic MPs, namely ozonation at high pH, O_3/H_2O_2, O_3/UV and O_3/catalysts [99, 111, 112]. However, as for all AOTs, the choice of the most suitable process depends on several factors related to the specific case of treatment, as available budget, location, and type of water.

AOTs aim to transform organic MPs into less toxic compounds, ideally yielding CO_2, H_2O, and inorganic ions as final products. For this reason, they can be used as a polishing treatment before water discharge/reuse, or applied before biological processes to reduce water toxicity and enhance biodegradability [7, 62]. The application of AOTs to treat industrial water and WW contaminated with organic MPs is a hot research topic in the scientific community; however, pilot and full-scale studies are still limited [113-115]. In the specific case of aquaculture, the need for water disinfection in facilities with water recirculation (*i.e.*, recirculating aquaculture systems (RAS)) has triggered interest in the use of AOTs [115, 116] and, more recently, the removal of organic MPs has also gained increasing attention [39, 117].

A general overview on the removal of organic MPs by AOTs, summarizing the most important information for its applicability, including for aquaculture aqueous matrices, is presented on *Chapter 2*.

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Chapter

2

State-of-the-art and Motivation

2.1. State-of-the-art

2.1.1. A review on the application of constructed wetlands for the removal of priority substances and contaminants of emerging concern listed in EU legislation

The removal of organic MPs from aquaculture water/wastewater is the central topic of this PhD thesis. Therefore, since CWs are a low-cost and environmentally friendly treatment solution for water depuration, a literature survey was performed in an early stage of this PhD project to gather relevant information on their promising applicability to remove MPs from aqueous matrices. PSs and certain other substances with EQS defined in the Directive 2013/39/EU and CECs of the watch list of Commission Decision 2015/495/EU were included in the research survey due to their relevance in the EU for SW monitoring. The survey comprised publications in the *Scopus* database (accessed until June 2016), using as keywords "*constructed wetlands*" and the "*name of EU PS/substance with EQS or CEC*". All types of articles dealing with the removal of at least one of the target organic MPs by CWs, were considered. Information was analysed according to the CWs removal efficiencies of PSs and CECs, as well as their studied inlet concentrations. The influence of CWs design and operational conditions on MPs degradation was also assessed. Since Decision 2015/495/EU was updated by the Decisions 2018/840/EU and 2020/1161/EU during the execution of this PhD project, a new revision of the literature was performed in November 2021 and included in this chapter. Studies were found for 20 PSs and 8 CECs of first EU Decision, and the data of the updated survey have not affected the conclusions drawn from the original search.

This sub-chapter is based on the following reference (original version available in **Appendix A1**, with permission of Elsevier):

Gorito, A.M., Ribeiro, A.R., Almeida, C.M.R, Silva, A.M.T. *A review on the application of constructed wetlands for the removal of priority substances and contaminants of emerging concern listed in recently launched EU legislation*, Environmental Pollution, 227 (2017) 428-443.

2.1.1.1. Organic PSs defined in Directive 2013/39/EU

The number of studies (n) published until June 2016 about the CWs removal of organic PSs and other substances with EQS to be considered listed in the Directive 2013/39/EU is shown in Figure 5, including the distribution by different classes. Moreover, Table 1 summarizes all the articles dealing with PSs, showing their respective range of removal by CWs and the initial concentration tested (encompassing spiked and non-spiked experiments).

Among the 41 target PSs/classes of PSs, only 20 were studied, with a quite significant number of works being focused on some specific compounds (*e.g.*, benzene and atrazine, followed by nonylphenol and chlorpyrifos). Pesticides are the most studied group of substances, including triazine (27%), organophosphorus (10%), organochlorine (8%), phenylurea (8%), and other classes of pesticides (3%). There are also several studies dealing with the removal of solvents by CWs (24%), with benzene showing a great representation. Industrial compounds represent 19% of the works testing CWs for their removal, in the following decreasing order of number of studies: nonylphenol > di(2-ethylhexyl)phthalate (DEHP) > octylphenol ≈ perfluorooctane sulfonic acid and its derivatives (PFOS). Polycyclic aromatic hydrocarbons (PAHs) are the PSs least reported (1%).

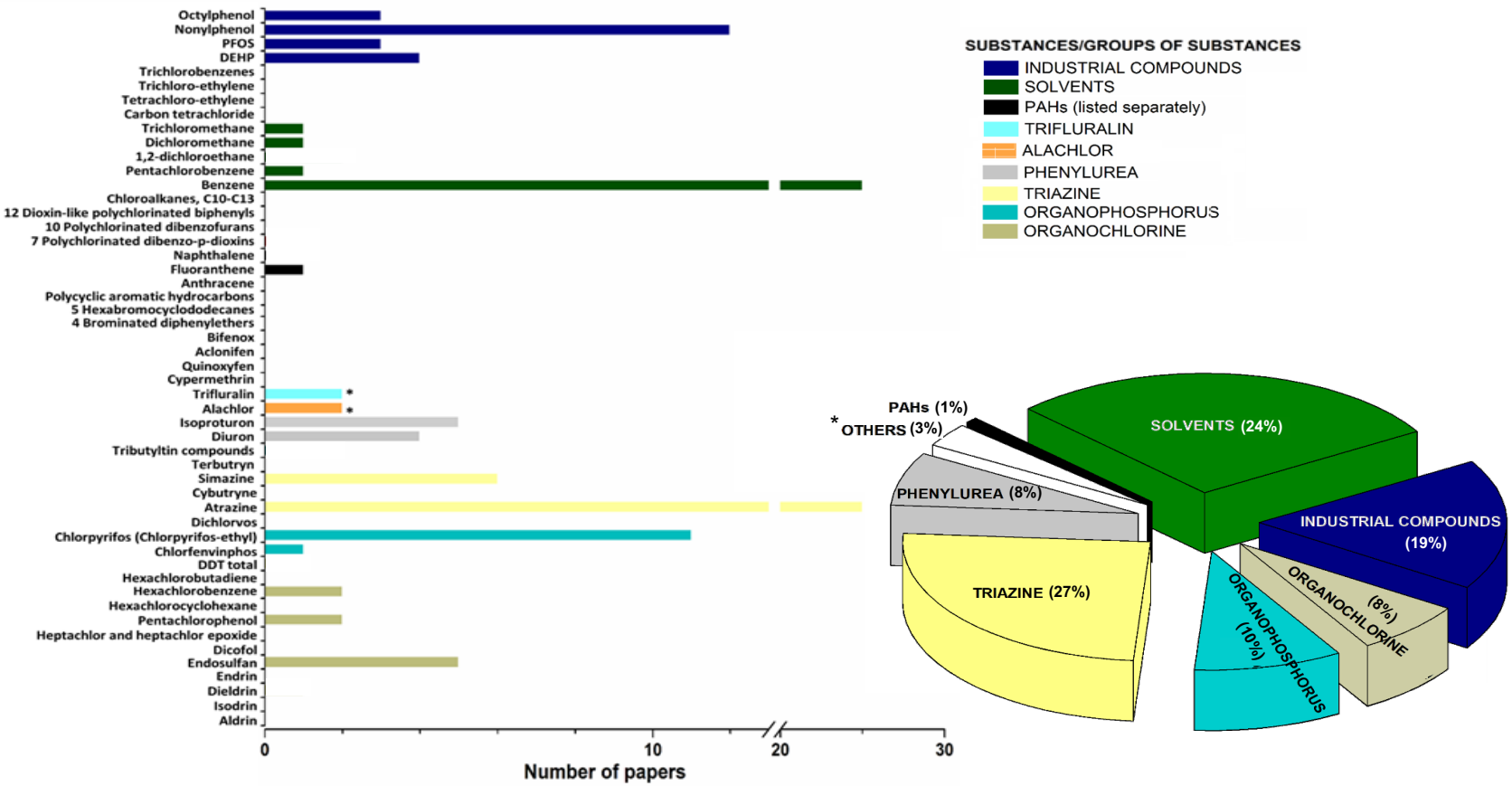


Figure 5. Number and percentage of studies published dealing with removal by CWs of organic PSs and substances with EQS listed in Directive 2013/39/EU, based on the *Scopus* database search employing as keywords "*constructed wetland*" and the "*name of each PS/substance with EQS*". *Others: trifluralin and alachlor (accessed until June 2016)

Table 1. Studies on the applicability of CWs for the removal of organic PSs defined in Directive 2013/39/EU, representing their respective range of removal by CWs and the initial concentration tested (grouped under spiked and non-spiked influents) in water/wastewater. No studies were found for the substances/classes of substances and certain other substances listed in footnote.

Class	Name of PS - (n = no. of studies)	Refs	Removal (%)	Initial concentration (µg L^{-1})	
				Non-Spiked	Spiked
Industrial compounds	Octylphenol - **(3)**	[1-3]	0–89	0.001–5.58	–
	Nonylphenol - **(12)**	[1,2,4-13]	0–90	0.001–1720	50
	PFOS – **(3)**	[14-16]	0–95	0.019–0.029	5000
	DEHP – **(4)**	[17-20]	19–49	12.5	–
Solvents	Trichloromethane – **(1)**	[21]	66	4.45	–
	Dichloromethane – **(1)**	[21]	63	0.87	–
	Pentachlorobenzene – **(1)**	[22]	99	–	2500
	Benzene – **(25)**	[23-47]	0–100	14–21000	999000
PAHS (listed separately)	Fluoranthene – **(1)**	[48]	97–99	–	0.001

Table 1. (Continued)

Phenylurea pesticides	Isoproturon – **(5)**	[49-53]	30–45	0.05–88	–
	Diuron – **(4)**	[22,54-56]	0–55	0.02–45	2500
Triazines pesticides	Simazine – **(6)**	[22,56-60]	20–60	0.006–0.169	2500–575000
	Atrazine – **(25)**	[51, 56, 58, 61-83]	0–100	0.005–5	0.00009–40000
Organophosphorus pesticides	Chlorpyrifos (chlorpyrifos-ethyl) – **(11)**	[8,84-93]	50–100	0.9–800	0.02–3
	Chlorfenvinphos – **(1)**	[94]	12–51	–	–
Organochlorine pesticides	Hexachlorobenzene – **(2)**	[95,96]	67–75	–	0.3–2.5
	Pentachlorophenol – **(2)**	[22,97]	89–94	1.1	2500
	Endosulfan – **(5)**	[22, 55, 85, 98, 99]	80–100	0.2–2.21	100-2500
Others pesticides:				–	
Dinitroaniline	Trifluralin – **(2)**	[83,100]	63–93		80–170
Chloroacetanilide	Alachlor – **(2)**	[22,83]	80	–	2500

No studies were found for the other 21 organic PSs/classes of PSs defined in Directive 2013/39/EU, namely: trichlorobenzenes, 1,2-dichloroethane and chloroalkanes (solvents); dioxin-like polychlorinated biphenyls, polychlorinated dibenzofurans and polychlorinated dibenzo-p-dioxins (dioxin-like compounds); naphthalene and anthracene (PAHs listed separately); polycyclic aromatic hydrocarbons (PAHs); hexabromocyclododecanes; brominated diphenylethers; bifenox; aclonifen; quinoxyfen; cypermethrin; tributyltin compounds (organotin); terbutryn and cybutryne (triazine); dichlorvos (organophosphorus); DDT total, hexachlorobutadiene, hexachlorocyclohexane, heptachlor and its epoxide and dicofol (organochlorine).

No studies were found for all substances with EQS to be considered, namely: dichlorodiphenyltrichloroethane, trichloro-ethylene, tetrachloro-ethylene, carbon tetrachloride, endrin, dieldrin, isodrin and aldrin.

PFOS, Perfluorooctane sulfonic acid and its derivatives; DEHP, Di(2-ethylhexyl)phthalate.

Focusing on the PSs inlet concentrations in CWs systems (Table 1), it is possible to verify a wide range of tested concentrations, either for the same compound, or between all of them. In non-spiked experiments, the removal of benzene, nonylphenol and chlorpyrifos was studied at the highest initial concentrations, up to 21 mg L^{-1}, 1.7 mg L^{-1} and 0.8 mg L^{-1} levels, respectively. Moreover, the pesticides isoproturon and diuron showed inlet concentrations up to 88 and 45 μg L^{-1}, respectively, while concentrations of only a few μg L^{-1} were found for atrazine, simazine, pentachlorophenol and endosulfan, as well as for the industrial compounds octylphenol, PFOS and DEHP, and for the solvents trichloromethane and dichloromethane. Comparing with non-spiked CWs inlets, spiked CWs inlet concentrations were quite higher and did not really match non-spiked magnitude levels. For instance, nonylphenol and chlorpyrifos are two of PSs which were found at the highest range of concentrations in the non-spiked CWs inlets, but they were studied at lower concentrations in spiked matrices. Isoproturon, also found at high concentrations in non-spiked CWs inlet solutions, was not considered in any spiked experiment. Typically, simazine is found in real WW at lower concentrations than the other triazine pesticide, atrazine; however, it has been studied at higher concentrations than atrazine in reports applying spiked inlet solutions. Overall, benzene and the triazine pesticides (atrazine and simazine) were the compounds spiked at the highest concentrations, at 1 g L^{-1} and up to 575 mg L^{-1}, respectively. Moreover, the removal of atrazine has been evaluated at the largest range of concentrations, ranging from 0.09 ng L^{-1} to 40 mg L^{-1}. It is important to realize that the concentrations of the pollutants can play an important role on the CWs performance, mainly in processes involving biodegradation and adsorption. In the case of biodegradation, high concentrations can destabilize the microbial community, whereas a saturation of the binding sites can occur in the case of adsorption [101].

Regarding CWs efficiencies, removal of organic PSs in both non-spiked and spiked solutions revealed good efficiencies for a considerable number of them, but very dissimilar removals have been obtained for the same compound (Table 1). The industrial compounds octylphenol, nonylphenol and PFOS were removed at poor to a high extent, whereas DEHP was eliminated between 19 and 49%. Regarding solvents, trichloromethane and dichloromethane were removed by around 65%, pentachlorobenzene by 99%, while benzene registered a broad spectrum of

removals between 0 and 100%. The pesticides pentachlorophenol, endosulfan and alachlor were highly removed by CWs (≥ 80%), and chlorpyrifos was eliminated by more than 50%; however, the concentration of isoproturon and hexachlorobenzene was only moderately reduced (from 30 to 75%, depending on the compound). The performance observed for the elimination of diuron and chlorfenvinphos varied from 0 to 55% and from 12 to 51%, respectively, while trifluralin registered a smaller interval between 63 and 93%. Still within the pesticides group, triazine had different behaviours, simazine was removed between 20 and 60%, whereas atrazine presented very dissimilar removals (either inefficient removal or removed to levels below the analytical detection limits). As referred above, a new survey was done to complement this study, covering the data published between 2016 and November 2021, which is not included in the review article that originated this chapter. Results showed that there were no significant differences in the data and conclusions herein presented. No other studies were found for the class of solvents, with the exception of 1,2-dichloroethane for which the first publication was launched in 2018. Likewise, the substances with EQS to be considered remained without information. Among the industrial compounds, PFOS was the PS with the higher number of new investigations (n=5). PAHs (listed separately) registered 11 additional studies, whereas naphthalene and anthracene were investigated for the first time. The class of pesticides still deserves great attention, these studies having increased by 52% (n=34) between 2016 and 2021.

2.1.1.2. CECs in the watch list of Commission Decision 2015/495/EU

The number of studies (n) on the CWs removal of CECs (Table 2) is represented in Figure 6 according to their class of substances. The most studied CECs were: diclofenac (with almost half of the studies), followed by the macrolide antibiotics (erythromycin, clarithromycin and azithromycin), 17-beta-oestradiol and estrone (respectively E2 and E1) with the same % of studies (15.6%) and then the synthetic hormone 17-alpha-ethinyloestradiol (EE2). It is interesting to note that diclofenac, E2 and EE2 suggested in Directive 2013/39/EU (*i.e.,* earlier in 2013) as compounds

to be included in the first watch list, now represent together almost 70% of the published studies on CWs, in contrast with the other compounds that were only identified in 2015, pointing a possible impact of the EU legislation on the research patterns. The data collected also show that the three macrolide antibiotics, erythromycin, clarithromycin, and azithromycin, are still poorly studied (15.8% for the three drugs) in comparison to diclofenac and the three estrogens, despite their known potential negative impact on the environment. Neonicotinoids (imidacloprid) were only reported in one study. The other CECs included in the first watch list were not considered in any study, namely the pesticides triallate, oxadiazon, acetamiprid, clothianidin, thiamethoxam, thiacloprid and methiocarb, the UV filter 2-ethylhexyl 4-methoxycinnamate (EHMC) and the antioxidant 2,6-di-tert-butyl-4-methylphenol commonly used as food additive.

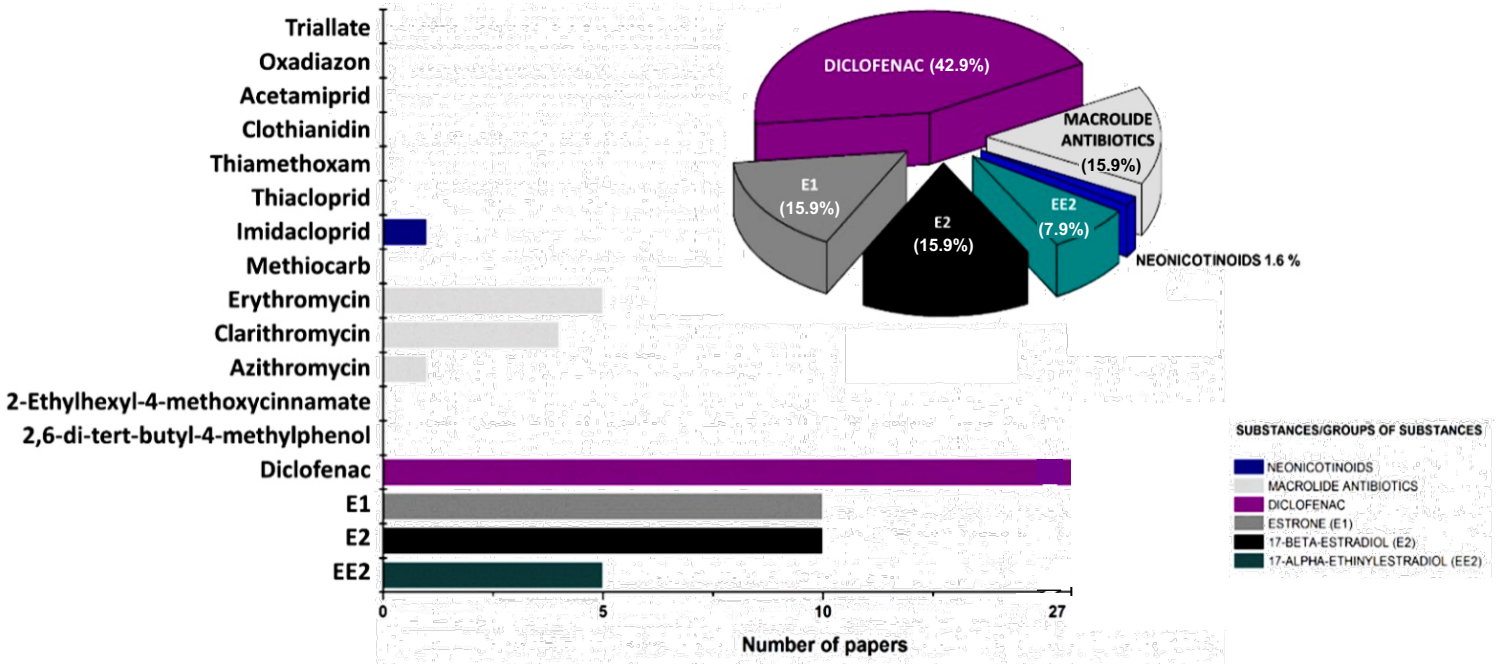


Figure 6. Number and percentage of studies published dealing with removal by CWs of CECs listed in the watch list of Decision 2015/495/EU, based on the *Scopus* database search employing as keywords "*constructed wetland*" and the "*name of each CEC*" (accessed until June 2016).

Table 2. Studies on the applicability of CWs for the removal of CECs defined in the watch list of Commission Decision 2015/495/EU, representing their respective range of removal and initial concentration (grouped under spiked and non-spiked influents) in water/wastewater. No studies were found for the substances/classes listed in footnote.

Class	Name of CEC - (n = no. of studies)	Refs	Removal (%)	Initial concentration (μg L^{-1})	
				Non-Spiked	Spiked
Neonicotinoids	Imidacloprid – **(1)**	[102]	70–100	–	60–1000
Macrolide antibiotics	Erythromycin – **(5)**	[103-107]	0–96	0.065–0.24	2.0
	Clarithromycin – **(4)**	[104-107]	0–89	0.07–0.3	0.4
	Azithromycin – **(1)**	[104]	100	–	0.1
Anti-inflammatory	Diclofenac – **(27)**	[10, 103, 105, 108-131]	0–96	0.19–36.9	2.5–5000
Estrogens	E1 – **(10)**	[13, 132-140]	0–100	0.0017–0.056	–
	E2 – **(10)**	[13, 132-136, 139-142]	0–100	0.001–0.189	2250
	EE2 – **(5)**	[108, 134, 135, 142, 143]	17–100	0.00094–0.025	–

No studies were found for the other 9 CECs, namely: triallate; oxadiazon; acetamiprid, clothianidin, thiamethoxam and thiacloprid (neonicotinoids); methiocarb; 2-ethylhexyl 4-methoxycinnamate (EHMC) and 2,6-di-tert-butyl-4-methylphenol.

E1, estrone; E2, 17-beta-oestradiol; EE2,17-alpha-ethinyloestradiol.

The CWs inlet concentrations showed that diclofenac was the compound found at the highest range of concentrations either in non-spiked or spiked investigations (Table 2). Diclofenac removal was evaluated in non-spiked inlet solutions up to 37 μg L^{-1}, but higher concentrations were also tested in spiked water/wastewater, *i.e.,* up to 5000 μg L^{-1}. The elimination of macrolide antibiotics clarithromycin and erythromycin was also assessed at lower concentrations in non-spiked experiments (0.065–0.3 μg L^{-1}); slightly higher concentrations (0.4 μg L^{-1}–2 μg L^{-1}) being employed in spiked experiments. In turn, the removal of macrolide azithromycin was only studied in spiked solutions (0.1 μg L^{-1}). The three estrogens, E1, E2 and EE2, were studied in non-spiked CWs inlets at concentrations lower than 0.2 μg L^{-1}, but only E2 was reported in spiked experiments, using concentrations quite higher (2250 μg L^{-1}). Overall, CECs listed in the watch list were found at lower concentrations in the realistic samples when compared with PSs (*Section 2.1.1.1*). The spiked experiments were also designed using inlet solutions with lower concentrations of CECs than those of PSs.

Regarding the performance of CWs to remove CECs (Table 2), the data reviewed showed very wide ranges for the removal of diclofenac (0–96%), the antibiotics erythromycin (0–96%), and clarithromycin (0–89%), and estrogens. The three estrogens were eliminated up to 100%, with a minimal removal of 0% for E1 and E2, and 17% for EE2. The spiked concentration of the neonicotinoid pesticide imidacloprid was reduced by ≥ 70%, whereas azithromycin was removed to levels below the analytical detection limits.

Although the discussion above was focused on CECs of Decision 2015/495/EU, in the last years this document was updated by Decisions 2018/840/EU [144] and 2020/1161/EU [145]. As a result, some CECs included in this survey were removed from the new watch lists due to sufficient monitoring data, while others were newly included. Presently, there is a group of 19 CECs flagged for EU-wide monitoring, namely metaflumizone, amoxicillin, ciprofloxacin, sulfamethoxazole, trimethoprim, venlafaxine, O-desmethylvenlafaxine, clotrimazole, fluconazole, imazalil, ipconazole, metconazole, miconazole, penconazole, prochloraz, tebuconazole, tetraconazole, dimoxystrobin, and famoxadone [145]. However, as expected, information on the performances of CWs for the new group of CECs is limited (November 2021): no studies were found for 7 CECs (metaflumizone,

clotrimazole, ipconazole, metconazole, tetraconazole, dimoxystrobin, and famoxadone), whereas other 8 CECs (amoxicillin, venlafaxine, O-desmethylvenlafaxine, fluconazole, imazalil, miconazole, penconazole, and prochloraz) were poorly investigated (≤ 5 studies for each one). Tebuconazole was described in 14 studies dealing with its removal by CWs, while sulfamethoxazole, ciprofloxacin and trimethoprim were the most investigated compounds (~ 30–80 studies). Interestingly, none of the CECs excluded from the first watch lists have been regulated so far as PSs, but some of these compounds may be included in the next update of the Directive 2013/39/EU.

2.1.1.3. Influence of CWs configuration and operating parameters on PSs and CECs removal

CWs are complex systems where physical, chemical, and biological processes may occur simultaneously, and these processes depend, among other factors, on the configuration and operating parameters of the CWs. By analysing the removal efficiencies, it is possible to verify that the studied PSs and CECs can be highly removed by CWs in most cases, exceptions being found for DEHP, isoproturon, diuron, simazine, and chlorfenvinphos, with removals consistently lower than or equal to 60%. However, it is important to mention that the comparison between different studies and the respective removal efficiency achieved for different compounds is merely indicative since different CWs systems and operating conditions were tested in most of these studies. Moreover, as abovementioned, the characteristics of the inlet solution may also influence the treatment occurring in CWs, which can also explain the different results for the same contaminant. Among the different parameters, wetland design can drastically affect the pollutants removal. For instance, nonylphenol removal rates may vary between 0 and 88% due to the CWs configuration [12]. The highest removal was observed in VSSF-CWs where aerobic conditions prevail and both planted and unplanted HSSF-CW systems originated, in most cases, higher concentrations of nonylphenol in the outlet than in the inlet. This fact might be explained by the biodegradation of its precursors nonylphenol ethoxylates that under anaerobic conditions leads to the formation of the parent compounds. However, in another study, a significant influence of the season in the removal

of nonylphenol was observed. Results demonstrated very different performances in winter and summer for the same SSF-CW system, respectively with removals of 25 and 80%. The lower removal observed in winter may be due to the colder temperature, which can inhibit microbial activity [13]. Wetland configuration also affected DEHP removal, which varied between 19 and 49%, being the highest removal also observed in VSSF-CWs [17]. In this configuration, the unsaturated media promotes higher O_2 diffusion inside the matrix pores, increasing the O_2 transfer capacity and, as consequence, the DEHP biodegradation and removal [17]. The different CW designs also influenced erythromycin and clarithromycin removals. In a study analysing the efficiency of SF-CWs and HSSF-CWs (with or without vegetation), erythromycin was only eliminated in a planted HSSF-CW system (64%), whereas clarithromycin was partially removed in a free water SF-CW (50%). For erythromycin, the results emphasized the generally better performance of SSF-CWs when compared with SF-CWs. In the case of clarithromycin, the SF-CW system performed better, maybe because photodegradation is the main elimination pathway in this case [111]. In another study, the removal efficiency of clarithromycin varied in a VSSF-CW system, depending on the season (0-17% in the summer and 89% in the winter), whereas the elimination of erythromycin was above 86% in all sampling campaigns [105]. However, the authors did not explain these different behaviours.

The HRT is another operating parameter that can affect the removal of pollutants. For instance, in a study including the two natural estrogens E2 and E1, no degradation occurred at HRT = 27.5 h, whereas the removals significantly increased (up to 59% and 85%, respectively) with an HRT of 137.5 h [140], probably due to a higher time of contact between the pollutant and CW substrate.

In general, the plants also have an important role in removing the studied pollutants, namely due to the stimulation of the microbial communities present in the CWs systems and consequent MPs degradation. Benzene removal was often higher in the presence of plants than in systems without them [46], whereas erythromycin was only removed in HSSF-planted systems [111]. The selection of plant species is also an important aspect. For instance, nonylphenol removal in a VSSF-CW planted with *Typha latifolia*, showed the highest removal capacity (87.5%) whereas the VSSF-CW unit planted with *Phragmites australis* showed an inadequate (< 0%) removal for this compound,

probably due to the presence of its precursor in the inlet solution [12]. Plant species also affected PFOS removal, with *Hygrophila pogonocalyx Hayata* showing higher PFOS uptake in comparison with other three aquatic plants, due to a strong adsorption of PFOS and the plant characteristics, such as its small-size root biomass, larger root surface area, faster root growth and shallower root distribution comparatively to the other tested plants [14]. Erythromycin was only removed in *Phragmites australis* planted CWs (removal up to 64%), whereas no removal of this CEC was observed in Typha angustifolia planted CWs [111].

Substrate type and its depth can also affect pollutants removal due to its sorption characteristics. As example, gravel appeared to be the most efficient filter material, in comparison to vesuvianite or zeolite (both with higher porosity) to remove DEHP from WW (a PS with a high K_{ow}), probably because that material favoured the retention of organic matter and the growth of biofilm on it, contributing for a higher microbial activity which biodegraded more efficiently the compound [17]. Different depth VSSF-CWs (7.5, 30, 60 cm) were tested to remove E1, E2 and EE2, which were more efficiently eliminated with a depth of 7.5 cm [134]. This result could be related to the highest root density and the higher penetration of O_2. Removal of atrazine from WW was also very variable (inefficient removal or elimination to levels below the analytical detection limits) and very dependent upon the specific CWs substrates [76].

Results obtained so far clearly indicate that the CWs parameters must be properly optimized for efficient removal of pollutants. A clear example is diclofenac, the most studied CEC and the second among all pollutants considered in this literature review. Table 2 shows that diclofenac removal varies between 0 and more than 96% (but often up to 60%), the removal depending on wetland configuration, substrate type, HRT, and plant species [115, 120]. Reported studies indicate that CWs are still viewed as a “black box” where only influent and effluent concentrations are measured to evaluate their performance, without details about the occurring processes, that affect the fate or transformation pathways of the contaminants. For instance, during degradation, pollutants can originate other compounds (transformation products) that can be more toxic than their parent compounds. In this regard, it is imperative to understand the basic elimination and transformation processes that drive the removal of pollutants in such “black box”, to optimize the CWs design and

operational parameters and consequently get better treatment efficiencies for a higher number of pollutants [146].

As shown above, most target PSs and CECs can be eliminated to levels below the analytical detection limits when using CWs. However, due to the refractory nature of some PSs and CECs, CWs can be limited in providing their adequate elimination from contaminated water/wastewater. Furthermore, CWs are dependent on several factors and treatment is characteristically slow. Combining or integrating CWs with other existent technologies, such as membrane processes and AOTs, with proved efficiency on the removal of PSs [147] and CECs [148], could be a good solution to enhance CWs global efficiency. Indeed, the combination of CWs with other treatment technologies was recently reviewed, targeting organic and nutrient removal, as well as the elimination of persistent organics pollutants and metals in general [149]. Considering the advantages, disadvantages, and respective operating costs of different alternatives (activated sludge, anaerobic reactor, membrane bioreactor, TiO_2-based photocatalysis, electrolysis, reverse osmosis), the authors also concluded that more research is required to propose optimal combinations for each type of water/wastewater.

2.1.1.4. Conclusions

Despite the recognized potential of CWs to remove pollutants from water/wastewater, studies dealing with organic pollutants defined in recently launched EU legislation are still limited to a few of these pollutants. In fact, only 20 out of a total of 41 organic PSs/class of PS and 8 CECs out of 17 of the first watch list, were studied.

Regarding PSs, benzene and atrazine are those receiving higher attention in the literature regarding their removal by CWs, followed by nonylphenol and chlorpyrifos. Although several reports deal with the removal of solvents, pesticides are the most studied group of substances. Diclofenac is the most investigated CEC, followed by the macrolide antibiotics and the estrogens E1 and E2. Thus, studies related to the application of CWs for the removal of many other organic PSs, certain other substances with EQS and some CECs are still missing, as well as reports dealing

with mixtures of several of these pollutants. These studies must be preferentially performed at realistic concentrations. Moreover, despite the need to extend the studies to other organic pollutants defined in EU legislation, studies regarding their removal in CWs must imperatively contemplate the understanding of the processes responsible for it. Only with that information one can optimize CWs design and operating parameters, consequently getting better treatment efficiency, and fully supporting CWs utility. In this sense, some important points can be addressed to extend and consolidate the research in this area, namely: (i) seasonal sampling campaigns to evaluate the most frequent PSs, certain other substances with EQS, and CECs in each type of water/wastewater; (ii) CWs experiments using different operating conditions (configuration, light penetration, and HRT), to assess their effects and achieve the best performance of CWs; (iii) CWs experiments with inlet solutions spiked with more PSs, certain other substances with EQS, and CECs at realistic levels to evaluate CWs removal of a wider set of pollutants when occurring at environmentally relevant concentrations; (iv) to study the transformation products of pollutants during CWs treatment, while simultaneously evaluating their possible elimination together with the parent compound; (v) evaluation of adequate approaches that can be coupled to CWs for the elimination of more recalcitrant compounds.

2.1.2. A brief review on the application of advanced oxidation technologies for the removal of organic micropollutants

The removal of organic MPs by CWs is definitely a good alternative because of their reduced costs and environmental friendliness. However, from the efficiency point of view, there are some concerns about the possible insufficient capacity of CWs to remove refractory compounds. Thus, AOTs which have a great potential to eliminate more organic MPs, can also be considered as option for the treatment of aquaculture water/wastewater (alone or combined with CWs). In contrast to CWs, AOTs have been extensively studied to remove organic MPs from water matrices, with high efficiencies registered. Therefore, the purpose of this chapter is to give a very brief overview on this topic, considering only information of review articles dealing with the application of AOTs for MPs removal. Review articles were retrieved from the *Scopus* database according to the following list of keywords: "*advanced oxidation processes*", "*advanced oxidation technologies*", "*UV*", "*ozone*", "*photocatalysis*", "*photocatalytic*", "*photocatalytic ozonation*", "*Fenton*", "*photo-Fenton*", "*electrochemical oxidation*", "*electrocatalysis*", "*sonolysis*", "*hydrodynamic cavitation*", "*catalytic wet air oxidation*", "*catalytic wet peroxide oxidation*", and "*micropollutants*", "*contaminants of emerging concern*" or "*priority substances*" (accessed until November 2021). The evolution of review articles published over the years is discussed, the main AOTs under study, and the representativeness of the water matrices and target MP/group of MPs. Therefore, the most important information concerning MPs removal by AOTs is herein summarized. O_3 and UV-based processes were the most investigated AOTs, but full-scale studies are still scarce for those and all other AOTs. Moreover, the influence of specific parameters on AOTs efficiency needs further research.

Considering the high capacity of AOTs to destroy organic pollutants, there are several research studies focused on its application to remove MPs from water and WW matrices. As a consequence, there are many review articles on AOTs for MPs elimination that summarize all the important information that has been published on the topic. As it is shown in Figure 7, a total of 98 review articles were published since 2011 [147, 148, 150-245], demonstrating the huge interest on AOTs to remove this type of contaminants. It should be noted that until 2011, only 3 reviews were found [246-248], which indicates the enormous recent developments in this research field. The number of publications revising AOTs significantly increased in 2020, a tendency that was maintained in 2021 with 37 review articles counted. At the end of this survey (*i.e.,* November 2021), 5 reviews were already published in journal issues of 2022 [232-236].

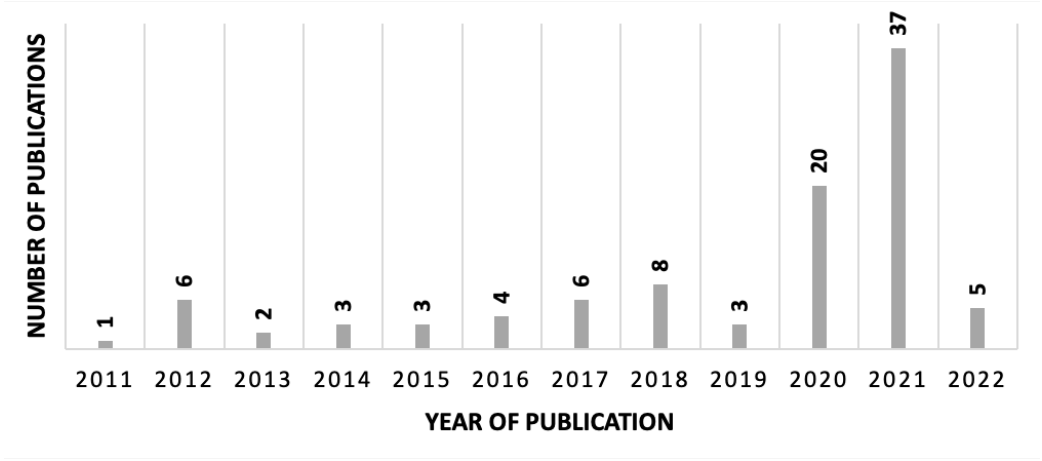


Figure 7. Number of review articles published since 2011, related to the application of AOTs to remove MPs. The reported studies were retrieved from the *Scopus* database, selecting "*Review*" in the document type field, and using as "*advanced oxidation processes*", "*advanced oxidation technologies*", "*UV*", "*ozone*", "*photocatalysis*", "*photocatalytic*", "*photocatalytic ozonation*", "*Fenton*", "*photo-Fenton*", "*electrochemical oxidation*", "*electrocatalysis*", "*sonolysis*", "*hydrodynamic cavitation*", "*catalytic wet air oxidation*", "*catalytic wet peroxide oxidation*", and "*micropollutants*", "*contaminants of emerging concern*" or "*priority substances*" (accessed until November 2021).

Among the reviewed AOTs, light-driven and O_3-based processes represent more than half of the total publications, accounting each one for 37 and 19% of the published reviews, respectively. In turn, electrochemical, Fenton, peracids, ultrasound (US) and plasma-based AOTs totalling 21% of the review articles published until November 2021. Furthermore, studies on AOTs in general (*i.e.,* involving the analysis of several types of AOTs) represent 23% of the review articles that were found (Fig. 8a).

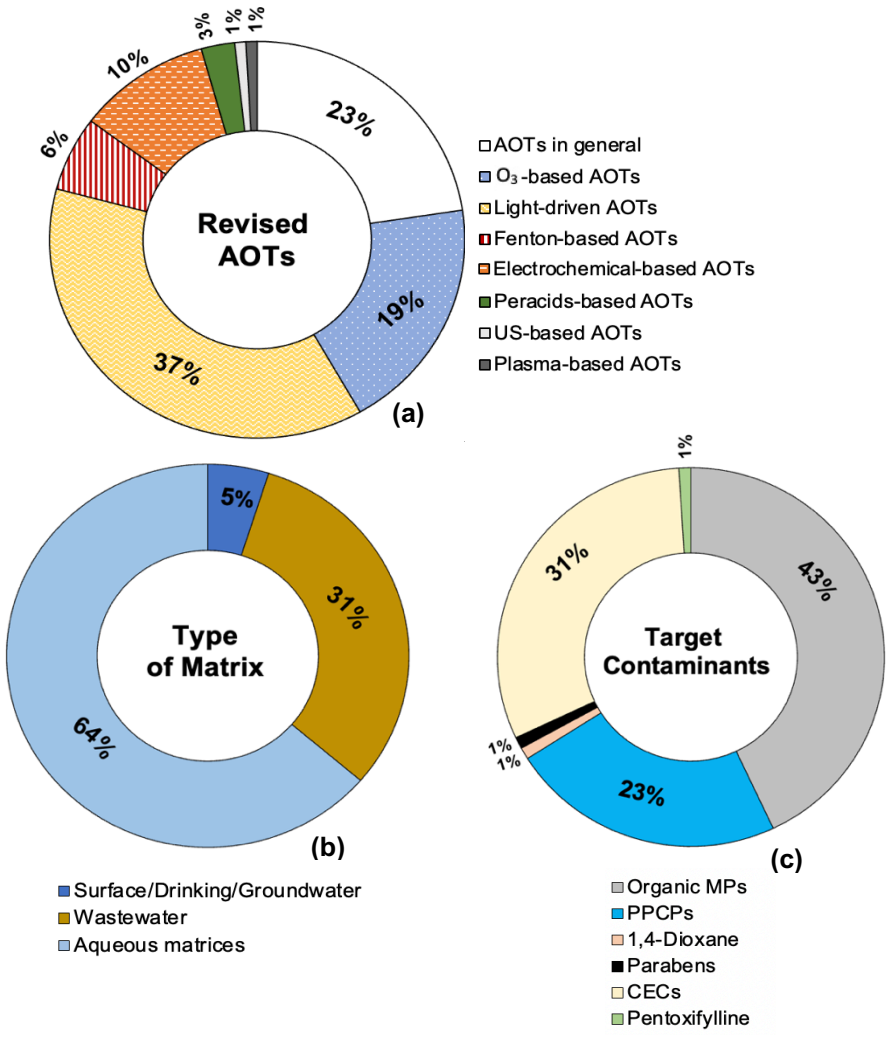


Figure 8. Distribution of the review articles found in the *Scopus* database dealing with the application of AOTs for organic MPs removal according to the: **(a)** analysed AOT, **(b)** type of water matrix and **(c)** target contaminants. The keywords used for the survey were the same as those described in Figure 7.

Since the photolytic degradation of MPs is highly dependent on the absorbance of each compound, reviewed information on light-driven AOTs is mainly related to UV light combined with an oxidant (in most cases H_2O_2) [157, 159, 236] and photocatalysis [155, 194, 195, 241, 244]. In addition, there is a lot of information dealing with TiO_2-assisted photocatalysis. For instance, alternatives such as TiO_2 doped catalysts have been studied to extend the absorption range of TiO_2 into the visible light and improve the TiO_2 efficiency under solar radiation [161, 163]. Reviews have also been published focusing on solutions for immobilizing TiO_2 to help overcome the challenge of catalyst separation from the water matrix after treatment (*e.g.*, TiO_2 nanotubes array, TiO_2 supported in ceramic materials, and TiO_2 magnetic photocatalysts) [168, 193, 219]. Moreover, the use of modified membranes, namely TiO_2-graphene oxide (GO) and polyethersulfone-TiO_2 nanofiber composite membranes, has gained attention since it can combine both membrane separation and photocatalysis processes for the removal of MPs [218, 234]. Traditionally, light-driven AOTs are based on the use of low-pressure mercury lamps; however, the light-emitting diodes (LEDs) technology has been pointed out as an alternative radiation source [180, 203]. Some reviews have already been focused on light-driven chemical and catalytic reactors, summarizing important information on their configuration and performance concerning MPs mitigation [188, 192, 210]. Regarding O_3-based processes, information of reviews includes reaction kinetics of MPs [162, 246], performance of ozonation for both MPs and microorganisms removal [198, 247], and possible combination of O_3 with other technologies (namely biological filters, catalytic membrane separation, and ultrasonication) to improve the efficiency of the process [183, 186, 189, 197]. In fact, conventional ozonation (*i.e.,* O_3 alone) can be insufficient to degrade recalcitrant pollutants, and some toxic transformation products can be generated, as is the case of bromate ion (BrO_3^-). This explains why O_3 has been combined with other technologies or radical promoters such as catalysts and chemical reagents [221]. Basically, combining catalysts or other chemicals with ozonation can promote a synergistic effect by transforming O_3 into $HO^\bullet$ radicals, which in turn lead to an efficient degradation of O_3-resistant compounds. Moreover, ozonation does not completely mineralize MPs, but it can oxidize organic MPs into smaller and more biodegradable compounds. Therefore, combining O_3 with post-biofiltration (sand biofiltration, biological activated carbon

filtration or biological aerated filtration) has shown interesting features to enhance the overall water quality, being a safe and affordable post-treatment methodology to remove oxidation by-products (including those with potential toxicity) and avoid bacteria regrowth [157, 166, 175, 197].

More recently, the application of electrochemical-based AOTs to remove MPs has received growing attention, since they are considered compact, scalable and automatable, and chemical-free systems. Reviews on electrochemical AOTs are mainly based on the discussion of operational parameters (*e.g.,* current density, pH, and MPs concentration) which have a great influence on the process efficiency [170, 199, 211, 215]. To overcome problems related to by-products generation, integrated solutions such as combination with biological digestion systems have been proposed [191]. Fenton-based processes have been described as a technology with a great capacity to remove organic MPs; however, since the optimum conditions are commonly achieved under acidic pH, it is hardly an option for full-scale systems just to remove MPs. The low pH (≤ 3) has a significant repercussion on the managing costs due to the acidification often needed, and subsequent neutralization before disposal/reuse, with concomitant formation of sludge [176, 202, 249]. Nevertheless, alternatives such as photo-Fenton at neutral pH and electro-Fenton have been studied to surpass the main drawbacks of this technology [176, 202, 226]. In addition, the application of peracids as "new" oxidants for MPs removal, namely peracetic acid (PAA), and peracid salts, *i.e.,* peroxymonosulfate (PMS) and persulfate (PS), has triggered more investigation on this topic. Oxidation of MPs by PAA is based on $HO^{\bullet}$, while oxidation of MPs by PMS and PS is based on $SO_4^{\bullet -}$.The reviews published so far in this topic (n=3) described the fundamental principles of these treatments, the factors influencing the oxidation process, and the effectiveness for MPs removal [171, 205, 222]. Besides all the abovementioned processes, plasma and US-based AOTs were recently reviewed as alternatives with potential to remove MPs [177].

Most reviews on MPs removal by AOTs comprise information dealing with different types of aqueous matrices (64%), but a significant percentage of studies is exclusively devoted to WW matrices (31%) (Fig. 8b). Domestic effluents are the most representative WW matrix, but hospital effluents are also widely studied because of the current concern related to the spread of pharmaceuticals in the aquatic environment [160, 179, 220, 249]. In addition, catalytic AOTs were

already revised for removing MPs from textile WW [225], and ozonation, Fenton-based techniques, and electrochemical AOTs were reviewed for degrading MPs from landfill leachate [235]. Regardless of the type of water matrix, AOTs intend to eliminate MPs which are ineffectively removed through conventional treatment processes. Thus, they have been considered to treat effluents from WWTPs, hospitals, industries, and landfill sites, but also to treat surface and ground waters supplying DWTPs [246, 247]. With this approach, the dissemination of MPs can be mostly controlled in their main sources of release, contributing to mitigate the MPs cyclical occurrence and consequent accumulation in the environment.

Although there are several classes of organic MPs, the majority of reviews does not deal with a specific class of these compounds (Fig. 8c). Despite this, PPCPs have received particular attention since they represent 23% of the review articles published so far. This agrees with the information mentioned above, which highlights a high apprehension related to the dissemination of this class of MPs. Moreover, some reviews are based on the use of AOTs to remove specific pollutants, namely 1,4-dioxane and pentoxifylline [178, 196], or other classes of MPs like parabens [209]. Currently, CECs is a largely applied term in the field of environmental pollution by MPs, and indeed, CECs represent 31% of the studies regarding the type of studied contaminant (Fig. 8c). Information of review articles shows that MPs have distinct behaviours when they are treated by AOTs, depending either on their chemical nature or the type of applied process, but in general high removals are achieved.

Overall, review publications indicate that studies on MPs removal by AOTs are still developed at lab and/or pilot scale and their possible application at full scale still lacks. However, ozonation and UV/H_2O_2 have already been applied at full scale [160, 164]. Furthermore, it is unanimous that there is a lot to explore regarding all the parameters that can affect the performance of AOTs (including pH, matrix, light intensity, and catalyst) [165, 176, 191]. Thus, it will be possible to establish the best treatment options for each type of water/wastewater, as well as the respective optimum conditions to be implemented. It is important to understand that it is not feasible to provide a global "best" technology to remove MPs. The decision on the best technology needs to be made for each water/wastewater depending on the local conditions (*e.g.*, available space and solar energy, cost

of electricity), the characteristics of the water to be treated and on the required effluent quality (reuse requirements, disinfection needs) [175]. Beyond MPs elimination, the toxicity of treated water must also be carefully evaluated, a still underexploited topic.

2.1.3. Advanced oxidation technologies and constructed wetlands in aquaculture farms: what do we know so far about micropollutant removal?

Aquaculture is the fastest-growing animal food-producing sector, and thus, preserving its water quality is an issue of major concern. Recently, the European Commission has launched a guiding document promoting more ecological aquaculture practices, but options for water treatment targeting MPs removal are still lacking. In this sense, after a survey on CWs (*sub-chapter 2.1.1*) and AOTs (*sub-chapter 2.1.2*) to remove organic MPs in any matrix, it was intended in the present section to consolidate the relevant information on the application of both these technologies in the specific case of aquaculture farms. For that, a search was done in the *Scopus* database (accessed until October 2020), using as keywords the name of a broad range of AOTs (*i.e.*, "*advanced oxidation technologies/advanced oxidation processes*", "*UV*", *"ozone*", "*photocatalysis*", "*photocatalytic*", "*photocatalytic ozonation*", "*Fenton*", "*photo-Fenton*", "*electrochemical oxidation*", "*electrocatalysis*", "s*onolysis*", "*hydrodynamic cavitation*", "*catalytic wet air oxidation*", "*catalytic wet peroxide oxidation*" *or "catalytic wet oxidation"*) and "*aquaculture*" or "*constructed wetlands*" and "*aquaculture*". All article types were considered dealing with the application of CWs and/or AOTs in aquaculture, studies being analysed according to the goal of the treatment: (i) removal of organic MPs; (ii) removal of conventional pollutants; (iii) removal of bacteria; and (iv) possible combinations between (i), (ii) and/or (iii). Since (i) and (iv) are related with MPs removal, they are the focus of this chapter. In this regard, only 34 studies were found (28 on AOTs and 6 on CWs) for MPs removal until October 2020. Using the same keywords above, but in November 2021, it was perceptible that studies on MPs removal by AOTs continue to increase. Moreover, both technologies in combination were not investigated yet for aquaculture waters.

This sub-chapter is based on the following reference (original version available in **Appendix A2**, with permission of Elsevier):

Gorito, A.M., Ribeiro, A.R.L., Pereira, M.F.R., Almeida, C.M.R, Silva, A.M.T. *Advanced oxidation technologies and constructed wetlands in aquaculture farms: What do we know so far about micropollutant removal?* Environmental Research, 204 (2022) 111955.

2.1.3.1. Treatment of aquaculture waters by CWs

A total of 59 studies on the application of CWs in aquaculture farms have been published until October 2020 (without any temporal restriction on the *Scopus* survey, Fig. 9). The elimination of conventional pollutants represents most of the published studies (91%); however, removal of organic MPs has gained increasing attention, representing nearly 9% of the total studies dealing with the treatment of aquaculture waters by CWs. In November 2021, 15 more studies were found in the *Scopus* database applying the same keywords *(i.e., "constructed wetlands"* and "*aquaculture"*), but none of them dealt with MPs elimination.

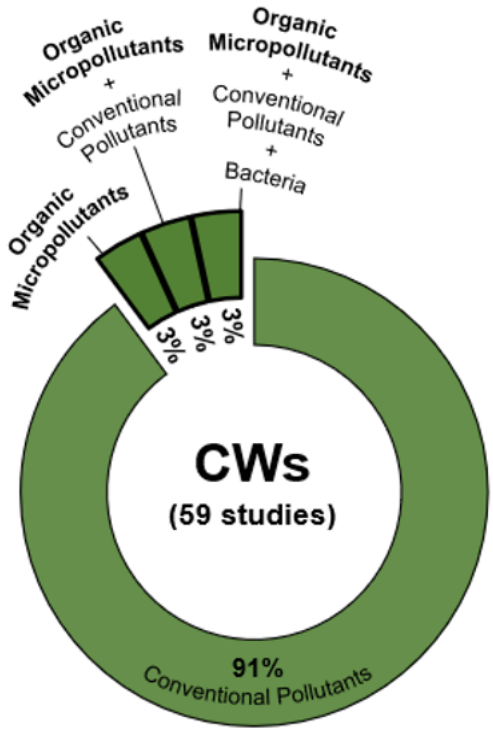


Figure 9. Total number of studies dealing with the application of CWs in aquaculture farms (59) and the distribution of studies regarding the type of contaminant studied (*i.e.,* organic MPs, bacteria, or more conventional pollutants – *e.g.*, organic matter, total suspended solids, and nutrients). The results were retrieved from the *Scopus* database using the keywords "*constructed wetlands*" and "*aquaculture*" (accessed until October 2020).

2.1.3.1.1 Removal of organic MPs

Among the studies dealing with the treatment of aquaculture effluents by CWs (Table 3), two have been conducted at the microcosm-scale [250, 251], one at the pilot scale [252] and the other three at full scale [1, 2, 253]. Among the last three references cited, two studies conducted by Hsieh, *et al.* [1, 2] focus on CWs to treat aquaculture effluents in combination with domestic and livestock WW. Thus, the CWs applied in these reports were not designed for their specific application in aquaculture farms, but instead for removing MPs from mixed effluents resulting from different activities.

Antibiotics are the most widely studied organic MPs, mainly the classes of fluoroquinolones, sulphonamides, and tetracyclines, which are the classes widely used in aquaculture facilities. Some studies have included other substances, such as endocrine disruptors [1, 2] and other compounds that can be found in aquaculture inlet waters [251]. Many of the target MPs that have been studied so far are included in Directive 2013/39/EU (alachlor, atrazine, chlorfenvinphos, isoproturon, nonylphenol, octylphenol, PFOS, and simazine) [254] and in the Decision 2018/840/EU (azithromycin, ciprofloxacin, clarithromycin, clothianidin, erythromycin, methiocarb, thiacloprid, thiamethoxam, E2, and EE2) [144]. Moreover, trimethoprim, venlafaxine, sulfamethoxazole, and ciprofloxacin were included in the recent Decision 2020/1161/EU [145]. Only one study reported the removal of geosmin and 2-methylisoborneol, which are taste and odour (T&O) compounds naturally occurring in aquaculture facilities. These substances represent a serious problem for the sector because the quality of the fish can be compromised by its unpleasant flavour, and the use of undesirable compounds (such as algicides) is required in some cases to avoid this situation [253].

The removal of target MPs by CWs has mainly been studied in freshwater aquaculture farms. Only a single study [250] has been performed with marine aquaculture effluents in non-recirculated aquaculture systems (non-RAS), and the high levels of salinity tested did not affect CW performance. All the investigations were conducted with non-synthetic water, independent of the scale of the study, demonstrating the concern for testing CWs under near-real conditions. The inlet concentrations of target pollutants studied in CWs ranged from a few to several μg L^{-1} (0.0001–

100 μg L^{-1}). Only two studies were performed under spiked conditions (non-spiked experiments also included) [250, 251]. Pollutant concentration can affect the removal efficiency of biodegradation and adsorption. For instance, high concentrations may destabilize the microbial community, disfavouring biodegradation, whereas saturation of the binding sites can limit adsorption [251]. In any case, the registered removal efficiencies did not show evidence of lower CW performance in the presence of more concentrated effluents [250-253].

A wide range of removals (0–100%) of the target MPs has been verified (Table 3), but it is important to consider that the number of compounds under analysis is also large and includes substances with very different characteristics. In general, CWs showed excellent performance in the removal of organic MPs. However, some of these compounds (EE2, EHMC, and sulfamethoxazole) were more resistant to the treatment, registering lower removal rates [2, 251, 252]. As mentioned above, the distinct conditions of CWs and the composition of effluents can lead to different removal efficiencies of organic MPs. For instance, by analysing alkylphenols and their ethoxylates, it was possible to verify that two studies of the same research group [1, 2] found very dissimilar removal of these compounds. When comparing the performance of CWs in removing nonylphenol diethoxylates, nonylphenol monoethoxylates, nonylphenol, and octylphenol in both reports, a series of three successive SF-CWs [2] proved to be much more efficient (76%–90%) than an individual SSF-CW (0–38%) operating with low concentrations of MPs [1]. In a study by Hsieh *et al*. [1], the ecological risks were evaluated by the risk quotients of nonylphenol and octylphenol derivatives before and after treatment; its decrease from influents to effluents indicates that CWs can effectively remove these phenolic endocrine disruptors. Furthermore, another study showed that CWs planted with single plant species are more efficient at removing enrofloxacin and sulfamethoxazole than CWs with mixed planting patterns [252]. In the same report, sorption was the main removal mechanism of enrofloxacin, favouring the use of *Phragmites australis* instead of *Iris pseudacorus* because of its deeper and denser root system that enlarges the area for biofilm growth. In contrast, sulfamethoxazole has a low sorption capacity, with degradation (and transformation) playing a significant role in its removal in comparison to substrate adsorption and plant uptake. In this case, *Iris pseudacorus* provided a better environment for the microbial

community to degrade or transform sulfamethoxazole [252]. Indeed, two other studies showed excellent performance of CWs removing enrofloxacin with a single plant species, specifically *Phragmites australis*, treating both freshwater [251] and marine [250] aquaculture effluents.

All the reports detailed in Table 3 (including SF and SSF-CWs) correspond to operating periods equal to or higher than 1 month, and the successive loads of pollutants did not affect the efficiency of the systems. Moreover, most of the studies dealing with the removal of organic MPs (4 out of 6) also considered the elimination of other types of pollutants (such as total suspended solids, organic matter, nitrogen, phosphorus, ARB&ARGs), which were also efficiently removed by CWs [250, 252, 253, 255]. The reported information shows encouraging results for the implementation of CWs to remove organic MPs from aquaculture waters; however, further investigation is needed to fully understand the mechanisms responsible for the elimination of these compounds. In this way, the design and operational parameters can be optimised through appropriate criteria to obtain better treatment efficiencies for a wider list of MPs.

Table 3. Studies on the applicability of CWs for the removal of organic MPs from aquaculture effluents.

Target Micropollutant	Type of aquaculture (category of water)	Concentration ($\mu g \cdot L^{-1}$)	Removal (%)	Ref.
Enrofloxacin	Non-RAS (Marine; non-synthetic)	100	> 99	[250]
Oxytetracycline		100	> 99	
Acetamiprid	Non-RAS (Freshwater; non-synthetic)	0.06–0.09	100	[251]
Alachlor		0.01–0.12	100	
Atorvastatin		0.08–0.43	100	
Atrazine		0.0003–0.08	94–100	
Azithromycin		0.03–0.05	90–98	
Bezafibrate		0.08–0.09	98–100	
Carbamazepine		0.11–0.13	100	
Cephalexin		0.02–0.07	100	
Ceftiofur		0.03–0.06	100	
Chlorfenvinphos		0.05–0.14	98–100	
Citalopram		0.06–0.13	100	
Clarithromycin		0.002–0.11	90–100	
Clindamycin		0.02–0.07	100	
Clofibric acid		0.06–0.09	97–100	
Clothianidin		0.06–0.09	100	
Diclofenac		0.07–0.09	83–100	
Diphenhydramine		0.12–0.18	90–100	

Table 3. (Continued)

EHMC	Non-RAS (Freshwater; non-synthetic)	0.3–1.8	26–87	[251]
Enrofloxacin		0.5–0.7	100	
Erythromycin		0.09–0.12	100	
Fluoxetine		0.00–0.08	100	
Isoproturon		0.0001–0.09	93–98	
Ketoprofen		0.04–0.12	100	
Methiocarb		0.6–0.9	100	
Metoprolol		0.07–0.11	100	
Norfluoxetine		0.007–0.12	100	
Ofloxacin		0.05–0.08	100	
PFOS		0.009–0.2	0–100	
Propranolol		0.1–0.13	88–100	
Simazine		0.057–0.064	91–100	
Thiacloprid		0.05–0.07	100	
Thiamethoxam		0.05–0.07	100	
Tramadol		0.03–0.06	94–100	
Trimethoprim		0.001–0.02	100	
Venlafaxine		0.05–0.08	100	
Warfarin		0.10–0.16	92–100	
Enrofloxacin	Non-RAS (Freshwater; non-synthetic)	0.03–0.06	75–82	[252]
Sulfamethoxazole		0.07–0.22	55–77	
Nonylphenol diethoxylates	Non-RAS (n.d; non-synthetic)	0.003–0.041	~ 0	[1]
Nonylphenol monoethoxylates		0.003–0.035	~ 0	
Nonylphenol		0.001–0.152	16	
Octylphenol		0.001–0.901	38	
Ciprofloxacin	Non-RAS (n.d; non-synthetic)	0.02	100	[2]
Chloramphenicol		0.06	98	
Chlortetracycline		not detected	n.d.	
Enrofloxacin		not detected	n.d.	
Leucomalachite green		n.d.	n.d.	
Malachite green		n.d.	n.d.	
Oxolinic acid		0.01	100	
Oxytetracycline		0.22	97	
Sulfamonomethoxine		0.09	87	
Sulfadimethoxine		0.08	91	
Sulfamerazine		not detected	n.d.	
Sulfamethazinte		not detected	n.d.	
Tetracycline		not detected	n.d.	
Bisphenol A		0.9	89	
Nonylphenol diethoxylates		0.2	85	
Nonylphenol monoethoxylates		0.3	77	
Nonylphenol		1.7	90	
Octylphenol		1.1	85	
E2		0.2	95	
E3		0.2	77	
EE2		0.02	32	
Geosmin	RAS (Freshwater; non-synthetic)	not detected	n.d.	[253]
2-methylisoborneol		not detected–0.08	67–100	

n.d., not defined; EHMC, 2-ethylhexyl 4-methoxycinnamate; PFOS, perfluorooctanesulfonic acid; E2, 17-beta-oestradiol; E3, oestriol; EE2,17-alpha-ethinyloestradiol.

2.1.3.1.2 Removal of other pollutants

The potential of CWs to eliminate more conventional environmental contaminants such as nitrogen, phosphorus, organic matter, and total suspended solids has been extensively described in the literature [256-258]. It is interesting to note that concerns about pollution caused by aquaculture, namely organic waste and nutrients released by their effluents, have increased over the last few years. As highlighted in the guiding document launched by the European Commission in 2016 [259], organic matter and nutrients (as excretory products and uneaten fish food) can cause eutrophication of receiving waters, jeopardising ecosystems, and natural dynamics of aquatic environments. Furthermore, even low concentrations of ammonia and nitrite ions (0.5 mg L^{-1}) can also be toxic to aquatic organisms [260]. Thus, it is important to implement measures to mitigate organic enrichment and nutrient input impacts, including treatment technologies such as CWs [259].

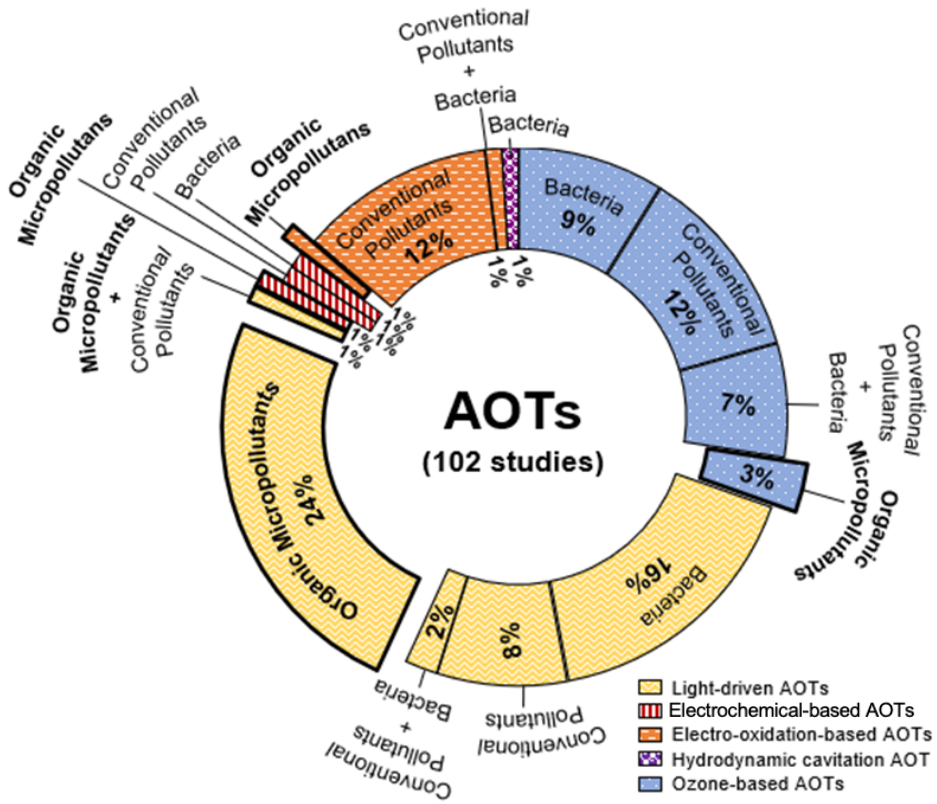
The removal of conventional pollutants represents 91% of CW studies in aquaculture farms (Fig. 9). These works include the treatment of both marine and freshwater aquaculture effluents in the laboratory, pilot (predominant) and full-scale CWs. Moreover, different configurations (SF and SSF-CWs) and operational conditions (such as nutrient loading rates, hydraulic regimes, and plant species) of the CWs were also tested. It has been found that these parameters can affect the performance of wetland systems, but the efficiencies were in general sufficient to guarantee appropriate levels of discharge of conventional pollutants owing to the high removal efficiencies achieved. As verified for organic MPs, the high salinity of marine aquaculture effluents did not affect the effectiveness of the biological treatment, with the *Salicornia* plant species being frequently used for the treatment of this type of effluent [261]. The removal of nutrients and organic matter (assessed through chemical oxygen demand, biochemical oxygen demand, and total organic carbon) strongly depends on the microbial activity. In fact, a considerable number of studies have focused on the composition and activity of the microbial consortium [261-264]. Most of the studies analysed the removal of nitrogen compounds from external carbon sources [263, 265] or iron addition [264] representing successful alternatives to enhance nitrification-denitrification processes. Despite the capacity of CWs to remove bacteria, only two studies have focused on the

elimination of ARB [250] and ARGs [252] from aquaculture waters, registering removals of approximately 95% and 57%, respectively.

Overall, the reviewed data indicate that CWs are highly efficient in removing conventional pollutants (such as organic matter, nitrogen, phosphorus, and total suspended solids) from aquaculture effluents. The functioning of CWs must be routinely controlled; if necessary, adaptations can be easily made to optimise the efficiencies obtained. Compared with other treatment technologies frequently applied in aquaculture farms (such as mechanical filters, trickling filters, submerged biofilters and rotating biological contactors), CWs may be an appealing alternative because of their attractive costs, environmental friendliness, and efficiency in removing either conventional pollutants or organic MPs.

2.1.3.2 Treatment of aquaculture waters by AOTs

A total of 102 studies from the database, accessed until October 2020, dealt with the application of AOTs in aquaculture farms (Fig. 2). UV (51%) and O_3-based (31%) treatments were the most representative AOTs, followed by electrochemical (14%), Fenton (3%) and hydrodynamic cavitation (1%) processes. Most reports (~70%) addressed the removal of bacteria and/or conventional pollutants from aquaculture effluents, mainly in RAS systems, to guarantee the quality of recirculating water and protect the farmed organisms. However, the removal of organic MPs already represents approximately 30% of the total studies published so far (mostly published since 2018; Table 4), showing emerging concerns about the presence of these substances in aquaculture waters. It should be noted that a literature revision performed in November 2021 has shown 20 more studies on AOTs treating aquaculture waters; 7 of them being precisely focused on MPs removal [266-272].



Additional Information:
Light-driven AOTs include UV, UV/H_2O_2, and UV photocatalytic processes
Fenton-based AOTs include Fenton, photo-Fenton and Fenton-like catalysis processes
Electrochemical-based AOTs include electro-oxidation, electrocatalysis, UV/electro-oxidation, electro-Fenton processes
O_3-based AOTs include O_3, O_3/H_2O_2, O_3/H_2O_2/UV, O_3 catalytic and O_3 photocatalytic processes

Figure 10. Total number of studies dealing with the application of AOTs in aquaculture farms (102) and the distribution of studies regarding the type of contaminant studied (*i.e.*, organic MPs, bacteria or conventional pollutants – *e.g.*, organic matter, total suspended solids, and nutrients). The results were retrieved from the Scopus database using the keywords "name of AOTs" and "aquaculture" (accessed until October 2020).

2.1.3.2.1 Removal of organic MPs

The collected information (Table 4) shows that most investigations have focused on heterogeneous photocatalysis to remove organic MPs from aquaculture effluents. TiO_2 is the most investigated photocatalyst among all the tested photocatalysts (TiO_2, porphyrin-TiO_2, Au-TiO_2, Zeolite-TiO_2,

TiO_2-rGO, TiO_2-Fe^0, Pd/WO_3, Zeolite-$TiCl_4$/$FeCl_3$, CN-TiO-CNT, GO/CN-TiO-CNT, and CuO/ZnO), whereas UV or solar radiation is generally applied to activate the photocatalyst. Experiments using TiO_2 nanotubes alone or combined with Au nanoparticles [273], TiO_2 with silica zeolite [274] and reduced graphene oxide (rGO) [275] are some examples of photocatalysts that have been developed to remove organic MPs from aquaculture waters. Alternatives to TiO_2-based photocatalysts have also been explored, in particular CuO/ZnO nanocomposites for the treatment of marine aquaculture effluents [276, 277]. A novel Pd/WO_3 catalyst was also investigated to remove organic MPs [278]. All photocatalytic studies were conducted at bench scale, except one performed at full-scale RAS [279] and two performed in pilot plants involving solar compound parabolic collectors [280] and packed-bed photocatalytic reactors [281]. Likewise, the other studied technologies were performed at lab scale, except for three studies on UV-and O_3-based processes implemented in RAS [282-284], which were conducted at pilot- and full-scale water treatment units. Antibiotics are the most studied substances, with an emphasis tetracyclines and sulfonamides classes. Tetracyclines, sulfonamides, trimethoprim, quinolones, β-lactams, fluoroquinolones and phenicols are pharmaceuticals authorized for administration to fish in Europe, although oxytetracycline gets particular attention due to its wide application in the aquaculture industry [275]. Among these, sulfamethoxazole, trimethoprim, and amoxicillin are included in the new EU watch list intended to improve knowledge of the occurrence and spread of antimicrobials in the environment [145]. E1, E2, and oestriol (E3) hormones were also studied as target compounds, with the first two being included in the prior list of CECs of Decision 2018/840/EU [144]. Although the use of hormones in aquaculture farms is prohibited in the EU territory, due to their possible impacts on farmed fish and human health, other countries such as Canada, Australia, New Zealand, Argentina, and the United States still permit their use for artificial fish reproduction and sex reversal [285]. Studies on AOTs also include the removal of T&O compounds such as geosmin and 2-methylisoborneol. The excessive eutrophication of fishponds promotes the development of cyanobacteria, and as a consequence, the generation of T&O compounds is frequently detected in aquaculture facilities [253].

The concentrations of the abovementioned substances in these studies, varied largely between 0.0042 and 200000 μg L^{-1}. In fact, AOTs have generally been investigated using matrices with concentrations of the target compounds higher than those found in the aquatic environment. Moreover, the effects of matrix constituents on the performance of AOTs (*e.g.*, removal efficiencies) are not considered when synthetic effluents are used. Consequently, the treatment efficiency is often overestimated when synthetic solutions are used. In general, studies that do not spike the effluents correspond to those that implement AOTs in aquaculture facilities [279, 282, 284, 286]. The selection of high concentrations in most studies is related to the unavailability of the highly sensitive analytical apparatus required to assess the treatment performance at ng–μg L^{-1} levels of MPs. Therefore, future research should involve testing concentrations of lower magnitudes, instead of studying conditions not adjusted to real scenarios. Investment in modern and powerful analytical technologies must be considered to enable the study of suitable conditions (*e.g.*, catalyst dose, treatment time, and intensity of radiation) favouring treatment scale-up.

Analysing the efficiencies of AOTs (Table 4), it is clear that each of the listed studies had a broad spectrum of removal efficiencies for the respective target MP. This is mainly due to the optimisation steps under different tested conditions (such as amount, composition and particle size of the catalyst, type and time of irradiation, initial concentrations of MPs, presence of inorganic ions, and treatment time). For instance, photolysis of oxytetracycline (in a real matrix) using simulated sunlight had significant removal variations (20–75%), with its degradation being influenced by total suspended solids, pH, and salinity of effluents [275]. On the other hand, higher removal (78– 100%) of oxytetracycline (in deionized water) was achieved by both solar photolysis and solar TiO_2 photocatalysis in pilot-scale compound parabolic collectors, requiring an accumulated UV energy of 7 and 1 kJ L^{-1}, respectively. However, additional experiments at the bench scale emphasised that the presence of PO_4^{3-} can affect the efficiency of the photocatalytic process [280]. Another study with deionised water indicated that the efficiency of solar photolysis of oxytetracycline and oxolinic acid was below 53%. Solar TiO_2 and porphyrin-TiO_2 photocatalysis showed considerably higher removals of oxolinic acid, but the enhancement in the removal of oxytetracycline was negligible [287]. These results are in accordance with those reported elsewhere [280]. The removal

of tetracycline was evaluated in a hybrid solution with Cu (II) using TiO_2 photocatalysis, showing that the initial concentration of the antibiotic can considerably affect the performance of the process. Increasing the concentration from 20 to 200 mg L^{-1} resulted in a reduction of photocatalytic performance by approximately 45% [288]. Moreover, the effect of pH on the photocatalytic degradation of the T&O compounds geosmin and 2-methylisoborneol was highlighted in another study, which also showed a negative effect of the real matrix on geosmin removal [286]. Likewise, the matrix has a significant influence on the degradation rate and cost effectiveness of UV/H_2O_2 and UV/O_3 removal of geosmin and 2-methylisoborneol [283]. In general, these compounds were highly removed after the total treatment time, except in a specific case of a UV-TiO_2 continuous reactor at bench-scale RAS [279]. Several operating parameters were identified as causing the limited performance, because a similar batch reactor implemented at full scale showed better removal of the same compounds [279]. When water containing formalin was treated using TiO_2-rGO UV photocatalysis, both inorganic ions and organic matter interfered with the treatment performance. This interference mainly occurs in salty water when compared with freshwater, due to the higher concentrations of inorganic ions that compete with the target contaminant for photogenerated HO• radicals and adsorption sites at the catalyst surface [289]. GO/CN-TiO-CNT photocatalytic membranes were more efficient in removing both sulfamethoxazole and bisphenol A (~ 80%) than CN-TiO-CNT [290]. For metronidazole removal, TiO_2 UV photocatalysis was better than UV photolysis, with the activity of the catalysts in terms of degradation following the order: Evonik-P90 ≈ Evonik-P25 > ECT-1023t > Hombikat. In this case, the results of the synthetic *vs.* non-synthetic matrix did not show any influence of the real matrix on the removal process [291]. AOTs mediated by $SO_4^{\bullet-}$ (UV/PS, UV/peroxydisulfate) also exhibited good removal of target MPs, with studies highlighting that inorganic ions such as Cl^-, HCO_3^-, and Br^- might play an important role in the treatment performance. The treatment of hormones by UV photolysis showed good removal percentages of these compounds mainly for higher UV doses (500–1000 mJ cm^{-2}), and the addition of H_2O_2 did not consistently improve the treatment efficiency [292]. In turn, O_3 could significantly eliminate amoxicillin, oxytetracycline, and oxolinic acid (O_3 doses: 20–41 μM), but higher removal of florfenicol was obtained under UV_{254} radiation [293]. For the degradation of

rifampicin antibiotic by the Fenton process, parameters such as the initial concentration of MP, temperature, pH, H_2O_2 dosage, and catalyst loading did not affect the performance; testing a real matrix, or catalyst deactivation being the main factors that decrease process efficiency [239].

In summary, the application of AOTs to remove MPs from aquaculture farms is at an early stage of investigation, most studies generally using very high concentrations of spiked MPs and/or synthetic solutions with lab-scale equipment. Nevertheless, the reported results demonstrate the potential of these technologies, and more studies are necessary at full scale to assess their performance under real conditions, as well as a comprehensive cost analysis. In fact, two studies involving AOTs (O_3, UV/O_3 and UV/H_2O_2) at full-scale RAS [282, 283], highlighted the capacity of these technologies to remove MPs, indicating that further research is needed for the possible integration of AOTs into the existing water treatment systems, creating a new treatment approach that can remove MPs.

Table 4. Studies on the applicability of AOTs for the removal of organic MPs from aquaculture effluents (only one study‡ refers to the treatment of both aquaculture influents and effluents).

Treatment technology	Target Micropollutant	Type of aquaculture (category of water)	Concentration (μg L^{-1})	Removal (%)	Ref.
Solar photolysis	Oxytetracycline	Non-RAS (Marine; synthetic and non-synthetic)	4000	20–75	[275]
Solar photolysis	Oxytetracycline Oxolinic acid	Non-RAS (Marine and freshwater; synthetic)	20000–40000 20000–40000	25–78 3–18	[280]
Solar TiO_2 photocatalysis	Oxytetracycline Oxolinic acid		20000–40000 20000–40000	18–100 20–100	
Solar photolysis	Oxytetracycline Oxolinic acid	n.d. (n.d.; synthetic)	13813 7837	35–53 8–28	[287]
Solar TiO_2 photocatalysis	Oxytetracycline Oxolinic acid		13813 7837	37–66 47–94	
Solar porphyrin-TiO_2 photocatalysis	Oxytetracycline Oxolinic acid		13813 7837	18–60 10–69	
Solar Pd/WO_3 photocatalysis	Geosmin	n.d. (n.d.; synthetic)	0.5–2	58–100	[278]
UV photolysis	Florfenicol	RAS (Marine; non-synthetic)	0.2–1.8	4–45	[284]
TiO_2 UV photocatalysis	Tetracycline	n.d. (n.d.; synthetic)	20000–200000	58–100	[288]

Table 4. (Continued)

TiO_2 UV photocatalysis	Geosmin 2-methylisoborneol	RAS (Freshwater; synthetic and non-synthetic)	≤0.04 ≤0.06	0–77% 0–70%	[279]
TiO_2 UV photocatalysis	Geosmin 2-methylisoborneol	RAS (n.d.; synthetic)	2 2	25–100 75–100	[294]
TiO_2 UV photocatalysis	Geosmin 2-methylisoborneol	RAS (Freshwater; synthetic and non-synthetic)	0.014–0.1 0.019–0.1	91–96 84–96	[281]
UV	Geosmin 2-methylisoborneol	RAS (Freshwater; synthetic and non-synthetic)	0.05 0.05	20–80 8–77	[286]
TiO_2 UV photocatalysis	Geosmin 2-methylisoborneol		0.05 0.05	17–90 10–62	
UV photolysis	Metronidazole	Non-RAS (Marine; synthetic and non-synthetic)	40000	4–47	[291]
TiO_2 UV photocatalysis	Metronidazole		40000	20–100	
TiO_2/Au-TiO_2 UV/VIS photocatalysis	Amoxicillin Ampicillin Doxycycline Oxytetracycline Lincomycin Vancomycin Sulfamethazine Sulfamethoxazole	Non-RAS (Freshwater; synthetic)	0.5 0.5 0.5 0.5 0.5 0.5 0.5 0.5	* * * * 32–100 * * *	[273]
TiO_2-rGO UV photocatalysis	Formalin	Non-RAS (Marine and freshwater; non-synthetic)	40000	16–64	[289]
UV	Ciprofloxacin	n.d. (n.d.; synthetic)	30000	15	[295]
TiO_2 photocatalysis	Ciprofloxacin		30000	13–60	
TiO_2-Fe^0 UV photocatalysis	Ciprofloxacin		30000	5–100	
Zeolite-TiO_2 UV photocatalysis	Sulfamonomethoxine	Non-RAS (Freshwater; non-synthetic)	100	96–100	[274]
Zeolite-TiO_2/USY UV photocatalysis	Geosmin	RAS (Freshwater; non-synthetic)	0.1	98–99	[296]

Table 4. (Continued)

Zeolite-$TiCl_4$/$FeCl_3$ UV photocatalysis	Florfenicol	n.d. (Freshwater; synthetic)	10000–50000	98–100	[297]
CN-TiO-CNT photocatalytic membranes	Sulfamethoxazole Bisphenol A	n.d. (n.d.; synthetic)	5000 5000	24–30 30–36	[290]
GO/CN-TiO-CNT photocatalytic membranes	Sulfamethoxazole Bisphenol A		5000 5000	54–78 80–84	
CuO/ZnO UV photocatalysis	Oxytetracycline	Non-RAS (Marine; n.d.)	10000–30000	43–89	[276]
CuO/ZnO UV photocatalysis	Chlortetracycline	Non-RAS (Marine; n.d.)	10000–30000	17–89	[277]
UV/peroxydisulfate	Ofloxacin	Non-RAS (Marine; synthetic)	18000	17–100	[298]
UV/persulfate	Sulfamethoxazole	Non-RAS (Marine; synthetic)	25000	6–100	[299]
UV	E1 E2 E3	RAS (Freshwater; synthetic and non-synthetic)	10 10 10	63–96 10–98 24–95	[292]
UV/H_2O_2	E1 E2 E3		10 10 10	65–96 19–98 22–96	
O_3	E2	RAS (Freshwater; non-synthetic)	0.475	~ 60	[282]
O_3 ǂ	Amoxicillin Florfenicol Oxolinic acid Oxytetracycline	Non-RAS (Marine; non-synthetic)	731 716 523 921	21–100 0–5 21–98 17–78	[293]
UV ǂ	Amoxicillin Florfenicol Oxolinic acid Oxytetracycline		731 716 523 921	10–59 36–85 3–10 5–25	
UV/H_2O_2	Geosmin 2-methylisoborneol	RAS (Marine; non-synthetic)	0.0042–0.0067 0.032–0.089	23–100 25–100	[283]
UV/O_3	Geosmin 2-methylisoborneol		0.0042–0.0067 0.032–0.089	5–90 20–100	
Fenton-oxidation with rGO@nFe/Pd nanocomposite	Rifampicin	n.d. (n.d.; synthetic and non-synthetic)	30000–60000	53–99	[239]
Electro-oxidation	Sulfadimidine Norfloxacin	(n.d. Marine; synthetic)	2000 2000	100 100	[300]

n.d.- not defined; *Removals for lincomycin. Most of the other target compounds showed maximum removal efficiencies close to 100% for photocatalysis. E1: estrone, E2: 17-beta-oestradiol, E3: oestriol.

2.1.3.2.2 Removal of other pollutants

Intensive aquaculture involves problems such as infrequent water renewal, overfeeding, fast growth, and inefficient removal of dead fish, promoting the proliferation of bacterial diseases [301]. Therefore, AOTs are mainly used in aquaculture for water disinfection, as shown by a considerable number of studies focused on bacterial removal (Fig. 10). Given that aquaculture with water recirculation have a higher risk of fish infection, AOTs are mainly applied in RAS to prevent bacterial dissemination and problems during fish production [302-304]. Considering the high costs of AOTs and the less stringent quality standards of released effluents in comparison to those that are recirculated in aquaculture activities, the application of AOTs is not very common in non-RAS. The use of antibiotics to control fish diseases caused by harmful bacteria is one of the major drawbacks of the aquaculture sector in terms of water pollution. However, the administration to fish can be greatly reduced (as encouraged by EU strategies for sustainable aquaculture) if AOTs are implemented as a measure to ensure water disinfection, which would correspondingly decrease the amount of antibiotics needed.

In addition to the removal of bacteria, AOTs have also been applied to eliminate other conventional pollutants from aquaculture waters (Fig. 10). O_3-based processes have often been used in full-scale RAS to enhance global water quality, and in general, high removal rates of organic matter, total suspended solids, colour, and inorganic compounds are achieved [305, 306]. Two publications reviewing the application of ozonation in aquaculture facilities with RAS to remove these and other conventional pollutants are already available in the literature [307, 308]. Both review articles highlight the high capacity of O_3 to inactivate a range of bacterial, viral, fungal, and protozoan fish pathogens. However, the O_3 concentration and its exposure length (contact time), pathogen loads, and levels of organic matter are parameters that require attention to avoid gross tissue damage and mortalities of cultured organisms. Summerfelt and Hochheimer [308] showed that even very low O_3 concentrations (9–80 μg L^{-1}) can be toxic to some fish species. In this sense, selecting the optimum O_3 dose is still a matter of concern in these facilities because of the high costs of generating this oxidant and the potential effects of residual O_3. Several studies have been performed to establish the proper criteria for selecting the most cost-effective amount of O_3 [309,

310]. Electrochemical AOTs have mainly been applied to remove ammonia from aquaculture waters; two studies have assessed pilot implementation in RAS [311, 312]. Studies on UV-based processes have also evaluated the removal of ammonia at the laboratory scale [313-315]. Regarding Fenton-based processes, a Fenton-coagulation batch reactor showed a good performance in removing organic matter, turbidity, and inorganic ions from aquaculture WW [316]. Thus, considering that AOTs have been used to eliminate bacteria and other conventional pollutants from aquaculture farms, and that other studies demonstrate their great potential to remove organic MPs when investigating different matrices, AOTs can be seen as an interesting alternative for the removal of both conventional contaminants and organic MPs from aquaculture waters.

2.1.3.3. Conclusions

The removal of organic MPs by AOTs and CWs from aquaculture waters is a recent area of study, and more research is needed to determine the feasibility of their full-scale application. The potential of both categories of technologies for the removal of MPs from contaminated water/wastewater is now recognised, but information specific to aquaculture is still limited, with only 28 and 6 published studies having focused on AOTs and CWs, respectively. Although these reports show attractive results in terms of MPs elimination, the investigated conditions are still far from reality, for example, testing very high concentrations of MPs, synthetic water samples, installations at micro-scale and batch treatments. In the specific case of AOTs, future research should be performed using lower concentrations of organic MPs, being directed to pilot and full-scale applications because the reviewed information is very focused on the pollutant itself, neglecting the influence of other aspects such as the real matrix, installation design, and costs. Since light-driven and O_3-based AOTs have been applied at full scale to remove other types of pollutants, investigating their performance regarding MP removal can be a valuable option. With this approach, it would be possible to reach valid conclusions about the treatment feasibility and the main parameters where improvement is possible. In relation to CWs, additional research is needed to increase the knowledge on the

removal mechanism of MPs. Groups of MPs with similar characteristics can be an interesting alternative; at the same time, several CW designs and operational factors should be extensively studied. Combining CWs and AOTs could also be an attractive alternative to remove MPs from aquaculture, since the individual advantages of both technologies can be eventually maximised; however, their application to aquaculture aqueous matrices has not been investigated yet.

2.2. Motivation

2.2.1. Scope

The state-of-the-art shows that both CWs and AOTs present encouraging results regarding the removal of organic MPs from aqueous matrices. However, information on CWs performance is still unknown for more than 30 EU PSs and CECs. In contrast, AOTs have been studied for a wide range of MPs, but most investigations are still conducted at lab-scale systems. Furthermore, the possible application of these technologies in aquaculture farms is only addressed in 34 studies. For both CWs and AOTs, investigations have been mainly focused on the evaluation of MPs removal efficiencies, but a more critical analysis of results foreseeing a full-scale implementation is needed. In addition, most studies have been conducted at conditions still far from the reality (*i.e.,* applying high spiked concentrations, synthetic matrices, among others), making it difficult to support the application of these technologies. Globally, there is still a lack of information about the current status of MPs occurrence in aquaculture waters, as well as specific guidance of proper treatments able to remove them. Thus, this PhD project intends to investigate the removal of organic MPs from aquaculture waters applying CWs and AOTs as treatments. This is a topic of major interest according to the EU priorities under Horizon Europe that include research and innovation on sustainable aquaculture.

2.2.2. Objectives

To achieve the main goal of this PhD project in selecting the best treatment solution (CWs, AOTs, or CWs-AOTs/AOTs-CWs) to mitigate the occurrence of organic MPs in aquaculture waters, specific objectives were defined, namely:

- To develop, optimize and validate solid-phase-extraction-ultra-high-performance liquid chromatography-tandem mass spectrometry (SPE-UHPLC-MS/MS) methods for analysis

of organic MPs, namely PSs, CECs, specific veterinary pharmaceuticals, recalcitrant compounds, and some possible metabolites, in water samples;

- To study bench-scale CW systems, applying real matrices and environmentally relevant concentrations, to remove organic MPs and other possible contaminants (*e.g.*, nitrogen and phosphorus) aiming to investigate and optimize the performance of CWs;
- To apply different AOTs through experiments designed as close as possible to realistic scenarios to evaluate the removal of organic MPs;
- To define the best treatment options according to the specific conditions of each aquaculture farm.

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Chapter

3

Constructed wetlands for the removal of organic MPs from freshwater aquaculture effluents

In this chapter, the performance of planted (*Phragmites australis*) VSSF-CWs was investigated, at microcosm scale, to remove organic MPs detected in non-spiked freshwater aquaculture effluents, namely atrazine, isoproturon, PFOS, clarithromycin, erythromycin, fluoxetine, and norfluoxetine. Moreover, the efficiency of these systems to remove a wider multi-component set of 35 MPs was studied by spiking them (alachlor, atrazine, chlorfenvinphos, isoproturon, PFOS, azithromycin, clarithromycin, erythromycin, diclofenac, methiocarb, acetamiprid, clothianidin, thiacloprid, thiamethoxam, simazine, atorvastatin, bezafibrate, carbamazepine, cephalexin, ceftiofur, citalopram, clindamycin, clofibric acid, diphenhydramine, enrofloxacin, fluoxetine, ketoprofen, metoprolol, norfluoxetine, ofloxacin, propranolol, tramadol, trimethoprim, venlafaxine, and warfarin) at 100 ng L^{-1} each into the same matrix. Removal percentages were weekly determined (during one month) by the difference of MPs concentrations in the CWs inlet and outlet. MPs concentrations were determined by SPE-UHPLC-MS/MS. Excellent removal rates (varying between 87 and 100%) of the target MPs were achieved in both non-spiked and spiked aqueous matrices, highlighting the potential of CWs to remove MPs from aquaculture effluents. This study contributes to increase knowledge regarding the application of CWs for the removal of a mixture of several organic MPs defined in EU legislation at realistic concentrations, a gap identified in *Chapter 2*.

This chapter is based on the following reference (original version available in **Appendix A3**, with permission of Elsevier):

Gorito, A.M., Ribeiro, A.R., Gomes, C.R., Almeida, C.M.R, Silva, A.M.T. *Constructed wetland microcosms for the removal of organic micropollutants from freshwater aquaculture effluents*, Science of the Total Environment, 644 (2018) 1171-1180.

3.1. Introduction

Attending to the dispersion of MPs in the aquatic environment, many food industries can end up using water polluted with residues of these compounds, as is the case of aquaculture farms. Moreover, since intensive aquaculture involves the artificial supply of food and medicines, MPs can also be introduced by these products, being posteriorly released in the effluents of this farm activity [1]. Thus, MPs can be found in both aquaculture influents and effluents and also in the resulting food products, affecting the environment and posing a risk for aquatic organisms and human health [2].

In this context, appropriate treatment processes to remove MPs are needed in aquaculture farms. Adsorption by activated carbon, membrane bioreactors and other membrane processes (*e.g.*, microfiltration, ultrafiltration, nanofiltration, and reverse osmosis), and AOTs are some examples of technologies already applied at full scale for removing MPs [3]. Nonetheless, in most cases, a high investment is required, emphasizing the need of exploring more affordable solutions [4], such as CWs [5]. Most reports dealing with CWs show the potential of these systems to eliminate specific types of contaminants, such as total suspended solids, organic matter, nutrients, and metals [3]. However, more recently, several studies also reported promising results for the removal of MPs, namely pesticides [6-8], veterinary medicines [9-11], PPCPs [6, 12, 13] and industrial compounds [14, 15]. Despite this, as shown in sub-*chapter 2.1.1*, the removal by CWs of a considerable number of relevant substances identified in EU documents (PSs and CECs) has not been investigated so far; most studies not being focused on the removal of MPs in multi-component mixtures [11, 16-18], nor on realistic concentrations. Furthermore, there are still few studies pointing out CWs as a possible option to eliminate MPs from aquaculture effluents (sub-*chapter 2.1.3. – section 1.1*).

In this work, aquaculture effluents were collected from a freshwater facility located in Portugal for posteriorly treatment in CW microcosms. Firstly, MPs were determined in the waters collected at the entrance and exit of an aquaculture freshwater facility to assess which of the selected MPs are present in its influents and effluents. Then, the efficiency of CWs to treat the raw (non-spiked) effluent, as well as the effluent spiked with 35 organic MPs, was investigated to evaluate the

potential of these biological systems to remove a mixture of a larger number of MPs. Effluents, instead of aquaculture influents, were studied because in most cases they have a more complex matrix and many MPs detected in aquaculture influents are expected to be present in the respective effluents. Target MPs in this study included PSs and CECs listed in Directive 2013/39/EU and watch list of Decisions 2015/495/EU, 2018/840/EU, and 2020/1161/EU respectively, as well as other relevant MPs selected due to their frequency of detection in water bodies, persistence and ecotoxicity. Two sets of experiments were carried out in microcosms simulating a VSSF-CW, one using spiked (100 ng L^{-1} of each selected MP) and another using non-spiked aquaculture effluents, during four one-week cycles of CWs treatment.

3.2. Materials and methods

3.2.1. Chemicals and materials

The 35 MPs targeted in this study included 6 PSs identified in the Directive 2013/39/EU (alachlor, atrazine, chlorfenvinphos, isoproturon, PFOS, and simazine) [19], 9 CECs defined in the Decision 2015/495/EU (azithromycin, clarithromycin, erythromycin, diclofenac, methiocarb, acetamiprid, clothianidin, thiacloprid, and thiamethoxam) [20], 8 CECs listed in the Decision 2018/840/EU (azithromycin, clarithromycin, erythromycin, methiocarb, thiacloprid, thiamethoxam, acetamiprid, and clothianidin) [21], and 2 CECs of the Decision 2020/1161/EU (trimethoprim and venlafaxine) [22]. Moreover, a set of 18 other CECs (atorvastatin, bezafibrate, carbamazepine, cephalexin, ceftiofur, citalopram, clindamycin, clofibric acid, diphenhydramine, enrofloxacin, fluoxetine, ketoprofen, metoprolol, norfluoxetine, ofloxacin, propranolol, tramadol, and warfarin) of environmental relevant concern was also analysed. The physicochemical properties of the analytes are present in Table D1.1. (Appendix D, Supplementary Material). All abovementioned reference standards, as well as the internal standards (atrazine-*d5*, acetamiprid-*d3*, methiocarb-*d3*, clothianidin-*d3*, diclofenac-*d4*, azithromycin-*d3*, ofloxacin-*d3*, fluoxetine-*d5*, and ketoprofen-*d3*), were of high purity grade (> 98%) and were purchased from Sigma-Aldrich (Steinheim, Germany).

Stock standard solutions with a concentration of approximately 1000 mg L^{-1} were prepared by dissolving known amounts of the standards in ethanol, methanol, or acetonitrile, depending on their solubility. Similarly, solutions of 1000 mg L^{-1} of each isotopically labelled internal standard were prepared, by dissolving the respective standard in methanol (except fluoxetine-*d5*, which was already in solution) or in 1% of NaOH solution in the case of ofloxacin–*d3*. Two working solutions were prepared using ethanol as solvent, one containing each target analyte at 2.5 mg L^{-1} and another containing the individual internal standards at 10 mg L^{-1}. Ethanol high performance liquid chromatography (HPLC) grade was acquired from Fisher Scientific (Leicestershire, UK), whereas methanol and acetonitrile (MS grade) were purchased from VWR International (Fontenay-sous-Bois, France). Formic acid and sulphuric acid were supplied by Merck (Darmstadt, Germany). Oasis® Hydrophilic-Lipophilic-Balanced (HLB) cartridges (150 mg, 6 mL) were acquired from Waters (Milford, Massachusetts, USA).

3.2.2. Sampling

Influent and effluent samples from a Portuguese freshwater aquaculture facility (through-flow system) were collected every week (approximately 15 L, grab samples) during February 2017 (1st, 8th, 16th and 22nd) to evaluate the presence of MPs, being the aquaculture effluents used to perform the CW microcosms experiments. To replicate the typical conditions of natural wetlands and their surrounding environment, *Phragmites australis* plants already grown, sediments in direct contact with plant roots and sand were collected in January 2017 at Lima river margins (Portugal), and immediately transported to the laboratory for subsequent assembly of microcosms.

3.2.3. CW systems

3.2.3.1. Set-up

CW microcosms were assembled at bench-scale in VSSF-CW configuration, as described elsewhere [9], and adapted to automatically operate with a recirculating water system (flow rate of approximately 10 mL min^{-1}), employing a pump and six outlets dripping on substrate top (Fig. 11a). Six microcosms were set up using plastic containers (40 cm × 30 cm × 30 cm) filled with a first layer of gravel (4 cm), a second layer of lava rock (2 cm) and, finally, a third previously prepared layer of the roots bed substrate (16 cm), into which *Phragmites australis* were transplanted (approximately 40 sprouts per microcosm). Gravel was used at the bottom to prevent clogging of the draining tubes, while lava rock was applied in the middle of layers to increase the porosity of the substrate, promote the retention of contaminants, and potentiate the development of biofilms, which have an important role in the degradation of micropollutants. Sediments in contact with plant roots and sand collected at Lima river were mixed (proportion 1:2, to increase substrate porosity) and homogenized to prepare the roots bed substrate for CW microcosms, the top layer. This layer of soil supports the plants (implementation of plants belowground tissues), also maintaining the native microbial consortium associated with this plant species. The *Phragmites australis* were arranged in accordance with their stems length, aiming at an equal distribution in their transplantation into the CW systems. As plants were fully developed, significant plant growth was not expected in the relatively short time of the experimental trial, and for that reason, a considerable plant density was used to maximize their effect on CW functioning. The plastic containers were wrapped with aluminium foil, to prevent natural light penetration through the container walls and the possible photodegradation of the compounds, mimicking VSSF-CWs natural conditions. Microcosms were kept under greenhouse-like conditions, namely natural day:night regime, with a temperature variation between 10 ºC and 20 ºC. Before the experiments, CW microcosms were acclimated for one week with a Hoagland nutrient solution (level just above the substrate surface),

in order to maintain suitable nutritional conditions for plants and substrate. Afterwards, the systems were filled with freshwater aquaculture effluents.

3.2.3.2. Operation and sampling

Six microcosms were assembled for spiked (3 microcosms) and non-spiked experiments (3 microcosms), each one treating 2 L of aquaculture effluents. This volume was established because it enabled the complete saturation of the substrate, maintaining the water level just above the substrate surface (saturation rate of approximately 100%). One set of triplicate CW systems was supplemented with 2 L of aquaculture effluents (as collected) and the other set of triplicate CW systems was supplemented with the same volume of aquaculture effluents spiked with 80 µL of a working standard solution containing each target MP at 2.5 mg L^{-1}. The resulting concentration (100 ng L^{-1} of each MP) was chosen taking into consideration our recent review addressing the occurrence of PSs and CECs in SW [23] and our previous studies dealing with MPs in aquatic systems [24, 25], where many of the selected MPs have been reported from ng L^{-1} to µg L^{-1} levels. After addition of the effluents on the substrate top of the CW systems (Fig. 11a), aquaculture effluents were treated for one week. This commonly used HRT is adequate for an efficient removal of pollutants, according to Zhang *et al*. [4]. During this time, effluents were automatically recirculated in the system as mentioned in *Section 3.2.3.1* (7 a.m.–7 p.m.), to prevent the development of anoxic areas and to promote the removal of MPs. The CWs performance was evaluated during one month, *i.e.,* four 1-week cycles. The regular addition of new aquaculture effluents into the CW microcosms, at the beginning of each week, allowed simulating the cumulative effect of a new load of pollutants added cyclically to the system, which may affect the CWs performance over time, as occurs in full-scale systems. In this sense, microcosms were weekly drained and refilled with spiked or non-spiked fresh aquaculture effluents, collected as indicated in *Section 3.2.2.* At the end of each week of treatment, the liquid phase of each CW microcosm was drained into glass bottles (Fig. 11b), to which deionized water was added to achieve a final volume of 2 L, equalizing the initial volume for a proper analysis (an average of 600 and 500 mL of deionized water was needed each week, in spiked and non-spiked CWs,

respectively, resulting from water losses of 30 and 25% for each one of the systems). Small amounts of deionized water were also added to CW microcosms along the week, whenever necessary, to maintain approximately 100% saturation of the substrate bed.

All samples collected during the CW experiments, as well as the aquaculture influent samples, were stored at 4 ºC and filtration was performed in less than 24 h, using 1.2 µm nylon membrane filters (Membrane Solutions, Texas, USA) to eliminate suspended solids before analysis.

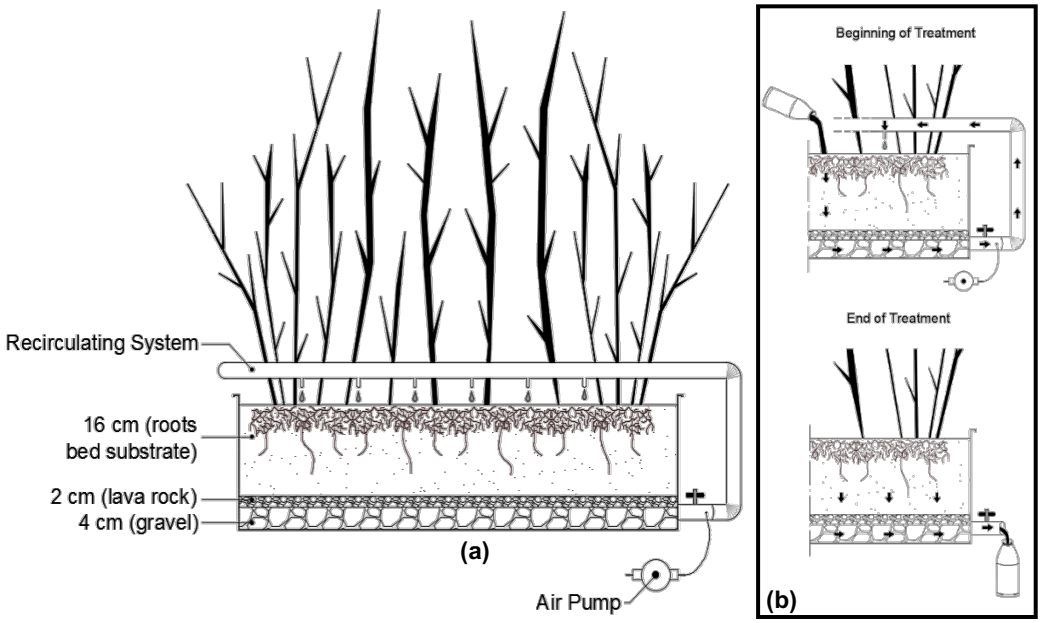

Figure 11. CW microcosm setup **(a)**, operation and sampling at the beginning and the end of each weekly treatment **(b).**

3.2.4. Chemical analysis (MPs, dissolved organic carbon and ions)

The concentration of the target MPs in the inlet and outlet samples of CW microcosms and in the freshwater samples of the aquaculture facility was determined using an analytical methodology that

couples SPE and UHPLC-MS/MS. Water samples were pre-cleaned and concentrated by using a SPE procedure described elsewhere [26]. Briefly, Oasis® HLB cartridges were used to extract 500 mL of sample adjusted to pH 3, with ethanol as eluting (4 mL) and reconstitution solvent (250 µL). MPs analysis was carried out in a UHPLC Shimadzu Corporation equipment connected to a triple quadrupole mass spectrometer detector (Ultra-Fast Mass Spectrometry series LCMS-8040). Methanol and water acidified with 0.10% formic acid (50/50, v/v) were used as mobile phases for the chromatographic separation. Capillary voltage, drying gas flow, nebulizing gas flow, desolvation and source temperature of the mass spectrometer were respectively 4.5 kV, 10 dm^3 min^{-1}, 3 dm^3 min^{-1}, 250 ºC and 400 ºC. Method detection limits (MDLs) and method quantification limits (MQLs) of the target analytes are presented in Table D1.2 (Appendix D, Supplementary Material).

The removal percentages of each of the 35 target MPs in the individual CW microcosms were determined using Eq. (1).

$$\text{Removal efficiency (\%)} = \frac{(C_{Inlet} - C_{Outlet})}{C_{Inlet}} \times 100 \quad \text{(Eq. 1)}$$

where C_{Inlet} and C_{Outlet} are the concentrations of the target MPs entering and leaving CW microcosms, respectively. The average weekly removal efficiency of each set of experiments (spiked and non-spiked aquaculture effluents) was determined by calculating the mean of each triplicate CW microcosms.

Dissolved organic carbon (DOC) was determined following the standard procedure 5310 B of the Standard Methods for Examination of Water and Wastewater [27], and using a Shimadzu TOC-L apparatus.

The concentrations of cations (sodium, potassium, ammonium, calcium, magnesium) and anions (chloride, bromide, bromate, nitrite, nitrate, phosphate, sulphate) in solution were determined by ionic chromatography using, respectively, Metrosep C4 Cationic Exchange and Metrosep A Supp 7 Anionic Exchange columns (250 mm × 4.0 mm) in a Metrohm 881 Compact IC Pro equipment. The pH values were measured using a pH meter pHenomenal® pH 1100L (VWR, Germany).

3.3. Results and discussion

3.3.1. Occurrence of target MPs in aquaculture facility influents and effluents

The influent and effluent samples weekly collected in the aquaculture facility (*i.e.*, non-spiked) were analysed to assess which of the selected MPs were present at the entrance and exit of the farm.

Nine MPs out of a target group of 35 were detected during the four weeks of sampling (Table 5), including 3 PSs listed in the Directive 2013/39/EU (atrazine, isoproturon, and PFOS), 3 CECs included in the Decisions 2015/495/EU and 2018/840/EU (azithromycin, clarithromycin, and erythromycin) and 3 other CECs (diphenhydramine, fluoxetine, and norfluoxetine). The pesticides and pharmaceuticals were the MPs found at lower concentrations (most of them at nearly 1 ng L^{-1}). Moreover, the industrial compound PFOS was quantified at a concentration ten times higher. With the exception of azithromycin and diphenhydramine, which were apparently removed from water in the aquaculture facility, no considerable differences between the inlet and outlet aquaculture water samples were observed in each sampling week, which means that this aquaculture farm is not a source of the MPs under study. Despite this, some differences were observed from week to week, which can be caused by the different anthropogenic activities and meteorological conditions occurring during the sampling period. The fact that a few MPs were only detected in the aquaculture influents may indicate their adsorption or degradation within the aquaculture facility or their bioaccumulation in aquatic biota. However, the distribution in the aquaculture facility is not a focus of this work. Notwithstanding, for most of the target MPs, the present results clearly indicate that MPs entering in the feed freshwater, were passing through the aquaculture facility, which emphasizes that water treatments are needed.

As abovementioned, the presence of MPs in SW is mainly related to the inefficient removal of these compounds in WWTPs and also to the industrial and agricultural activities (common practices near the sampling site).

Table 5. Concentration of the target MPs (ng L^{-1}) in the influents (I) and effluents (E) of the selected freshwater aquaculture facility (mean and standard deviation, n = 2); * or ** represent one measurement of the target analyte, being the other below the MDL or MQL, respectively. The compounds listed in footnote were not detected in any sampling week.

Organic MPs	Sampling period			
	Week 1 (***February 1st, 2017***)	**Week 2** (***February 8th, 2017***)	**Week 3** (***February 16th, 2017***)	**Week 4** (***February 2nd, 2017***)
Pesticides				
Atrazine	**I =** 0.43 ± 0.01	**I =** 0.19 ± 0.04	**I =** 0.12 ± 0.01	**I =** 0.4 ± 0.2
	E = 0.42 ± 0.01	**E =** 0.5 ± 0.2	**E =** 0.26 ± 0.01	**E =** 0.2 ± 0.1
Isoproturon	**I** < 0.13 (MQL)	**I** < 0.13 (MQL)	**I** < 0.04 (MDL)	**I** < 0.04 (MDL)
	E < 0.13 (MQL)	**E** < 0.13 (MQL)	**E** < 0.04 (MDL)	**E** < 0.04 (MDL)
Industrial Compound				
PFOS	**I =** 10.0 ± 0.7	**I =** 10 ± 1	**I =** 11± 2	**I** < 0.91 (MDL)
	E = 11 ± 2	**E =** 9.54 ± 0.07	**E =** 11 ± 2	**E** < 0.91 (MDL)
Pharmaceuticals				
Azithromycin	**I =** 1.12 ± 0.09	**I =** 2.8 ± 0.2	**I =** 1.30 ± 0.03	**I =** 1.03 ± 0.09
	E < 0.15 (MDL)	**E** < 0.15 (MDL)	**E** < 0.15 (MDL)	**E** < 0.15 (MDL)
Clarithromycin	**I =** 0.40*	**I =** 1.1 ± 0.4	**I** < 0.39 (MQL)	**I =** 3.28**
	E < 0.39 (MQL)	**E** < 1.92**	**E** < 0.39 (MQL)	**E** < 0.39 (MQL)
Diphenhydramine	**I =** 1.91 ± 0.01	**I** < 0.02 (MDL)	**I =** 1.4 ± 0.6	**I** < 0.02 (MDL)
	E < 0.02 (MDL)	**E** < 0.02 (MDL)	**E** < 0.02 (MDL)	**E** < 0.02 (MDL)
Erythromycin	**I** < 0.13 (MDL)	**I** < 0.13 (MDL)	**I** < 0.13 (MDL)	**I** < 0.13 (MDL)
	E < 0.13 (MDL)	**E** < 0.39 (MQL)	**E** < 0.39 (MQL)	**E** < 0.13 (MDL)
Fluoxetine	**I** < 0.10 (MDL)	**I** = 1.93 ± 0.08	**I** < 0.10 (MDL)	**I** < 0.10 (MDL)
	E < 0.10 (MDL)	**E** = 2.71**	**E** < 0.10 (MDL)	**E** < 0.10 (MDL)
Norfluoxetine	**I** < 1.61 (MDL)	**I** = 8.1 ± 0.2	**I** < 1.61 (MDL)	**I** < 1.61 (MDL)
	E < 1.61 (MDL)	**E** = 6.52*	**E** < 1.61 (MDL)	**E** < 1.61 (MDL)

The other 26 target MPs (alachlor, chlorfenvinphos, simazine, diclofenac, methiocarb, acetamiprid, clothianidin, thiacloprid, thiamethoxam, atorvastatin, bezafibrate, carbamazepine, cephalexin, ceftiofur, citalopram, clindamycin, clofibric acid, enrofloxacin, ketoprofen, metoprolol, ofloxacin, propranolol, tramadol, trimethoprim, venlafaxine, and warfarin) were not detected in any sample. PFOS, Perfluorooctane sulfonic acid.

3.3.2. Removal of MPs in CW microcosms

Aquaculture effluents were used in CWs experiments, since in most cases the effluents have a more complex matrix than the freshwater entering into the aquaculture facilities (these effluents can contain higher levels of organic matter or nutrients, which can affect CWs performance), and also because most of the MPs determined in the influents were also present in the effluents.

A set of experiments was conducted using effluents as collected (Fig. 12) and another set by spiking the same effluents with the selected 35 MPs at 100 ng L^{-1} each (Fig. 13), with the aim of simulating a worst-case scenario and because the MPs not found in the realistic effluent under study may be found in the effluents of other aquaculture farms. In Figures 12 and 13, the closed circles refer to the initial MP concentration in the effluent. Some deviations from 100 ng L^{-1} in spiked experiments were observed due to the presence of these compounds in the collected effluent samples and/or matrix effects. The removal efficiencies (grey bars) of the investigated MPs throughout the four weeks of treatment demonstrated a high elimination performance for most of the target compounds, in both non-spiked and spiked experiments.

In the particular case of the non-spiked experiments (Fig. 12), high removal efficiencies were obtained for the majority of the detected MPs. The pesticide atrazine was fully eliminated in all cycles of treatment (*i.e.,* to concentrations below the MDL, 0.05 ng L^{-1}). In the case of PFOS, its removal was almost complete, except in the first week of treatment, which may be related to the acclimation of the biological systems on the initial stage. In fact, these biological systems may require a period of adaptation before attaining their full performance, as observed in other studies [28]. Removals nearly 100% were also observed for the pharmaceuticals clarithromycin, fluoxetine and norfluoxetine. However, these compounds were only quantified in the effluent samples collected in the second week, hampering a detailed analysis and the comparison of the removal efficiencies between different cycles. These preliminary experiments using non-spiked effluents (as collected), demonstrated the potential of the CWs to eliminate most of the MPs present in the studied effluent samples.

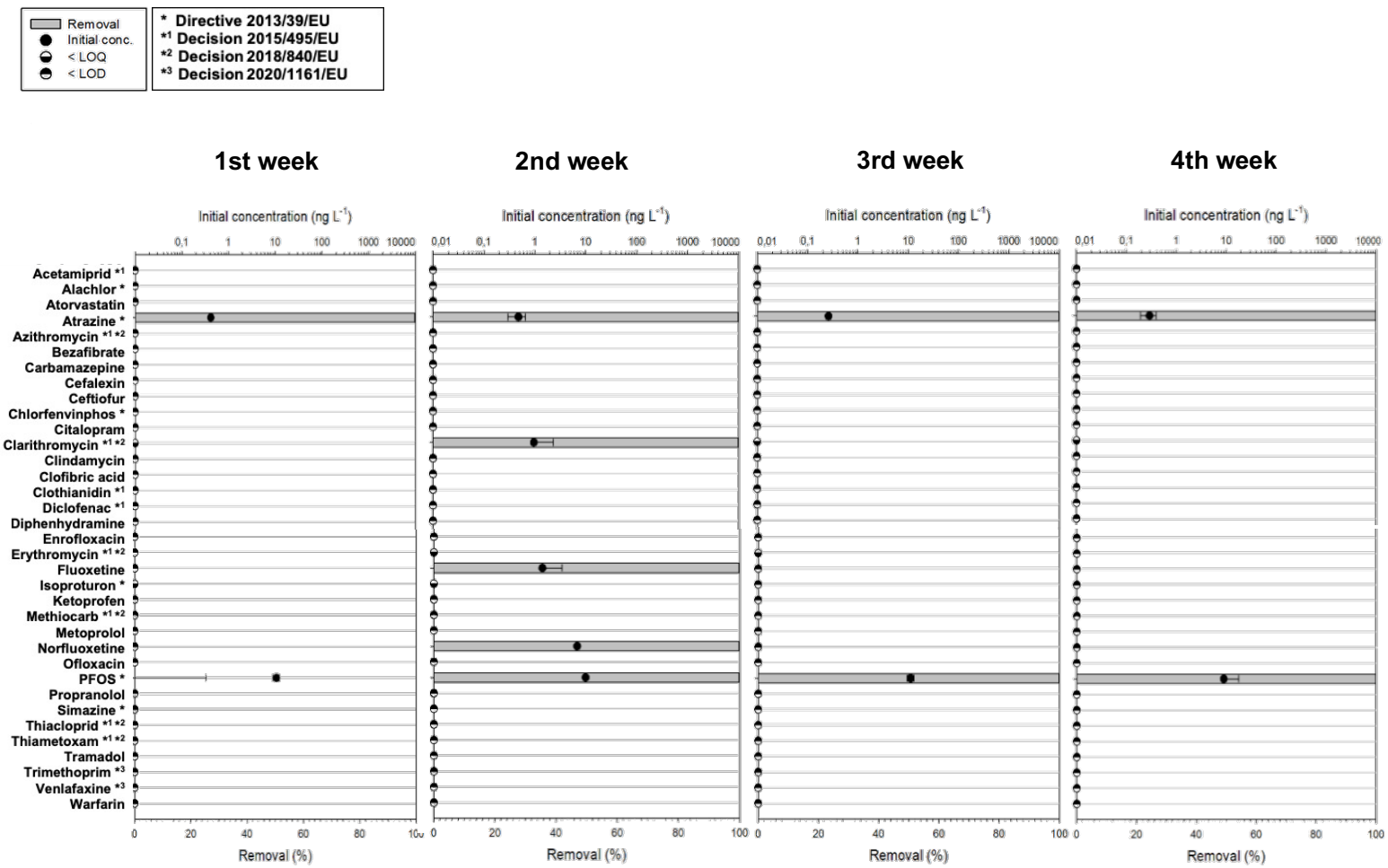

Figure 12. Weekly removal efficiencies of the 35 target MPs during the four 1-week cycles of CW microcosms treatment, using non-spiked aquaculture effluents.

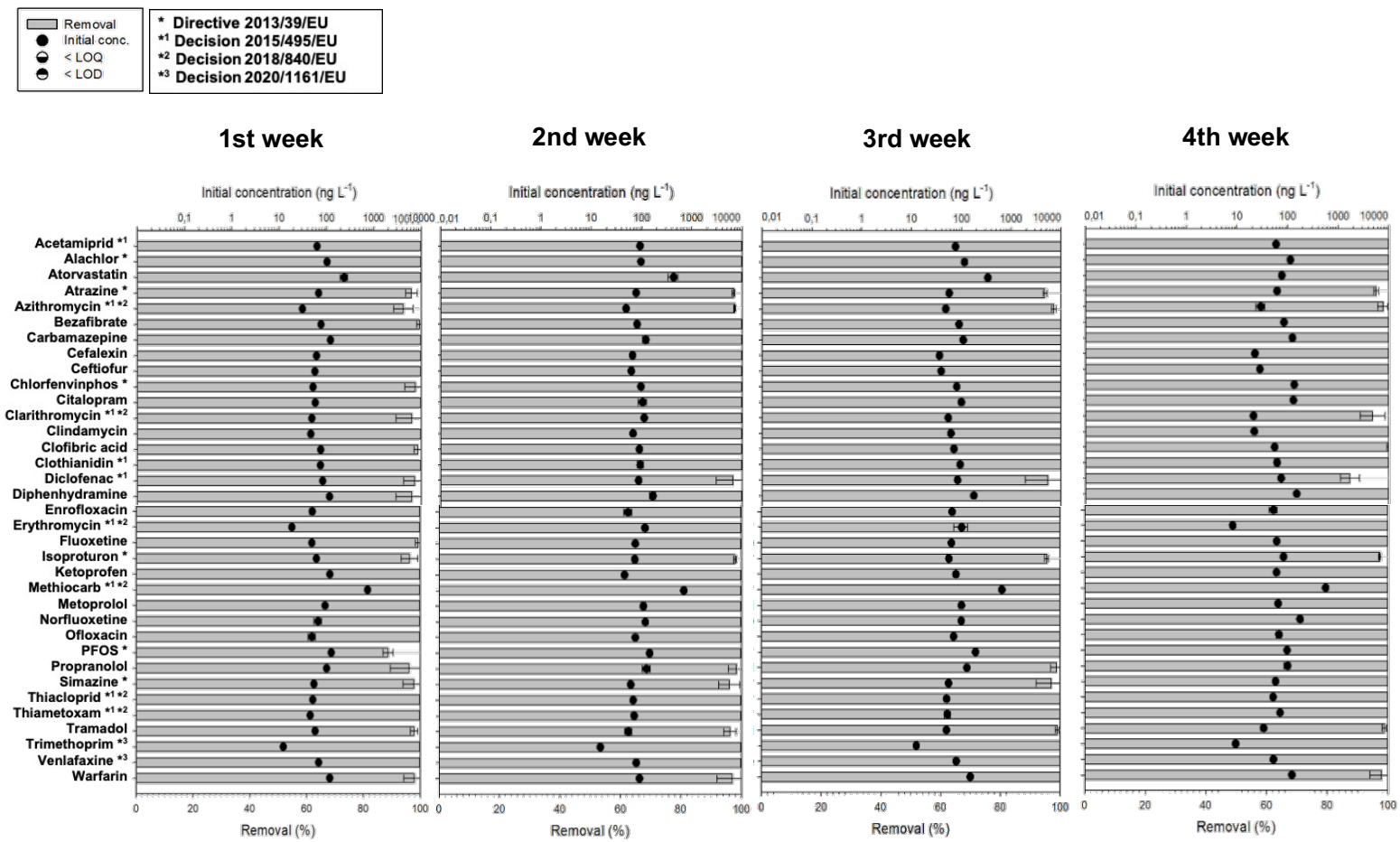

Figure 13. Weekly removal efficiencies of the 35 target MPs during the four 1-week cycles of CW microcosms treatment, using spiked aquaculture effluents.

For spiked experiments (Fig. 13), it is noticeable the adaptation period of the CW systems at the beginning of the experiments, with an overall removal efficiency slightly lower for many MPs, as suggested by the results obtained with PFOS in the non-spiked experiments. However, the overall efficiencies in the four 1-week cycles were higher than 87% for all compounds. Specifically, 21 out of the 35 MPs were totally eliminated (*i.e.*, to levels below the MDL, meaning removal rates ≈ 100%) in all weeks of spiked experiments, namely acetamiprid, alachlor, atorvastatin, bezafibrate, carbamazepine, cephalexin, ceftiofur, citalopram, clindamycin, clothianidin, enrofloxacin, erythromycin, ketoprofen, methiocarb, metoprolol, norfluoxetine, ofloxacin, thiacloprid, thiamethoxam, trimethoprim, and venlafaxine.

In the case of acetamiprid, atorvastatin, cephalexin, citalopram, clothianidin, methiocarb, norfluoxetine, thiacloprid, and thiamethoxam, no studies were found in the literature regardless of the matrix, invalidating a possible comparison with the obtained results. For the other MPs (alachlor, bezafibrate, carbamazepine, ceftiofur, clindamycin, enrofloxacin, erythromycin, ketoprofen, metoprolol, ofloxacin, trimethoprim, and venlafaxine), studies in the literature showed lower removal rates than those described in this work [7, 29-33]. However, a direct comparison is difficult because the tested operating conditions were considerably different, as well as the type of matrix employed (which are known to significantly affect the removal rates achieved in CWs). In the case of the MPs bezafibrate, metoprolol, venlafaxine, erythromycin, ceftiofur, and enrofloxacin, the following removals were respectively obtained for conditions similar to those tested in this work: 66%, 80%, 57%, > 92% [34], > 85% and > 98% [35], but using, respectively, domestic and livestock WW as matrix. The higher inlet concentrations of bezafibrate and venlafaxine studied by Rühmland *et al.* [34]) might justify the lower removal rate (66 and 57%, respectively), when compared to the removals obtained in this study. Regarding carbamazepine, the published works generally reported poor performances of CWs to remove it, including studies employing VSSF-CWs, the same configuration herein reported [32, 36]. Therefore, a more detailed analysis would be needed to understand these marked differences.

For the MPs that were not eliminated from water to levels below the MDL (Fig. 13), atrazine, azithromycin, diclofenac, isoproturon, propranolol, simazine, tramadol, and warfarin, the removal

efficiencies were always higher than 87%. The review presented in *Chapter 2.1.1.1* shows very dissimilar removals for the PSs atrazine (0-100%) and simazine (20-60%) and removals up to 50% for isoproturon [3]. Therefore, the present study exhibited a better performance of CWs to remove isoproturon, *i.e.,* almost total elimination. In turn, removal efficiencies for the watch list CEC azithromycin (94-98%) were similar to those described by Berglund *et al.* [29]). Although that study applied different conditions, namely a SF-CW instead of the configuration used in the present study (VSSF-CWs), the removal performances were similar. These results may indicate that photodegradation (that can occur in SF-CWs) and biodegradation (that can occur in VSSF-CWs) were not probably the main elimination processes for this specific MP. High values of K_{ow} and the acid dissociation constant (*i.e.*, pK_a in negative base-10 logarithm) of azithromycin suggest its ability to sorb into soil and/or to be uptaken by plants, common processes in both configurations. For diclofenac, removal rates between 88 and 98%, slightly lowering over time, were observed. Published works dealing with its removal by CWs reported very different results, ranging from 0 to 96% but typically below 60% [3]. For the same configuration used in the present study, Reyes-Contreras *et al.* [37] achieved a maximum efficiency of 55% and observed that CWs performances decreased over time, as well as in cold seasons. Studies applying SF-CWs configurations have been demonstrating better results for the removal of diclofenac, probably due to its susceptibility to photolysis when exposed to sunlight irradiation, in comparison with other elimination processes [34, 38]. Although the configuration used in this study does not promote photodegradation, diclofenac was efficiently removed, suggesting its microbial degradation, as already evidenced in other studies [36, 39]. Lastly, for the pharmaceuticals propranolol, tramadol, and warfarin, very similar removal rates were observed, always higher than 96%. According to Vystavna *et al.* [40], propranolol tends to accumulate in the sediments and plants of CWs due to its hydrophobicity. In that study, removals up to 80% were obtained using a combined VSSF and HSSF-CWs, being the removal of this pollutant favoured by an increase of HRT and plants cover. Similar efficiencies were reported in other studies [6, 37]. For tramadol, the literature describes removals up to 80% (VSSF-CWs; HRT = 11 days) [34] and for warfarin, removals up to 44% (HSSF-CWs; HRT = 6 days) [33].

Other MPs (chlorfenvinphos, clarithromycin, clofibric acid, diphenhydramine, fluoxetine, and PFOS) also had removal rates lower than 100% in some periods of treatment (between 89% and 99%), mainly in the first week corresponding to the adaptation phase (Fig. 13). Thus, this group of compounds was more susceptible to the adaption period, but totally eliminated in the course of treatment. Information about removal of chlorfenvinphos and diphenhydramine by CWs is limited and unclear [41, 42]. Removal efficiencies of clarithromycin, fluoxetine and PFOS are reported in several studies, presenting dissimilar values depending mainly on the operating conditions [3, 32]. Clofibric acid has been classified as recalcitrant in CW systems, phytoremediation and adsorption being the main processes contributing to its removal [4]. Most studies on the removal of clofibric acid were performed in HSSF-CWs, and for that reason, more research on the VSSF-CW configuration is needed for a reliable comparison [4].

The obtained results highlight the potential of CWs to remove a significant number of MPs; however, research is still needed to fully understand the processes behind these removals. As discussed above, the CW configuration has a significant impact on the achieved performance. So, additional studies are being carried out in which different configurations, plants, substrate composition and HRT are tested to identify and intensify the processes responsible for the removal of recalcitrant MPs, as well as other often detected at hundreds or thousands of ng L^{-1} in SW (such as alachlor, azithromycin, clarithromycin, clothianidin, diclofenac, erythromycin, thiacloprid, thiamethoxam, and simazine) [23].

3.3.3. DOC and ions monitoring

The DOC contents and concentrations of nutrients are relevant parameters which may largely affect the natural interactions inherent to biological systems. Mean DOC contents (Fig. 14) of the influents were 4.9 ± 0.8 mg L^{-1} and 9.0 ± 0.9 mg L^{-1} for non-spiked and spiked experiments, respectively. The higher values in the spiked experiments, in comparison to the non-spiked ones, are due to the addition of the methanolic solution (containing all the target MPs) to the aquaculture effluents. Moreover, the marked differences between non-spiked influents and effluents treated

during the first week, in terms of DOC contents, were probably due to plants and microorganisms activities. After the first week, the DOC content in CW microcosm effluents was similar (approximately 10 mg L^{-1}) for both non-spiked and spiked experiments. Although organic carbon can be used by microbial communities and by plants for cellular respiration and biomass growth, DOC also can be leached into the water flowing through CW systems due to plants and bacteria grow, die and decay [43]. The few studies published on DOC variation during CWs treatment indicate very dissimilar results, but the potential of these biological systems as a possible source of DOC has recently received considerable attention [43].

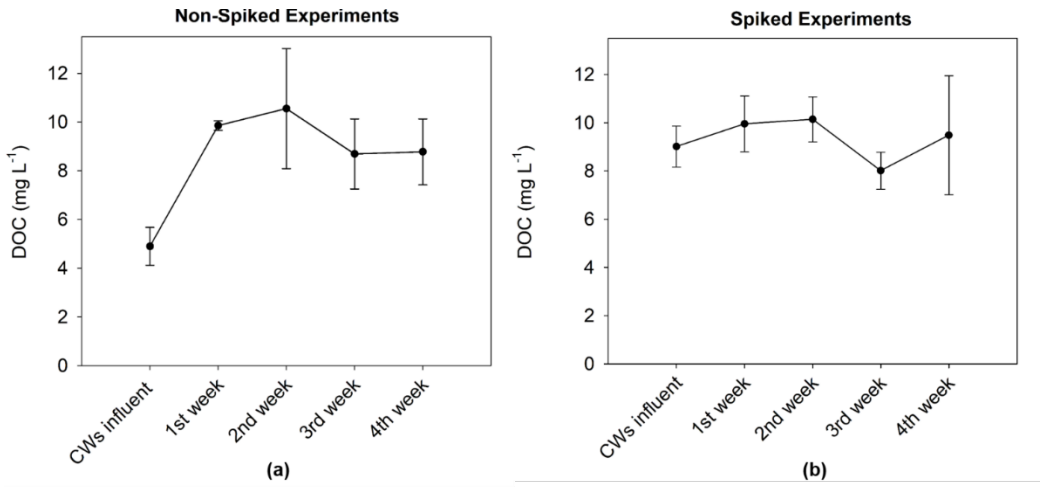

Figure 14. DOC contents in CW microcosm influents and effluents during the four 1-week cycles of treatment, using non-spiked **(a)** and spiked **(b)** aquaculture effluents.

Studies dealing with the removal of nutrients by CWs have been particularly focused on nitrogen and phosphorus. In the present work, twelve ions species, including sodium (Na^+), potassium (K^+), ammonium (NH_4^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), chloride (Cl^-), bromide (Br^-), bromate (BrO_3^-), nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{3-}), and sulphate (SO_4^{2-}), were selected to monitor during all the weekly cycles (Fig. 15).

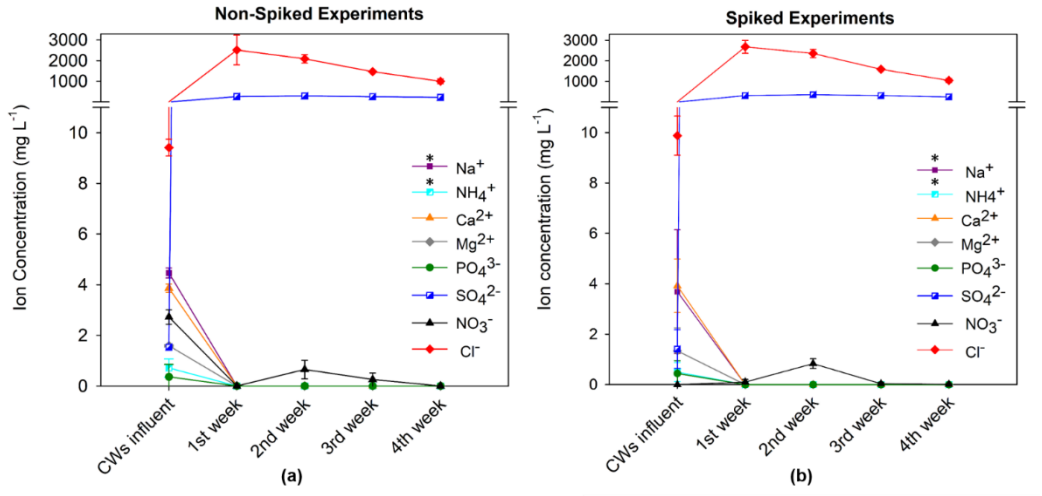

Figure 15. Concentrations of ions in CW microcosm influents and effluents during the four 1-week cycles of experiments, using non-spiked **(a)** and spiked **(b)** aquaculture effluents. In both cases, K^+, BrO_3^-, Br^- were always below MDL (0.30, 0.08 and 0.08 mg L^{-1}, respectively) and NO_2^- was always below MQL (0.25 mg L^{-1}). *Cations Na^+ and NH_4^+ were only determined in CW influents, due to matrix effects verified after CW treatment.

The nutrients are typically eliminated by CWs through biotic and abiotic processes, specially occurring around the rhizosphere of vegetation. Therefore, they are adsorbed, absorbed, transformed, trapped, and subsequently removed from water in CW systems [44]. Most of the analysed ions, namely Ca^{2+}, Mg^{2+}, PO_4^{3-} and NO_3^- (with inlet concentrations under 4 mg L^{-1}), were considerably removed by the CW microcosms during the four cycles of treatment in non-spiked (Fig. 15a) and spiked (Fig. 15b) experiments. In both cases, as expected, the removal trend over the weeks was very similar (≈ 100%). Although it was not possible to determine the NH_4^+ concentrations in CW microcosm effluents by using ionic chromatography due to matrix effects (high concentration of Na^+), a preliminary analysis by a spectrophotometric method (as in Bôto *et al.* [28]) showed high removal rates (> 70%) in both non-spiked and spiked experiments (data not shown). For K^+, BrO_3^-, Br^- and NO_2^-, removals were not evaluated because the concentrations of

these ions were below the MDL (respectively 0.30, 0.08 and 0.08 mg L^{-1}) or below the MQL (0.25 mg L^{-1}), respectively.

The removal of nutrients is affected by several factors, such as their properties, availability of O_2, temperature, vegetation type, HRT, substrate composition and nutrient loading rate [44]. Primary macronutrients nitrogen and phosphorus (which include NH_4^+, NO_2^-, NO_3^- and PO_4^{3-}) are predominantly removed by nitrification-denitrification processes and adsorption and/or precipitation into soil/CW substrates, respectively [44, 45]. In contrast, Ca^{2+} and Mg^{2+} (secondary macronutrients) are mainly absorbed by plants [46]. Although nutrients elimination rates vary considerably with the operating conditions used in CWs and influent loads, the removal yields obtained in the present study were quite similar to those already reported for bench-scale [35] and full-scale [47] CW systems. Therefore, the assembled CW microcosms were working properly, simulating a real CW system along the experimental period.

The concentrations of the anions SO_4^{2-} and Cl^- considerably increased from few mg L^{-1} in the influents to hundreds and thousands of mg L^{-1} in the respective effluents. Little attention has been paid to the removal of these ions in CWs, being the information about these two micronutrients scarce in the literature [44]. Anaerobic respiration is the main process responsible for SO_4^{2-} removal, and for that reason, this occurs always followed by NO_3^- depletion [48, 49]. Whitmire and Hamilton [48] suggested that SO_4^{2-} production during the treatment may be due to the NO_3^- removal processes, which stimulate sulphur oxidation or, on other hand, inhibit SO_4^{2-} reduction by denitrification. Regarding Cl^-, the existing studies are inconclusive [50, 51] and thus, more research is needed on this topic. For Na^+, although quantification by ionic chromatography was not possible due to matrix effects (partial co-elution with NH_4^+), a clear increase in the peak area was observed, indicating a significantly higher concentration of Na^+ in CW microcosm effluents in comparison to the influents. In fact, the root bed substrate could release some of these ions (by a washing effect), as the sediment and sand were collected in a location of the river that can still suffer from salty water intrusion, resulting from the influence of the sea, into which this river drains.

Overall, these results showed that the performance of CW microcosms to remove nutrients was not affected by the presence of a significant number of MPs in the feeding water. Therefore, besides

the removal of MPs, these biological systems can remove conventional pollutants from aquaculture effluents, like nutrients, and contribute for a more environmentally responsible and sustainable aquaculture sector.

3.4. Conclusions

The investigated VSSF-CW system demonstrated a great potential to remove organic MPs from freshwater aquaculture effluents. In non-spiked experiments, most MPs quantified in aquaculture effluents (atrazine, PFOS, clarithromycin, fluoxetine, and norfluoxetine) were highly removed (≈ 100%), the PFOS in the first week of operation being the only exception. Furthermore, the spiked experiments with all the target MPs added simultaneously to the freshwater aquaculture effluents confirmed these good performances (weekly removal rates above 87% for all target MPs). The comparison of the results with those obtained in other studies is difficult, because the tested conditions are variable according to the aim of each one, but removal percentages obtained for some of target MPs were higher than values that have been described in the literature. Although the DOC content was not decreased by the CW systems, high removals of nutrients were generally obtained, revealing that the large number of MPs in spiked effluents did not affect the performance of these systems. Further research is still needed to better understand the processes that are responsible for the removal of MPs in CWs, in order to optimize these systems and improve their efficiency.

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Chapter

4

Quenchers in AOTs for analysis of MPs by liquid chromatography coupled to mass spectrometry: sodium sulphite or catalase?

After evaluating the performance of CWs to remove organic MPs (*Chapter 3),* AOTs were investigated for the same purpose (including a set of 26 organic MPs with a wide range of polarity and pK_a). However, when treatment trials with UV/H_2O_2 were conducted, for which sodium sulphite (Na_2SO_3) was used to quench residual H_2O_2, analytical interferences were observed during the quantification of a group of compounds. This triggered the need to further investigate this effect. Therefore, the possible effect of H_2O_2 and their commonly quenchers used in H_2O_2-based AOTs, *i.e.*, Na_2SO_3 and catalase, were investigated on the analytical signal of 3 detectors coupled to liquid chromatography (LC), namely tandem mass spectrometry (LC-MS/MS), fluorescence detection (LC-FD) and LC-diode array detection (LC-DAD).

MPs monitored by LCM-MS/MS after electrospray ionization (ESI) under positive ionization (PI) mode and presenting a pK_a higher than 5.9 revealed a great signal suppression when using Na_2SO_3 as quencher. In this sense, to further explore this effect, 2 MPs, metoprolol (with signal suppression) and diclofenac (without interference), were selected. These MPs were analysed before and after addition of H_2O_2, H_2O_2/Na_2SO_3 and H_2O_2/catalase in reaction vials, using: (i) different detectors coupled to LC, namely LC-MS/MS (with ESI under either PI or negative ionization (NI) modes), LC-FD and LC-DAD; (ii) different environmental matrices (SW, drinking water (DW), WW and ultrapure water (UPW)); and (iii) different MPs concentrations (0.1–10 mg L^{-1}). A remarkable signal suppression was verified for metoprolol only in samples with H_2O_2/Na_2SO_3 analysed by LC-MS/MS, reinforcing some interference in this detector for a specific group of compounds when H_2O_2/Na_2SO_3 was used. To the best of our knowledge, the effect of Na_2SO_3 as quencher of residual H_2O_2 was never reported before for LC-MS/MS analysis and the results presented in this chapter support the recommendation to use catalase instead of Na_2SO_3 as quencher.

This chapter is based on the following reference (original version available in **Appendix A4**, with permission of Elsevier):

Gorito, A.M., Barbosa, M.O., Ribeiro, Almeida, C.M.R, Pereira, M.F.R., Silva, A.M.T., Ribeiro, A.R.L., *Quenchers in advanced oxidation technologies for analysis of micropollutants by liquid chromatography coupled to mass spectrometry: Sodium sulphite or catalase?*, Science of the Total Environment, 692 (2019) 995-1004.

4.1. Introduction

Attending to the analytical limitations and costs to quantify organic pollutants at very low concentrations in environmental matrices, most studies dealing with AOTs have been performed: (i) with ultrapure water (UPW), distilled water or synthetic solutions mimicking environmental waters; and (ii) spiking one or a few model pollutants at mg L^{-1} levels (concentrations well above those occurring in the environment). In this type of studies, liquid chromatography (LC) coupled to the simple and widely available UV or diode array detection (DAD) detectors has been employed to quantify these pollutants. However, the great advances on analytical methodologies allowed to apply more realistic approaches, by extending the research to multiple MPs in real environmental matrices, at the concentration levels that they are actually occurring [1].

Nowadays, LC-MS/MS by atmospheric pressure ionization is a key analytical tool for quantification of MPs [2-5]. This technique combines the advantages of LC as a versatile separation methodology for polar, non-volatile, or thermally unstable compounds, and mass spectrometry (MS) as an extremely sensitive detection and identification technique [6, 7]. MS is based on the formation of ions from the analytes, which are then fragmented and analysed according to their mass to charge ratio (m/z). Electrospray ionization (ESI) of target analytes by a strong electric field applied in the liquid sample has been often used in environmental samples analysis [8]. With this, charged droplets are originated, from which the ions at the surface are ejected to the gaseous phase and fragmented for quantification [9]. During this complex process, factors such as surface activity (affinity for electrospray droplet surfaces) and solvation energy can affect the response of analytes to ionization [10], often resulting in the suppression or, less frequently, in the enhancement of their signals [7, 11]. In this regard, the main drawback of the LC-ESI-MS technique is the matrix effect in the ionization, which must be considered to avoid erroneous interpretation of results [7, 12]. This effect results from the competition between the analytes and the co-eluting interfering species [13], and thus the following aspects are able to cause them: (i) the matrix itself (*e.g.*, shown by comparison of UPW and WW); (ii) the sample pre-treatment procedure; (iii) the reactants needed for the AOT under study (*e.g.*, oxidants, catalysts); and/or (iv) the reactants used to quench the

radicals in order to stop the oxidative reaction (*e.g.*, H_2O_2 or O_3 quenchers). The traditional methods for quenching residual H_2O_2 after oxidative reactions are based on the addition of reactants as free chlorine, catalase, Na_2SO_3 or sodium thiosulfate ($Na_2S_2O_3$), depending on the post-AOT water analysis [14, 15]. Given the complexity of reaction between H_2O_2 and $S_2O_3^{2-}$ (slow reaction, proceeding through multiple intermediate steps and exhibiting oscillatory properties under certain conditions) [14], in several studies Na_2SO_3 is usually selected instead of $Na_2S_2O_3$ [16-19]. Nonetheless, the application of these two inorganic scavengers has been recently discouraged for quenching residual H_2O_2 after AOTs due to several possible undesirable effects, namely the undesirable high concentrations of salts for mass spectrometry analysis [14]. However, other interferences can occur.

In this work, possible analytical interferences of the oxidant H_2O_2 and respective quenchers, catalase and Na_2SO_3, were studied, using different detectors coupled to LC. Initially, a SW spiked with a group of 26 MPs, each at a concentration of 100 ng L^{-1}, was treated by continuous mode UV/H_2O_2 process, but the detection signals were completely suppressed for more than half of the compounds and respective internal standards in both control (H_2O_2 without UV, where removal is not expected) and UV/H_2O_2 experiments, being their removal rates inconclusive. We hypothesized that a possible interference of Na_2SO_3 used as quencher could take place in the LC-MS/MS. In this sense, a second approach consisting in batch experiments using reaction vials was performed to assess the effect of the oxidant (H_2O_2) and combined reactants (H_2O_2/Na_2SO_3, H_2O_2/catalase) on the LC-MS/MS, LC-DAD and LC-FD signals of 2 MPs with distinct characteristics and responses when using Na_2SO_3. These 2 compounds were selected out of the target 26 MPs: metoprolol (positive interference) and diclofenac (without interference). The effect of each component (H_2O_2, H_2O_2/Na_2SO_3, H_2O_2/catalase) was also investigated considering different water matrices (SW, DW and WW, vs UPW) and different concentration levels: 0.1 mg L^{-1} (LC-MS/MS and LC-FD); 1.0 mg L^{-1} (LC-MS/MS, LC-DAD and LC-FD); and 10 mg L^{-1} (LC-MS/MS, LC-DAD and LC-FD).

4.2. Materials and methods

4.2.1. Chemicals and materials

All reference standards of the MPs under study (acetamiprid, atrazine, azithromycin, bezafibrate, carbamazepine, citalopram, clarithromycin, clindamycin, clofibric acid, diclofenac, diphenhydramine, fluoxetine, isoproturon, ketoprofen, metoprolol, norfluoxetine, ofloxacin, PFOS, propranolol, simazine, thiacloprid, thiamethoxam, tramadol, trimethoprim, venlafaxine, and warfarin; >98% purity), were purchased from Sigma-Aldrich (Steinheim, Germany). The physicochemical properties of the analytes can be found in Table D2.1 (Appendix D, Supplementary Material). The respective deuterated compounds used as internal standards (atrazine-*d5*, acetamiprid-*d3*, diclofenac-*d4*, azithromycin-*d3*, ofloxacin-*d3*, fluoxetine-*d5*, ketoprofen-*d3*; >98% purity), catalase from bovine liver powder (2,000- 5,000 units/mg protein) and H_2O_2 (30%, w/w) were obtained from Sigma-Aldrich (Steinheim, Germany). Na_2SO_3 (98.5% purity) was acquired from Acros Organics (New Jersey, USA). Stock solutions of ca. 1000 mg L^{-1} were prepared for each reference and internal standard and 2 working solutions were prepared by their dilution in ethanol, one containing all target MPs at 2.5 mg L^{-1} and another containing all internal standards at 10 mg L^{-1}. Stock solutions of approximately 10 mg L^{-1} were also prepared by dissolution of known amounts of diclofenac and metoprolol in each matrix under study (*i.e.,* UPW, SW, DW and WW).

Formic acid was purchased from Merck (Darmstadt, Germany). Methanol and acetonitrile (MS grade) were acquired from VWR International (Fontenay-sous-Bois, France), whereas ethanol HPLC grade was obtained from Fisher Scientific UK Limited (Leicestershire, UK). UPW (resistivity > 18 mΩ cm) was supplied by a Milli-Q water system. Oasis® HLB cartridges (150 mg, 6 mL) were acquired from Waters (Milford, Massachusetts, USA).

4.2.2. SW, DW, and WW samples

All water samples were collected in April 2017 using pre-rinsed amber glass bottles, which were then transported at 4 °C to the laboratory and kept refrigerated until use. WW was collected from a secondary clarifier of a municipal WWTP located in Northern Portugal, SW samples were collected from Crestuma-Lever reservoir, and DW was collected from the water supply network of Porto. WW and SW samples were previously filtered through 1.2 µm glass microfiber filters GF/C, 47 mm (Whatman™, United Kingdom) and DW (chlorine content: 47.5 mg L^{-1}) was used without any pre-treatment.

4.2.3. UV/H_2O_2 experiments (A): set-up and procedure

Continuous UV/H_2O_2 experiments to treat SW (Fig. 16A) and the respective controls (H_2O_2 without UV) were conducted in a column (70 cm height × 3 cm i.d.) packed with glass rings, continuously fed with SW sample (15 mL min^{-1}) and pure O_2, and connected to a loop column to simulate a perfectly mixed reactor, as described elsewhere [20]. The main column was equipped with eight 10 W UV LEDs (λ_{max} = 382 nm).

The experiments were conducted in duplicate, applying an HRT of 41 min to treat 6 L of SW spiked with 100 ng L^{-1} of each target analyte . The selection of this concentration was based on our recent review reporting that many of the target MPs have been found worldwide in SW at ng L^{-1}–µg L^{-1} levels [21]. For each experiment, the column was pre-filled with distilled water. Afterwards, the SW was pumped, and the treatment started when the steady state (t_s = 180 min) was reached, *i.e.,* when no more dilution effect occurs, and the outlet solution has reached a steady concentration. Thus, after t_s (t_1), H_2O_2 was added according to the stoichiometric amount with respect to the content of DOC in SW and the LEDs were switched on in the case of UV/H_2O_2 experiments. After t_1, another t_s of 180 min was considered before collecting the last samples at t_2 (triplicates of 500 mL, meaning t_{2a}, t_{2b}, t_{2c}). A stoichiometric excess of Na_2SO_3 with respect to H_2O_2 (5% excess in

relation to the stoichiometric amount) or 5 mL of fresh catalase solution (0.1 g L^{-1}) based on a previous work [22], were added to each triplicate sample, to quench the residual H_2O_2. It should be noted that the addition of catalase was tested in a second round of control experiments, after analytical problems with Na_2SO_3 have been detected.

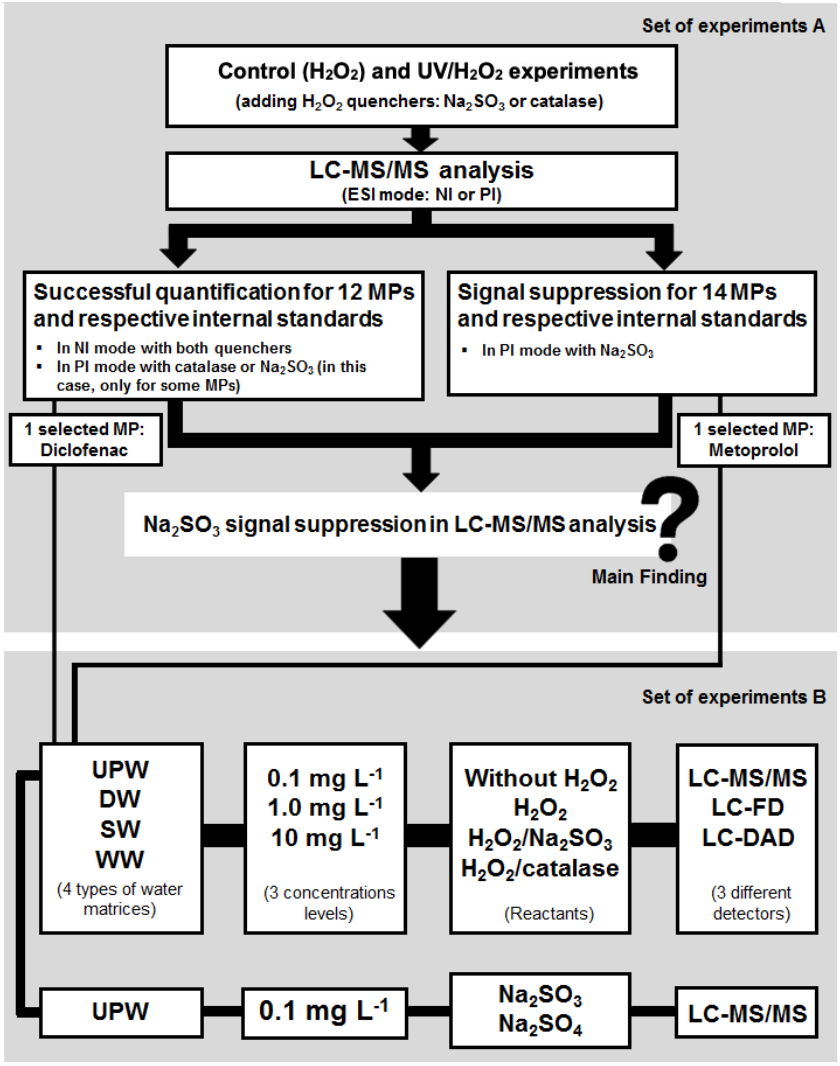

Figure 16. Scheme of the experimental approach, including continuous control (H_2O_2) and UV/H_2O_2 experiments (A) and batch experiments (B) to evaluate the matrix effects and interference of reactants used in (A). Electrospray ionization (ESI); negative ionization (NI); positive ionization (PI).

4.2.4. Study of the analytical interference of reactants (B)

With the aim of understanding the interference related to the addition of H_2O_2/Na_2SO_3 in the analysis of more than half of the MPs by LC-MS/MS in the set of experiments (A) (Fig. 16A), which could result either from Na_2SO_3 residual presence or the product of its oxidation (sodium sulphate (Na_2SO_4), Eq. 2), experiments were performed using UPW spiked with the 2 MPs (diclofenac and metoprolol) at 0.1 mg L^{-1}, by adding only Na_2SO_3 or Na_2SO_4 solutions to the samples, without any sample preparation (pre-filtration or pre-concentration) (Fig. 16B).

$$Na_2SO_3 + H_2O_2 \rightarrow Na_2SO_4 + H_2O \quad \text{(Eq. 2)}$$

The possible interference of H_2O_2, H_2O_2/Na_2SO_3 and H_2O_2/catalase was further investigated, evaluating the signal obtained in 4 matrices (SW, UPW, DW, and WW) spiked with 2 MPs at 3 concentration levels (0.1, 1.0, 10 mg L^{-1}) by LC-MS/MS, LC-FD, and LC-DAD (Fig. 16B), without any sample pre-concentration. Metoprolol and diclofenac were the MPs selected for this set of batch experiments due to their different behaviour in the LC-MS/MS analysis, after quenching the samples of (A) with Na_2SO_3. All experiments were performed in 1.5 mL reaction vials, under dark conditions in triplicate.

Solutions containing the 2 MPs (diclofenac and metoprolol) at 10 mg L^{-1} in UPW, DW, SW and WW (*Section 4.2.1*) were diluted in the respective matrix to obtain concentrations of 0.1 and 1.0 mg L^{-1} in the 4 matrices. H_2O_2, H_2O_2/Na_2SO_3 or H_2O_2/catalase were added to 1.5 mL vials (batch mode) containing 1 mL aliquots of each matrix spiked at the 3 concentration levels. A stoichiometric excess of H_2O_2 (5% excess in relation to the stoichiometric amount) was added to each sample, which was determined based on the DOC content of UPW, DW, SW and WW (0.5 ± 0.1, 1.6 ± 0.1, 2.6 ± 0.5 and 16.8 ± 0.9 mg L^{-1}, respectively). Posteriorly, a volume of 100 µL of a Na_2SO_3 solution of 3.3 mg L^{-1} or of a 0.1 g L^{-1} catalase solution [22], was added to evaluate the effect of the reactants H_2O_2/Na_2SO_3 and H_2O_2/catalase, respectively. The effect of the oxidant itself (H_2O_2) was also assessed, by adding to aliquots containing H_2O_2, 100 µL of the respective matrix to obtain the same

dilution factor. Blanks were prepared by adding the same volume of each matrix, instead of H_2O_2, H_2O_2/Na_2SO_3 or H_2O_2/catalase. All samples were analysed by LC-MS/MS, LC-FD and LC-DAD, as triplicate measurements. The effects on the signal, namely the matrix effects (DW, SW and WW) and the interference caused by the addition of the oxidant (H_2O_2) and the reactants (H_2O_2/Na_2SO_3 and H_2O_2/catalase) were determined using the Eqs. 3–5.

$$\text{Matrix effect [DW, SW and WW] (\%)} = \frac{A_{(\text{Spiked Matrix}-\text{Blank Matrix})}}{A_{(\text{Spiked UPW}-\text{Blank UPW})}} \times 100 \quad \text{(Eq. 3)}$$

$$\text{Oxidant effect [}H_2O_2\text{] (\%)} = \frac{(A_{(\text{Spiked Matrix}-\text{Blank Matrix})} - A_{(\text{Spiked Matrix with Oxidant}-\text{Blank Matrix})})}{A_{(\text{Spiked Matrix}-\text{Blank Matrix})}} \times 100 \quad \text{(Eq. 4)}$$

$$\text{Reactant effect [}H_2O_2/Na_2SO_3\text{, }H_2O_2\text{/catalase] (\%)} = \frac{(A_{(\text{Spiked Matrix}-\text{Blank Matrix})} - A_{(\text{Spiked Matrix with Reactant}-\text{Blank Matrix})})}{A_{(\text{Spiked Matrix}-\text{Blank Matrix})}} \times 100 \quad \text{(Eq. 5)}$$

where $A_{\text{Spiked UPW}}$ and $A_{\text{Blank UPW}}$ are the peak areas of the spiked and non-spiked UPW samples, respectively; $A_{\text{Spiked Matrix}}$ and $A_{\text{Blank Matrix}}$ are the peak areas of the spiked and non-spiked matrices, respectively, without addition of H_2O_2; $A_{\text{Spiked Matrix with Oxidant}}$ is the peak area of spiked matrices after addition of H_2O_2; and $A_{\text{Spiked Matrix with Reactant}}$ is the peak area of spiked matrices, after adding H_2O_2/Na_2SO_3 or H_2O_2/catalase. These measurements were made for each matrix and MP concentration. Values of 100% indicate absence of interference, while values higher or lower than 100% indicate a signal enhancement or suppression, respectively.

4.2.5. Analytical methodologies

The concentration of the target MPs in the first set of experiments (A) was determined using a validated analytical method described elsewhere [3], based on offline SPE-UHPLC-MS/MS. Briefly, SW samples (500 mL) previously filtered (*Section 4.2.2*) were acidified to pH 3 with sulphuric acid,

followed by pre-cleaning and extraction with SPE using Oasis® HLB cartridges and ethanol as eluting (4 mL) and reconstituting solvent (250 µL). Chromatographic analysis was carried out in a UHPLC Shimadzu Corporation equipment connected to a triple quadrupole mass spectrometer detector (Ultra-Fast Mass Spectrometry series LCMS-8040). A Kinetex™ XB-C18 100 Å column (100 × 2.1 mm i.d.; 1.7 µm particle diameter) supplied by Phenomenex, Inc. (California, USA) was used for chromatographic analysis. A gradient of methanol and water acidified with 0.1% formic acid was used as mobile phase at 0.20 mL min^{-1}. Capillary voltage, drying gas flow, nebulizing gas flow, desolvation and source temperature of the mass spectrometer were respectively 4.5 kV, 10 dm^3 min^{-1}, 3 dm^3 min^{-1}, 250 ºC and 400 ºC. Retention time, MDLs, MQLs and recoveries of the target analytes are presented in Table D2.2, Appendix D.

Regarding the second set of experiments (B), metoprolol and diclofenac were determined in the samples without any preconcentration step (only filtration of WW and SW, as described in *Section 4.2.2*, by UHPLC-MS/MS and also with a LC System coupled to DAD (Shimadzu SPD-M20A) and FD (Shimadzu RF-20Axs). For these analyses, the mobile phase consisted on a mixture of 0.1% aqueous formic/acetonitrile (50:50, v/v) at isocratic mode and at a flow rate of 0.20 mL min^{-1}. Excitation and emission wavelengths (λ_{Exc};λ_{Em}) of the FD were set as follows: λ_{Exc} 230 nm/λ_{Em} 290 nm from 0 to 4.5 min; λ_{Exc} 285 nm/λ_{Em} 360 nm from 4.5 to 6.5 min; and λ_{Exc} 230 nm/λ_{Em} 290 nm from 4.5 to 6.5 min.

A pH meter pHenomenal® pH 1100L (VWR, Germany) was required for the pH adjustments. DOC was measured using a Shimadzu TOC-5000A analyser (Shimadzu Scientific Instruments, Japan), following the standard procedure 5310 B of the Standard Methods for Examination of Water and Wastewater [23].

4.3. Results and discussion

4.3.1. UV/H_2O_2 experiments (A): removal of organic MPs and interference of reactants

SW samples spiked with a set of 26 MPs at 100 ng L^{-1} were treated by UV/H_2O_2, being MPs concentrations determined afterwards. The final concentrations and removals rates (not shown) were inconclusive for 14 compounds (azithromycin, carbamazepine, citalopram, clarithromycin, clindamycin, diphenhydramine, fluoxetine, metoprolol, norfluoxetine, ofloxacin, propranolol, tramadol, trimethoprim, and venlafaxine), since the analytes and their surrogates used as internal standards had a complete or large signal suppression in the LC-MS/MS analysis, in both control (H_2O_2 without UV) and treated samples (UV/H_2O_2) which were quenched with Na_2SO_3 after passage in the continuous-flow reactor. Control samples allowed confirming that the absence of signal for the target MPs was not related to their elimination during UV/H_2O_2, which could have resulted in concentrations below the MDLs. Indeed, in the control samples, the decrease on the initially spiked concentrations (Fig. 18, black diamonds) was not expected due to the inability of H_2O_2 to degrade MPs in the absence of an UV irradiation source or other oxidants as O_3. Moreover, this suppression effect was not observed in the initial concentration of spiked samples (*i.e.,* without H_2O_2 and Na_2SO_3) in which all target MPs could be analysed, giving an insight that some interference could have resulted from the treatment process, namely after the addition of H_2O_2 and Na_2SO_3 in both control and treated samples. Interestingly, this analytical interference was only observed for part of the MPs, corresponding to those eluted first in the chromatographic column and ionized in ESI under positive ionization (PI) mode. Although the exogenous suppressor resulting from addition of H_2O_2/Na_2SO_3 has been verified for a considerable number (14) of MPs, other 12 compounds including diclofenac did not show any analytical interference. In this sense, and taking into account the behaviour of the 26 target MPs included in this set of experiments (A), the specific properties of these compounds may be related to the described interference.

Many studies have investigated the phenomenon of matrix effect. However, its cause is still not fully understood [24]. Several factors contributing to analyte signal suppression or enhancement have been pointed out, including: (i) the competition between analytes and interfering compounds for the available charges and access to the droplet surface; (ii) the formation of solid particles due to the use of non-volatile additives or, in the case of less-volatile target compounds, the difficulty to convert the charged droplets in gas-phase ions; and (iii) the formation of analyte ions or neutral complexes when additives or matrix components act like ion pairing reagents; (iv) among others [25, 26].

According to Tan *et al.* [26], compounds with high mass, polarity and basicity are more likely to be impacted by matrix effect. The distribution of the 26 target MPs according to their pK_a and log K_{ow} (Fig. 17) shows that pK_a is the dominating factor, describing the respective response of these compounds in the presence of Na_2SO_3 as H_2O_2 quencher. In fact, pK_a was already described as one of the most influential parameters in predicting the ionization in ESI source via monoprotonation, under PI mode [27]. MPs with low pK_a (< 5.9) showed no interference of this reactant (#1-12 in Fig. 17; Table D2.1, Appendix D), whereas MPs with higher pK_a (> 5.9) were affected by it (#13-26 in Fig. 17; Table D2.1, Appendix D). It is interesting to mention that acid analytes (*i.e.,* with low pK_a) have a natural tendency to originate deprotonated forms, being expected to be ionized in the negative ionization (NI) mode. In turn, basic analytes (*i.e.,* with high pK_a) tend to create cations and their ionization is likely to occur in the PI mode [28]. It was noticeable that the MPs analysed under PI mode and eluted first, which are basic, had a detection interference in the samples containing H_2O_2/Na_2SO_3 (Fig. 18), suggesting that the alkaline pH of the samples containing Na_2SO_3 might reduce their ionization, or a matrix effect of co-eluting substances could be the origin of such interferences. Some exceptions were observed for several neutral and acidic MPs analysed under PI mode, namely for pesticides acetamiprid, atrazine, isoproturon, thiacloprid, thiamethoxam, simazine and the anticoagulant warfarin, which did not have analytical interference. In contrast, all acidic MPs monitored under NI mode (clofibric acid, PFOS, bezafibrate, diclofenac, and ketoprofen) had a good performance of MS detector in the presence of H_2O_2/Na_2SO_3.

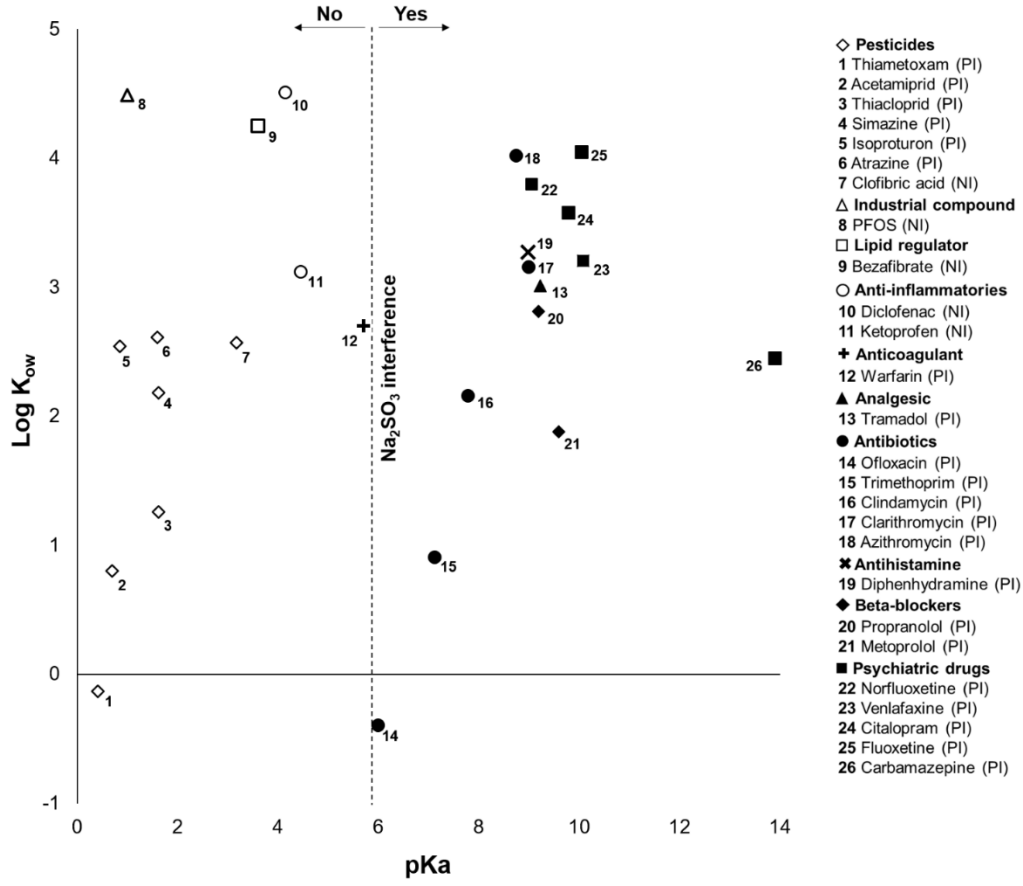

Figure 17. Representation of target MPs according to their pK_a and log K_{ow}, grouped by absence (No) or presence (Yes) of interference by adding H_2O_2/Na_2SO_3 to the SW sample spiked at 100 ng L^{-1}. PI and NI are positive and negative ionization modes, respectively.

Therefore, we hypothesized that: (i) the remaining Na_2SO_3 in solution used as reaction quencher (in excess) or the product of the reaction between H_2O_2 and Na_2SO_3 (Na_2SO_4), could interfere with the PI taking place in the ESI interface of the LC-MS/MS; (ii) raising the sample pH after addition of H_2O_2 and Na_2SO_3 might reduce the ionization of basic molecules; (iii) some adduct could be produced, resulting from the presence of the reactants in the sample solution; or (iv) less likely the

reaction between H_2O_2 and Na_2SO_3 in solution might originate some reactive species, which slightly altered the structures of MPs.

In this sense, control experiments (H_2O_2 without UV, where degradation of MPs was not expected) were repeated to understand the role of Na_2SO_3, by testing the addition of Na_2SO_3 or catalase quenchers and the absence of any quencher (*i.e.,* H_2O_2 alone) (Fig. 18). The obtained data showed a clear interference in the quantification of some MPs when using H_2O_2/Na_2SO_3, specifically in the case of the compounds abovementioned, all eluted at the very beginning of the chromatographic run and ionized under PI mode. In turn, H_2O_2 alone and H_2O_2/catalase did not cause any interference in the analysis of MPs by LC-MS/MS, with similar initial and final concentrations of MPs being registered (Fig. 18). Although the treatment efficiency was not the focus of this particular work, it should be mentioned that UV/H_2O_2 (HRT of 41 min) using catalase as quencher had removal rates below 21% for all compounds (not shown). Considering that MPs were spiked at 100 ng L^{-1} and that the validation of the method was performed with the same type of water collected at different times, the inaccuracy found for the initial concentration of some MPs (Fig. 18) was ascribed to a slightly different matrix effect between samples collected in different days.

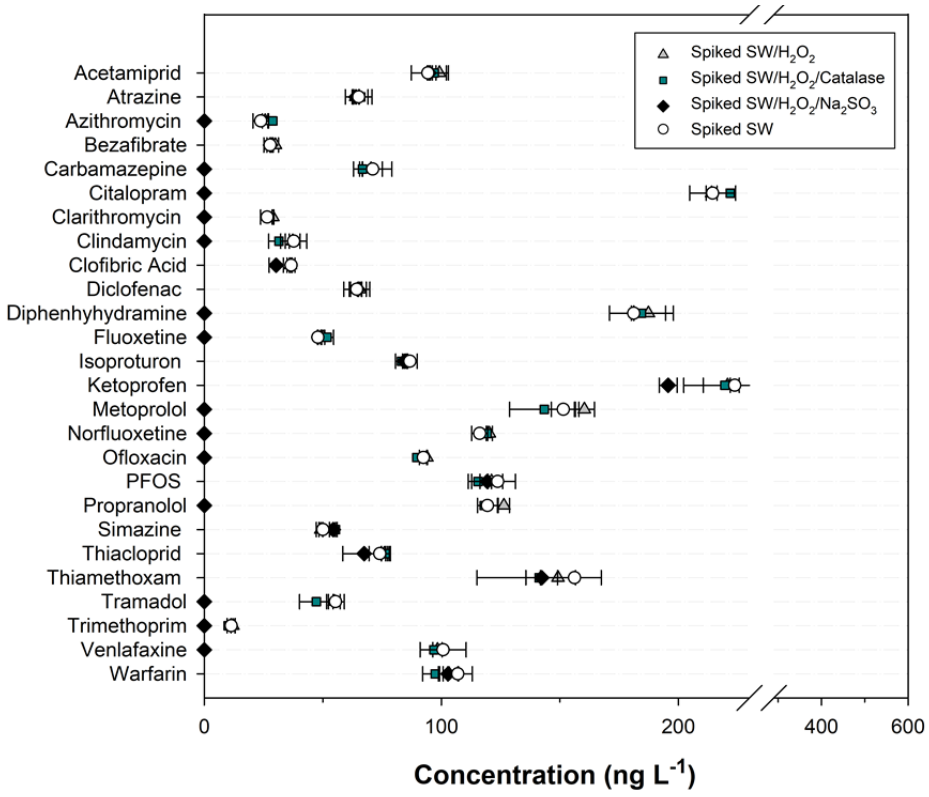

Figure 18. Variation of MPs concentrations (ng L^{-1}) in control (H_2O_2) experiments, including initial samples (Spiked SW) and samples analysed, before (Spiked SW/H_2O_2) and after addition of H_2O_2-quenchers (Spiked SW/H_2O_2/Na_2SO_3 and Spiked SW/H_2O_2/Catalase).

These results reinforce that the matrix-matched validated analytical methods using internal calibration methods to calculate removal efficiencies are crucial to avoid misinterpretations of the results, particularly bias conclusions about treatment efficiency of AOTs. Moreover, these results suggest that Na_2SO_3 is not a good option as quencher in AOT experiments when LC-MS/MS is employed, maybe due to a suppression effect taking place in the ESI interface of LC-MS/MS (matrix effect or pH effect) or due to the formation of adducts or by-products. To test these hypotheses, reinforced by the absence of such effect when using catalase instead of Na_2SO_3 as H_2O_2 quencher, the effects of each single component (H_2O_2, H_2O_2/Na_2SO_3, and H_2O_2/catalase) on the LC-MS/MS signals of 2 MPs selected from the set of the 26 target MPs, one analysed after ESI under PI mode

and with verified analytical interference (metoprolol) and another analysed after ESI under NI mode and without interference (diclofenac), were studied (*Section 4.3.2*), as shown in Fig. 16B. The analytical interference on other detection modes (LC-DAD and LC-FD) was also evaluated.

4.3.2. Matrix effects and interference of reactants (B) in chromatographic analysis

As referred above, metoprolol (ESI+) and diclofenac (ESI-) were selected as model compounds due to their different behaviour, when using Na_2SO_3 as quencher of H_2O_2, leading to signal suppression only in the case of metoprolol (Fig. D2.1, Appendix D, Supplementary Material). In this set of experiments (B), the sample pre-treatment was restricted to the pre-filtration of WW and SW and SPE was not used as sample pre-treatment in order to confirm that the analytical interference resulted from some effect related to the addition of H_2O_2/Na_2SO_3. To understand whether this effect was due to Na_2SO_3 or to the product (Na_2SO_4) that could be formed by reacting with H_2O_2 (Eq. 2), UPW spiked with the MPs at 0.1 mg L^{-1}, and either Na_2SO_3 or Na_2SO_4, were analysed by LC-MS/MS (Fig. 16B). The results showed that neither Na_2SO_3 nor the product Na_2SO_4 in solution, nor the sample pH (≈ 9) due to the addition of Na_2SO_3 were responsible for this effect. This suggested that another product of the reaction between H_2O_2 and Na_2SO_3 could be causing an analytical interference, but more research will be needed to elucidate which species might be formed and cause this effect. The matrix effect (SW, DW and WW, vs UPW), the impact of adding the oxidant (H_2O_2) and the quenchers (H_2O_2/Na_2SO_3, H_2O_2/catalase) were deeply investigated using 3 detection modes (MS, DAD and FD) and 3 initial concentrations of MPs (0.1 mg L^{-1}, 1.0 mg L^{-1} and 10 mg L^{-1} for LC-MS/MS, LC-DAD and LC-FD). This strategy aimed at representing 3 levels of concentrations in the different water matrices and understanding the effects on the detection modes, including MS/MS, and on the 2 conventional detectors DAD and FD.

In general, a similar trend was found for matrix effects occurring in the 3 detectors for diclofenac (Fig. 19), which was a slight signal enhancement in both spiked DW at the 2 highest concentrations

tested, as well as at all concentrations in SW. This fact can be attributed to matrix effects in the ESI source of co-eluting species present in the matrix in the case of LC-MS/MS and to species absorbing in the same wavelengths selected to analyse diclofenac, in the case of LC-DAD and LC-FD. In turn, WW led to a small suppression of the signal for all the concentrations and detectors. Regarding the effects of the oxidant (H_2O_2) and the quenchers (H_2O_2/Na_2SO_3, H_2O_2/catalase), a very slight loss of signal was observed for all detectors that can be ascribed to the addition of H_2O_2 and not to these quenchers, since the effect was similar in the presence and absence of quenchers. Additionally, the DW samples had a large signal suppression when H_2O_2 was added, more notorious at the lowest concentration of diclofenac, which can be justified by the chlorine present in the matrix that might react with H_2O_2. This effect was mitigated when Na_2SO_3 was added, since this reactant is known as a dechlorinating agent able to reduce the residual chlorine used as a disinfectant in the DW supply [3, 29] and consequently prevent the suppression. In fact, the average chlorine content of DW samples (spiked and non-spiked) was 47.5 mg L^{-1}, whereas it decreased to values below the quantification limit (< 0.18 mg L^{-1}) in those samples to which Na_2SO_3 was added. Thus, these results confirm that diclofenac is not impacted by the addition of Na_2SO_3 to the treated samples containing H_2O_2.

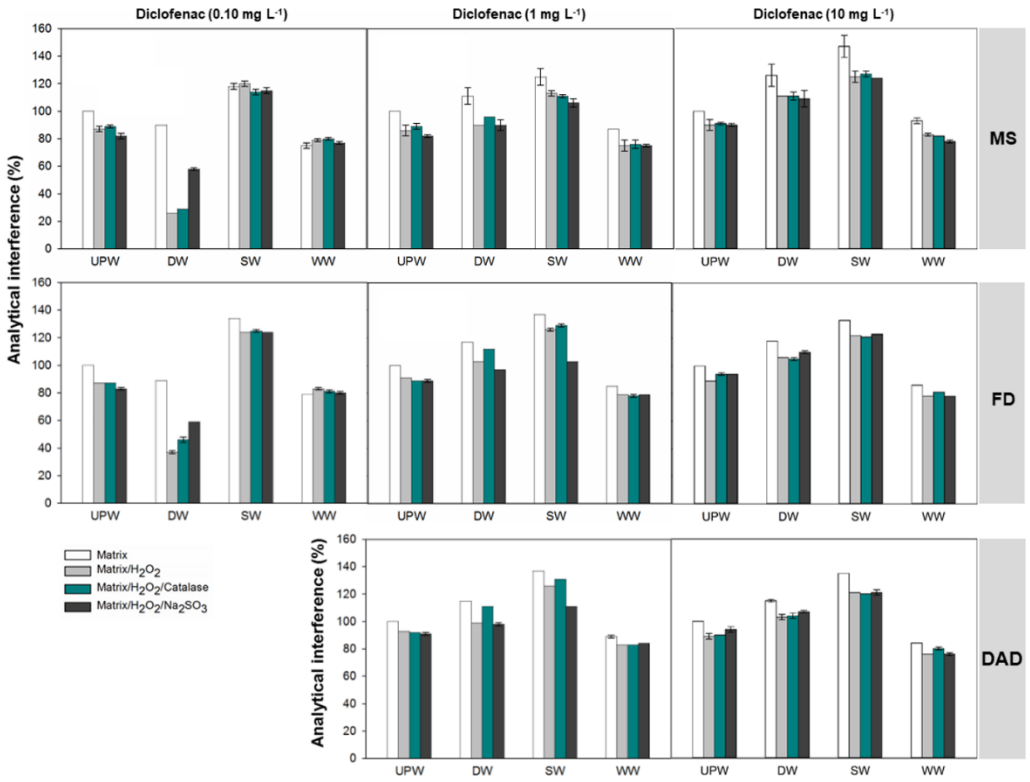

Figure 19. Analytical interference (%) resulting from the matrix, the oxidant (H_2O_2) and the quenchers (H_2O_2/Na_2SO_3, H_2O_2/catalase) in MS, FD, and DAD detectors, for diclofenac spiked at 0.1 mg L^{-1} (left), 1.0 mg L^{-1} (centre) and 10 mg L^{-1} (right) in UPW, DW, SW and WW samples.

In the case of metoprolol (Fig. 20), the matrix effect in the LC-MS/MS occurred mostly for DW, with 32% at 0.1 mg L^{-1}, 63% at 1.0 mg L^{-1} and 93% at 10 mg L^{-1}, suggesting that this effect in DW was concentration-dependent. The results would be different if any quencher of chlorine was used as pre-treatment of DW, before the experiments. Moreover, this suppression of the signal was also verified in spiked DW analysed by using DAD (60% at 1 mg L^{-1} and 91% at 10 mg L^{-1}) and FD (62% at 0.1 mg L^{-1}, 84% at 1 mg L^{-1} and 93% at 10 mg L^{-1}), maybe due to the chlorine occurring in tap water, showing the importance of matrix-matched validation of analytical methods. In the case of WW, the analytical interferences in the LC-MS/MS were negligible. In turn, it was observed a slight

enhancement of the signal for metoprolol in the case of SW (106–144% for DAD and 100–111% for FD) and WW (118–124% for DAD and 121–131% for FD), in the same detectors (DAD and FD), maybe due to some species absorbing in the same wavelengths selected to analyse metoprolol, namely organic matter.

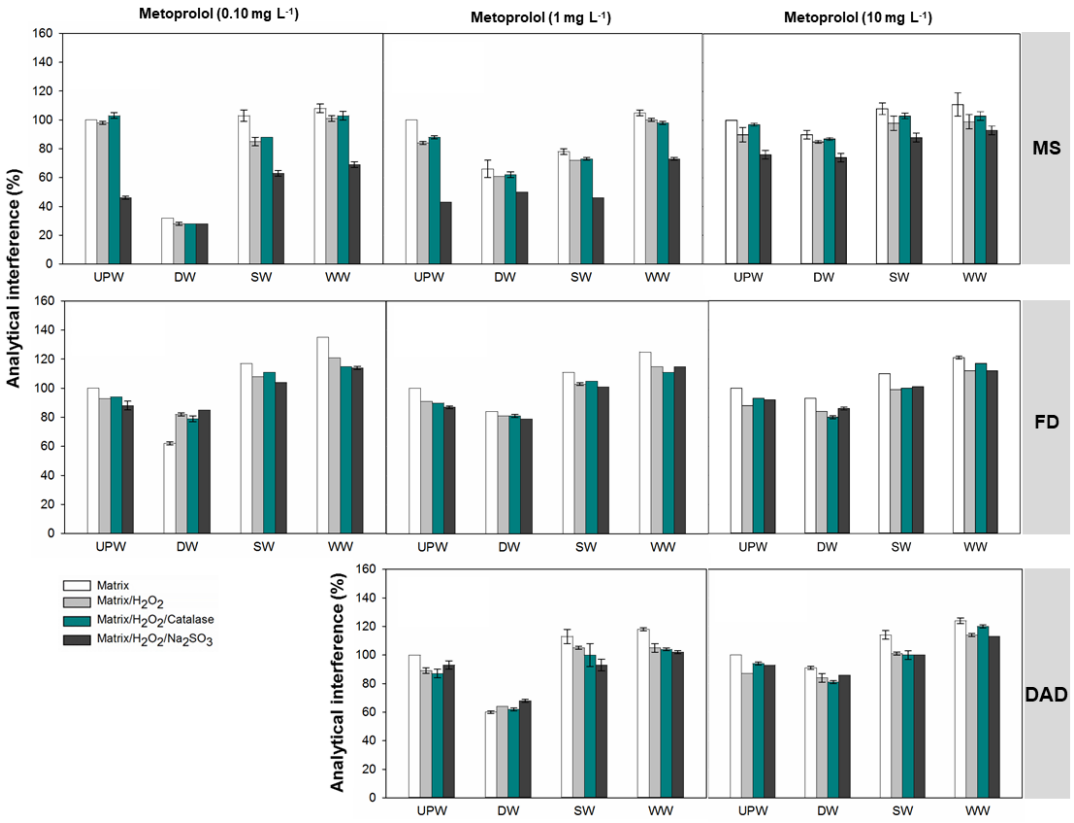

Figure 20. Analytical interference (%) resulting from the matrix, the oxidant (H_2O_2) and the quenchers (H_2O_2/Na_2SO_3, H_2O_2/catalase) in MS, FD, and DAD detectors, for metoprolol spiked at 0.1 mg L^{-1} (left), 1.0 mg L^{-1} (centre) and 10 mg L^{-1} (right) in UPW, DW, SW and WW samples.

For all matrices and concentrations, H_2O_2/Na_2SO_3 led to a decrease on the signal of metoprolol in the MS detector, which was more evident when the spiked metoprolol concentration was lower. This signal decrease may be ascribed to some species resulting from the addition of H_2O_2/Na_2SO_3,

leading to a suppression of ionization in the ESI source or adducts formation, which was confirmed by the absence of signal reduction in both DAD and FD detectors. This fact showed that the interference observed was not related to the sample preparation nor chromatographic steps, otherwise it would be expected in all detectors. The interference of Na_2SO_3 and H_2O_2/Na_2SO_3 on the signal of paraquat in LC-DAD was reported before [30] as dependent on the concentration of Na_2SO_3 and attributed to the shift effect in the maximum wavelength or in the absorbance signal. However, this effect of the quencher both in the presence and absence of H_2O_2 was only verified at concentrations of Na_2SO_3 one order of magnitude higher than those here tested.

The signal suppression was attenuated in the case of DW, maybe due to the consumption of Na_2SO_3 by the chlorine present in tap water, which was thus less or not available to interact and cause suppression in the ionization source. Interestingly, the addition of oxidant or quencher in the presence of chlorine, did not originate the signal suppression verified for diclofenac in DW when H_2O_2 was added.

The obtained results confirmed the interference of H_2O_2/Na_2SO_3 in the LC-MS/MS analysis of metoprolol, as had previously observed in the set of experiments (A) (*Section 4.3.1*). The formation of adducts cannot be disregarded, but the fact that all the affected compounds are ionized under PI mode have a pK_a > 5.9 and are eluted in the same range of time corresponding to the beginning of the chromatographic run, points out that matrix effects can justify the analytical interferences observed. Nonetheless, the suppression effect arising from the use of H_2O_2/Na_2SO_3 in MS detector is still not described in the literature, being addressed for the first time in this study. Overall, the present report clearly indicates that the application of Na_2SO_3 as H_2O_2 quencher should be replaced by catalase, when it comes to samples to be analysed by LC-MS/MS. These findings highlight the crucial role of the matrix effect interpretation in order to avoid wrong conclusions.

4.4. Conclusions

This study shows for the first time the suppression effect in the MS detection of a wide set of compounds having different chemical moieties, caused by Na_2SO_3 commonly added as a H_2O_2 quencher to samples resulting from experiments with AOTs. In the case of LC-MS/MS, the signal of the MPs monitored under NI mode and of those with lower pK_a was not affected, whereas MPs monitored under PI mode and with higher pK_a, had a strong signal suppression. This suggests that analytes pK_a may be the governing parameter on this effect. This is in accordance with previous studies reporting pK_a as one of the most important factors to predict the ionization in ESI source via monoprotonation, under PI mode. For instance, diclofenac, selected as the model MP with low pK_a, did not have noteworthy suppression effects, whereas metoprolol, as the model MP with high pK_a, had suppression in the ESI source of the LC-MS/MS, which seemed to be concentration-dependent. For all matrices and concentrations of metoprolol tested, H_2O_2/Na_2SO_3 led to a suppression of the ionization in the ESI source and a consequent decrease on the signal in the MS detector, which was more noticed for the lowest concentration. The results suggest that some product of the reaction between H_2O_2 and Na_2SO_3 might cause this suppression in the ionization source of compounds with higher values of pK_a or some adducts can be formed, but more investigation is required to understand this effect. Moreover, the decrease on the signal was mitigated in the case of DW, due to the consumption of Na_2SO_3 by the chlorine present in tap water, which avoided the suppression in the ionization source.

These conclusions are relevant for studies focused on treatment processes using H_2O_2 or O_3, which have to consider that: (i) Na_2SO_3 is not a good option as quencher, due to an analytical interference taking place in the LC-MS/MS; (ii) catalase should be used instead to avoid interferences in the MS detection; and (iii) matrix-matched validation and internal calibration are crucial to calculate removal efficiencies and avoid bias conclusions.

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Chapter

5

Ozone-based water treatment (O_3, O_3/UV, O_3/H_2O_2) for removal of organic MPs, bacteria inactivation and regrowth prevention

Studies on AOTs for removing organic MPs have been mainly focused on O_3-based processes because of their huge potential to oxidize these compounds and common acceptance in the water treatment industry (so far mainly for disinfection). However, as indicated in *Chapter 2*, selecting the best operating parameters need to consider the characteristics of the water/wastewater to be treated, the specific characteristics of applied treatment, and the required effluent quality in order to establish the best treatment technology. Therefore, in this chapter, O_3-based water treatments to simultaneously remove organic MPs and bacteria from SW samples collected in a river reservoir supplying a DWTP were investigated. Initially, 21 environmentally relevant organic MPs were screened in the selected SW. Of those, 15 were detected at low (< 20) ng L^{-1} level. SW samples were then spiked with these 21 MPs (100 ng L^{-1}each) and O_3-based treatments with excess of O_3 and high HRT (41 min) were tested. Regardless of the treatment applied (O_3, O_3/UVA-LEDs, and O_3/H_2O_2), removal efficiencies above 85% were always obtained for all MPs, except PFOS which was removed by less than 50%. Thus, single ozonation was considered the best treatment solution, afterwards being investigated in terms of water disinfection at milder operating conditions (3 mg O_3 L^{-1} and HRT of 10 min). Under these conditions, the removals of spiked MPs were also higher than 85% (except for PFOS), whereas bacteria (*i.e.,* total heterotrophs at 37 and 22 ºC, *Escherichia Coli*, and enterococci) decreased to values lower than the permissible levels for DW, even upon storage for 3-days. However, tests with lower O_3 doses (1.5 mg O_3 L^{-1}) showed it could not prevent the regrowth of total heterotrophs incubated at 37 ºC, which reached levels five times higher than those recommended. The results herein described point out the importance of using O_3 doses capable of accomplishing both MPs elimination and microbial inactivation for long periods when single ozonation is applied also aiming disinfection.

This chapter is based on the following reference (original version available in **Appendix A5**, with permission of Elsevier):

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5.1. Introduction

It is known that many organic MPs are not completely removed in conventional WWTPs, resulting in a permanent entry and consequent persistence in watercourses [1, 2]. Since MPs persist in aquatic systems, several water related activities might use waters containing residual amounts of MPs (*e.g.*, aquaculture farms, dairy industries, and DWTPs), rising environmental and health concerns [3-5]. In the particular case of DWTPs, some MPs in SW might end up in tap water, leading to possible human exposure, bioaccumulation and consequent adverse effects [1, 6]. In this context, it is important to apply effective water treatment technologies for the removal of MPs from SW [7].

In addition, microbial contamination of DW contributes to the dissemination of water-borne diseases and, therefore, its control through effective treatments in DWTPs is indispensable [8]. For this reason, in the EU, the Directive 98/83/EC (amended by Directives 2015/1787 and 2020/2184) sets microbiological quality standards for potable water, including indicators of faecal contamination, namely *Escherichia coli* and enterococci, and total heterotrophs as target bacteria [9]. Chlorination is the most common disinfection treatment for DW, but the by-products that chlorine originates, by reacting with natural organic matter, are of concern due to their possible carcinogenicity, teratogenicity, and genotoxicity [10]. Adsorption using activated carbon [11-14] and membrane technologies (such as microfiltration, ultrafiltration, nanofiltration and reverse osmosis) [13, 15] have shown good removal efficiencies of MPs in DWTPs, and some of those can also be employed for disinfection [16]. However, in the first case the adsorbent must be regenerated [17, 18], and a proper treatment or disposal of the concentrate generated is needed in the second case [15]. AOTs, such as ozonation, have been shown as an interesting option in DWTPs for disinfection [19-21]. Moreover, MPs can be oxidized by the highly reactive species that are generated by these processes, rather than transferring these pollutants from one to another phase [22, 23]. Combining O_3 with H_2O_2 or UV radiation may be beneficial options, since both may accelerate the decomposition of O_3 into highly reactive species [22, 24]. Although O_3 based-processes might form reaction by-products, the overall quality of the water is often improved [22, 24].

In fact, AOTs are promising technologies that can be used for many applications like purification and disinfection of water, gases, and soils, sterilization of medical rooms and instruments, disinfection of plant and animal products, among others [25-30]. However, regarding DWTPs, studies on the removal of MPs are still very limited when more realistic conditions are considered (*e.g.*, real matrices, wide range of MPs, and treatment operating in continuous mode). Furthermore, when the simultaneous removal of MPs and disinfection is considered to treat SW in particular, only three studies were found in the literature for this type of water matrix [7, 31, 32]. Two of these studies were focused on UV/H_2O_2 without O_3 [31, 32], and another investigated photocatalytic ozonation [7], but none of these reports targeted the disinfection to comply with national and EU regulations on water for human consumption. Thus, the novelty of the present study relies on the comparison of three O_3-based treatments to remove multi-class organic MPs from SW and conclude about the potential of the best cost-effective treatment to simultaneously inactivate bacteria according to the EU limits for water consumption.

The main research objectives were: (i) to evaluate the occurrence of MPs in a SW reservoir supplying a DWTP in Northern Portugal (*i.e.,* 5 PSs of EU Directive 2013/39 [33], 8 CECs included in the watch lists of EU Decisions [34-36], and 8 other CECs of particular interest due to their occurrence and persistence in the environment); (ii) to compare O_3 -based treatments (O_3, O_3/UVA-LEDs and O_3/H_2O_2) operating in continuous mode for the removal of all target MPs spiked in SW; and (iii) to investigate milder operating conditions to achieve a simultaneous removal of organic MPs and water disinfection.

5.2. Materials and methods

5.2.1. Chemicals and materials

All reference standards of organic MPs under study (alachlor, atrazine, azithromycin, carbamazepine, clarithromycin, clindamycin, clothianidin, diclofenac, diphenhydramine, fluoxetine, isoproturon, methiocarb, metoprolol, norfluoxetine, PFOS, propranolol, simazine, thiacloprid,

thiamethoxam, tramadol, and venlafaxine; Table D3.1 Appendix D, Supplementary Material) were purchased from Sigma-Aldrich (Steinheim, Germany). The respective deuterated compounds used as internal standards (atrazine-*d5*, acetamiprid-*d3*, clothianidin-*d3*, diclofenac-*d4*, fluoxetine-*d5*, methiocarb-*d3*, propranolol-*d7*; >98% purity) were also obtained from Sigma-Aldrich (Steinheim, Germany). Stock solutions of each reference and internal standard (*ca.* 1000 mg L^{-1}) were prepared in methanol, ethanol or acetonitrile, depending on the solubility of each substance. Two working solutions, one containing all reference MPs (2.5 mg L^{-1}) and another including all internal standards (5 mg L^{-1}), were prepared by dilution of the individual stock solutions in ethanol.

Ethanol HPLC grade was acquired from Fisher Scientific (Leicester- shire, UK), whereas methanol and acetonitrile MS grade were purchased from VWR International (Fontenay-sous-Bois, France). Formic acid and sulphuric acid were supplied by Merck (Darmstadt, Germany). Oasis® HLB cartridges (150 mg, 6 mL) were acquired from Waters (Milford, MA, USA). H_2O_2 (30%, w/ w) and catalase from bovine liver powder (2000–5000 units/mg protein) were obtained from Sigma-Aldrich (Steinheim, Germany). UPW (resistivity > 18.2 MΩ cm at 25 ºC) was supplied by a Milli-Q water system from Millipore (Massachusetts, USA).

The culture media Slanetz and Bartley (SB) and Plate Count Agar (PCA) were purchased from VWR International (Fontenay-sous-Bois, France). Chromogenic Coliform Agar (CCA) was acquired from Scharlab (Barcelona, Spain) and 2,3,5-triphenyltetrazolium chloride solution (TTC) from Sigma-Aldrich (Steinheim, Germany). For confirmatory tests, Bile Esculin Azide Agar (BEA, ISO 7899–2) and N,N,N′,N′ -tetramethyl-p- phenylenediamine (TMPD) were provided by Sigma-Aldrich (Steinheim, Germany).

5.2.2. SW samples

All experiments were performed using SW samples collected from Douro River (41◦ 04'39.4"N 8◦ 28'21.0"W) between 2017 and 2019, over five sampling periods (January, April and October 2017, June 2018, and September 2019; n = 5). The physico-chemical parameters, namely pH, dissolved organic/inorganic carbon (DOC/DIC), ions dissolved concentrations, are described in the Appendix

D, Supplementary Material (Table D3.2). This waterbody supplies a DWTP that serves 1.39 million people and treats an average flow of 270,000 m^3 day^{-1}. After collection, the samples were stored in pre-rinsed amber glass bottles, transported at 4 ºC to the laboratory and kept refrigerated until use. In the case of microbiological assays, samples were collected in sterile flasks and immediately processed in the same day of sampling. For determination of the target MPs in SW, the samples were analysed as collected. O_3-based (O_3, O_3/UVA-LEDs and O_3/H_2O_2) and control (UVA-LEDs) experiments were performed with SW spiked with the target MPs at 100 ng L^{-1} each to start the runs with similar concentrations of MPs, because SW was collected in different sampling periods, and also to simulate a worst-case scenario, *i.e.,* the occurrence of all target 21 MPs at hundreds ng L^{-1}. The SW spiking was conducted as described elsewhere [37]. Briefly, the ethanolic solution containing all target MPs was added to an empty volumetric flask and the residual solvent was evaporated using a nitrogen flow. Afterwards, the SW was added into the flask, which was stirred and sonicated for 30 s. With this approach, we aimed to minimize the variations in DOC content resulting from the addition of the stock ethanolic solution of MPs to SW.

5.2.3. Experimental set-up and procedure

Screening (O_3, O_3/UVA-LEDs or O_3/H_2O_2) and control (UVA-LEDs) experiments were performed in a flow-through glass bubble column reactor operating in continuous mode. Spiked SW was fed at the bottom of the reactor (Fig. 21a) by using a peristaltic pump (Watson-Marlow, UK; Fig. 21b), and constantly collected after treatment at the outlet stream located at the top of the reactor (Fig. 21c). This bubble column reactor (3.0 cm internal diameter × 70 cm height; Fig. 21d) was packed with glass Raschig rings (diameter: 3 mm and length: 3 mm; filling volume of 385 mL after packing) to promote the O_3-water mass transfer, as demonstrated in our previous publication [38]. A BMT 802X O_3 generator (BMT Messtechnik, Germany) (Fig. 21g) was used to produce O_3 from pure O_2 at a constant inlet concentration (50 g Nm^{-3}) and flow rate (15 Ncm^3 min^{-1} in the gas phase), and introduced into the reactor with a ceramic diffuser [7]. O_3 in the gas phase was monitored by using a BMT 964 O_3 analyser (BMT Messtechnik, Germany) (Fig. 21h). To remove the remaining

O_3 from the gas phase leaving the reactor, gas washing bottles filled with potassium iodide solution were employed (Fig. 21i). A Q45H/64 dissolved O_3 probe (Analytical Technology, USA; Fig. 21k) was calibrated by using the Indigo colorimetric method [39] and a T60 UV-Visible Spectrophotometer for absorbance measurements (PG Instruments; Lutterworth, UK). In the case of light-driven experiments, eight 10 W UVA LEDs (15.5 mm × 23.0 mm) with a dominant emission line at 382 nm and long service life (intensity remains above 70% after 10,000 h work) were set along the reactor with a distance of 5 cm (Fig. 21f) [7, 40]. The LEDs medium nominal irradiation was 390 W m^{-2} determined with a UV-Vis spectroradiometer (USB 2000 +, Ocean-Options, USA). In H_2O_2-based experiments, H_2O_2 was directly added to the spiked SW as a single pulse, and the dosage of H_2O_2 was selected according to the stoichiometric amount with respect to the DOC content (Fig. 21j).

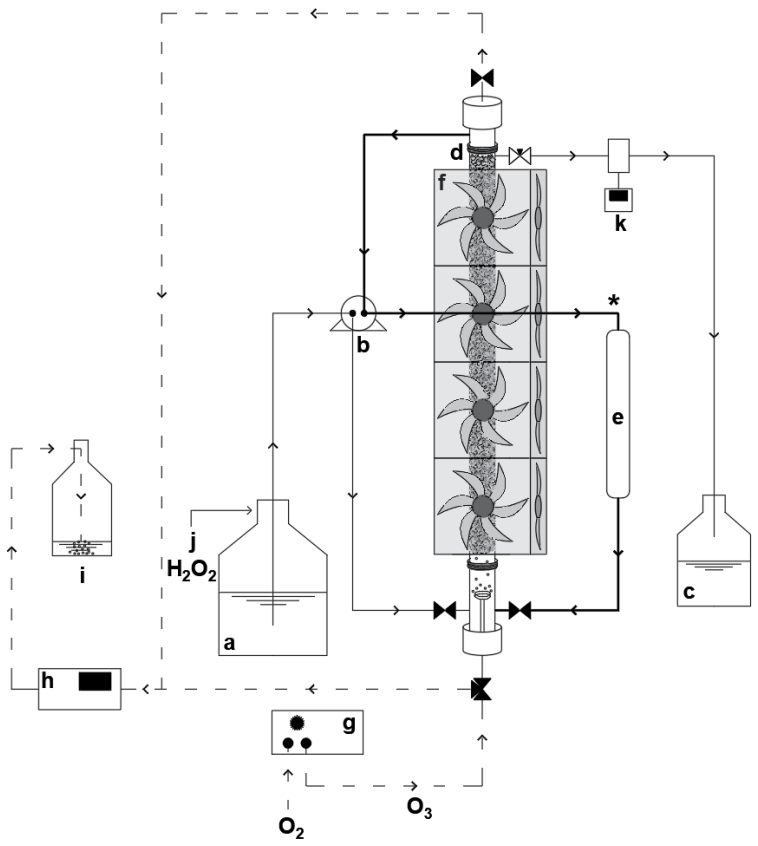

Figure 21. Schematic representation of the experimental set-up of the bubble column reactor operating in continuous mode: **(a)** inlet of SW; **(b)** peristaltic pump; **(c)** outlet of SW; **(d)** glass column, packed with glass Raschig rings; **(e)** loop column*; **(f)** UVA-LEDs; **(g)** ozone generator; **(h)** O_3 analyser; **(i)** gas washing bottles; **(j)** H_2O_2 addition; and **(k)** Q45H/64O_3 probe. **Bold line*: loop used in screening experiments of different treatment processes *(Section 5.3.2)* and not employed in those experiments conducted at milder operating conditions *(Section 5.3.3).*

In a typical run, the reactor was initially filled with distilled water. When spiked SW starts to be pumped into the reactor (t_0), a dilution effect is observed until steady-state conditions are achieved. Considering our previous studies conducted in the same installation – one focused on photocatalytic ozonation of SW collected in the same location [7] and another dealing with O_3/H_2O_2 treatment of urban WW and where different operating conditions were studied [41] – the liquid

phase (SW) was pumped at 15 mL min^{-1} corresponding to an HRT of 41 min and a steady-state time (t_s) of 180 min. It is worthy to note that, for each type of treatment, the spiked SW was pumped through the reactor and once t_s was achieved, the treatment started (t_1) by bubbling O_3, switching on the LEDs (O_3/UVA-LEDs) or adding H_2O_2 (O_3/H_2O_2), according to the treatment under study. After t_1, another t_s of 180 min was considered until collection of the final samples (t_2, triplicates of 500 mL, meaning t_{2a}, t_{2b}, t_{2c}). For H_2O_2-based experiments, 5 mL of a fresh catalase aqueous solution (0.1 g L^{-1}) was added to each sample to quench any residual oxidant, as described elsewhere [42]. Each experiment was performed in triplicate and the efficiency was estimated considering the average of MPs concentration abatement between t_1 (before treatment) and $t_{2(a, b, c)}$ (three samples collected after treatment, in steady state). Based on previous works [7, 42], all these screening experiments were performed using a loop column (Fig. 21e) (details in *Section 5.3.2*). After selecting the best performing treatment, and in order to mimic a real single passage bubble column reactor, this loop column/recirculation (bold line, Fig. 21) was not employed in a second set of experiments performed under milder conditions (details in *Section 5.3.3*).

5.2.4. Chemical analysis

A SPE-UHPLC-MS/ MS analytical method was used to determine the concentration of the target MPs in SW samples (including spiked and non-spiked SW) before and after treatment. The SPE protocol was performed as described elsewhere [43]. Briefly, the SW samples (500 mL) were filtered through 1.2 µm glass-fiber filters (47 mm GF/C, WhatmanTM, UK) and acidified to pH 3 with sulphuric acid. Oasis® HLB cartridges were used to pre-clean and extract the OMPs from the samples in a manifold provided by Waters (Milford, USA) and a Labconco® CentriVap (Kansas City, USA) was applied to dry the extracts. Ethanol was used as conditioning and eluting solvent of the cartridges (4 mL) and as restorative solvent of the dried sample extracts (250 µL). For chromatographic analysis, a Shimadzu Corporation apparatus (Tokyo, Japan) with LC (UHPLC, Nexera) and MS/MS detection (triple quadrupole mass spectrometer detector, LCMS-8040) was used. A KinetexTM 1.7 µm XB-C18 100 Å column (100 × 2.1 mm i.d.) supplied by Phenomenex,

Inc. (California, USA) was employed and the mobile phase (at a flow rate of 0.2 mL min^{-1}) consisted of UPW and acetonitrile acidified with 0.1% formic acid, operating at gradient mode (15% of organic phase during 3 min, a linear gradient during 18 min up to 85%, which was kept during 2 min, after which the initial conditions were set again in 0.1 min to condition the column during 9.9 min). Column oven and autosampler temperatures were set at 40 ºC and 4 ºC, respectively. The volume of injection was 5 µL. Capillary voltage, drying gas flow, nebulizing gas flow, desolvation and source temperature of the mass spectrometer were 4.5 kV, 12.5 dm^3 min^{-1}, 2.5 dm^3 min^{-1}, 250 ºC and 400 ºC, respectively. The analysis was based on a triplicate matrix-matched calibrations curve, by adding selected amounts of each reference standard and a known and constant amount of internal standards. Retention time, range, MDLs and MQLs of the target analytes are available in Table D3.3. (Appendix D, Supplementary Material).

A TOC-L analyser (Shimadzu Scientific Instruments, Japan) was used to determine the DOC content, following procedure 5310B of the Standard Methods for Examination of Water and Wastewater [44]. The concentration of cations (sodium, potassium, ammonium, calcium, magnesium) and anions (chloride, bromide, bromate, nitrite, nitrate, phosphate, sulphate) in solution were respectively determined by ionic chromatography using Metrosep C4 Cationic Exchange and Metrosep A Supp 7 Anionic Exchange columns (250 mm × 4.0 mm) in a Metrohm 881 Compact IC Pro equipment. The pH values were measured using a pH meter pHenomenal® pH 1100 L (VWR, Germany).

5.2.5. Microbiological assays

Microbiological analyses were performed considering the guidelines of the Portuguese legislation regarding water quality for human consumption [45] (transposing the Directive 2015/1787/EU [9]). Two independently collected SW samples were analysed (i) before, (ii) immediately after treatment, and (iii) after three days of storage in the dark at room temperature (for regrowth assays). Total heterotrophs, *E. coli* and enterococci were isolated and enumerated by the membrane filtration method, following EN ISO 6222, EN ISO 9308-1, and EN ISO 7899–2, respectively [9]. Adequate

serial dilutions of each sample were prepared in sterile saline solution (0.85% NaCl). Volumes ranging from 100 mL to 1 mL of samples or dilutions thereof were filtered in duplicate, through cellulose nitrate membranes (0.22 µm pore size; Millipore, UK), which were further placed onto the culture medium and incubated according to the bacterial group: PCA for total heterotrophs (37 ºC, 2 days, and 22 ºC, 3 days); CCA for E. coli (37 ºC, 1 day); and SB for enterococci (37 ºC, 1 day). Oxidase test with 1% TMPD and incubation in BEA at 44.0 ± 0.5 ◦C for 4 h was used to confirm *E. coli* (oxidase negative) and enterococci (blackening of BEA), respectively.

5.3. Results and discussion

5.3.1. Occurrence of target MPs in SW samples supplying a DWTP

Considering all collected samples (n = 5), 15 out of 21 target MPs were detected at ng L^{-1} levels at different frequencies (Fig. 22), namely (i) 5 PSs of EU Directive 2013/39 (alachlor, atrazine, isoproturon, PFOS, simazine) [33]; (ii) 5 CECs included in the watch lists of EU Decisions 2015/495 (azithromycin, clarithromycin, thiacloprid, and diclofenac) [34], 2018/840 (azithromycin, clarithromycin, thiacloprid) [35], and 2020/1161 (venlafaxine) [36]; and (iii) 5 other CECs (carbamazepine, clindamycin, diphenhydramine, fluoxetine, tramadol). Clothianidin, methiocarb, metoprolol, norfluoxetine, propranolol, and thiamethoxam, were never detected in these samples (Fig. 22).

Most of the MPs found in SW are pharmaceuticals (azithromycin, clarithromycin, carbamazepine, clindamycin, diclofenac, diphenhydramine, fluoxetine, tramadol, venlafaxine), and their occurrence is probably related to their wide usage in medicine and ineffective elimination by conventional treatment of urban WW. In particular, the highest concentrations were observed for carbamazepine and tramadol (12.6 and 20.8 ng L^{-1}, respectively), whereas the lowest concentration was registered for fluoxetine (< 0.11 ng L^{-1}). A similar concentration was reported for tramadol (17 ng L^{-1}) in another

study performed in the same river [46]. Carbamazepine was detected at a lower concentration (1 ng L^{-1}) in areas nearby the sampling site of the present study [47].

Other detected MPs were pesticides (alachlor, atrazine, isoproturon, simazine, and thiacloprid), and their occurrence can be related to agriculture runoff. Atrazine (already banned) and isoproturon were always detected, the highest concentrations of this group of substances being found for isoproturon (between 3.2 and 9.2 ng L^{-1}), but still below the limit of 0.1 µg L^{-1} set for pesticides in EU legislation on the quality of water intended for human consumption [48]. Likewise, the sum of all pesticides concentrations was also below the maximum admissible (0.5 µg L^{-1}) by the same regulation [48].

The industrial compound PFOS was quantified up to 12.0 ng L^{-1}, and it has been detected in several watercourses due to its wide commercial application during the past sixty years, high solubility and recalcitrance to WW treatment [49, 50].

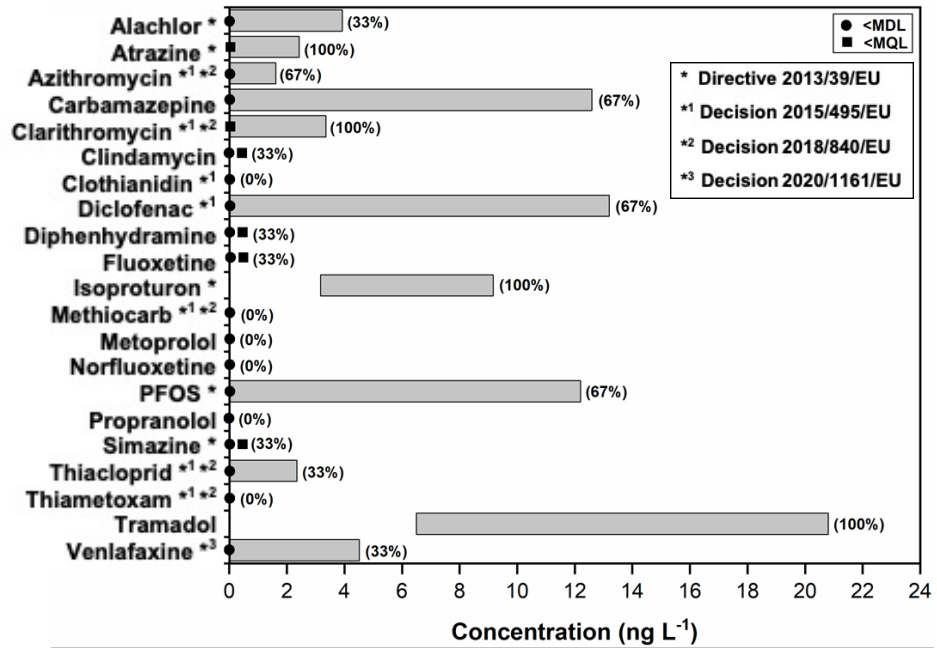

Figure 22. Concentrations range (ng L^{-1}) and detection frequency (%) of each target MP in SW collected during the sampling campaign.

Overall, all the PSs included in the present work were detected during the sampling campaign, highlighting the concern about the possible effects of these substances in the aquatic environment. However, the concentrations of these MPs were always below the Maximum Allowable Concentration – EQS (MAC-EQS) set in Directive 2013/39/EU [33]. Moreover, the detection of 10 out of 16 CECs, 5 of which already flagged in EU Decisions, urges the importance of monitoring studies as well as risk assessment for these substances that are still not included as PSs with EQS in any regulation. Considering the high variability of MPs occurrence in SW (*i.e.,* susceptible to the quality and volume of the discharged urban WW, weather conditions, population habits, among others), spiked SW was used in the following studies simulating a worst-case scenario where all target MPs would be present. The spiking was conducted with a known concentration (100 ng L^{-1}) of each of the 21 target MPs, this concentration being selected taking into account the range of concentrations typically reported for MPs in monitoring studies of SW [51].

5.3.2. Degradation of MPs: selection of the best performing treatment

The removal rates of MPs for each studied treatment (after reaching steady state, t_2), as well as the initial concentrations of analytes at t_1, are shown in Fig. 23. Slight deviations from the original spiked concentration (100 ng L^{-1}) were registered for some compounds. These deviations are mainly attributed to matrix effects and to the inherent occurrence of these MPs in natural SW. As expected, low removals (< 10%) were obtained in the control experiment with UVA-LEDs (Fig. 23a). In contrast, very high removal efficiencies (> 85%) for most of the studied MPs were obtained with the O_3-based treatments (Fig. 23b–d). PFOS was the exception and the most recalcitrant MP regardless of the O_3-based treatment applied.

It is well known that UVC lamps are more effective to promote the direct photolysis of organic compounds than UVA and UVB lamps [52-54]. However, traditional UV mercury lamps are characterized by a short lifetime, low energy conversion efficiency and high pollution potential. For this reason, UV-LEDs have gained increasing interest presenting short warm-up time, no mercury

disposal issues, stability and easy handling, operation at low voltage, and low power requirement [54]. LEDs have been investigated to remove organic pollutants from water matrices, but high-intensity UVC-LEDs are still very expensive and only considered in few studies [55, 56]. Looking for a less costly alternative, UVA-LEDs have been more often employed [57, 58]. Photosensitizers contained in waters (*e.g.*, humic substances dissolved in natural organic matter and inorganic species like nitrates) can generate highly reactive species, leading to indirect photochemical oxidation of MPs, especially those for which indirect photolysis is the main reactive pathway [52, 59]. Nonetheless, this phenomenon had no considerable impact for the SW under treatment, as can be observed by the low degradation of the MPs in the control experiment with UVA-LEDs (Fig. 23a) and by comparison of the results obtained with O_3/UVA-LEDs and O_3 (Fig. 23b and c). Indirect photolysis of MPs is very dependent on the water matrix, high removal efficiencies of some MPs being reported by photolysis in other studies when using UVA-LEDs and urban WW as matrix [59].

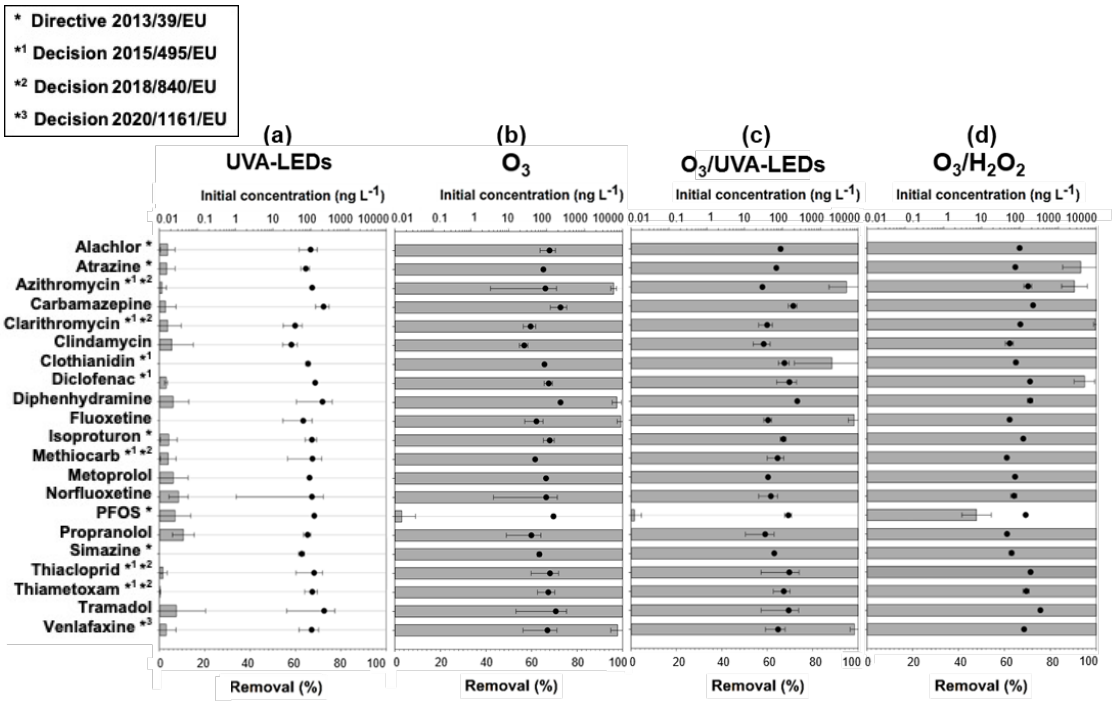

Figure 23. Removal of MPs in continuous mode with loop (simulating a perfectly mixed reactor): **(a)** UVA-LEDs (control), **(b)** O_3, **(c)** O_3/UVA-LEDs and **(d)** O_3/H_2O_2. Experimental conditions: spiked SW (100 ng L^{-1} of each MP), HRT: 41 min, t_s: 180 min, O_3 in excess: 50 g Nm^{-3} in gas phase, and pH: 8.5.

Regarding the O_3-based experiments (O_3, O_3/UVA-LEDs and O_3/H_2O_2), 20 out of 21 MPs were removed to levels below the MQL, regardless of the applied treatment, PFOS being the exception. Nonetheless, it is important to bear in mind that the high or partial degradation of MPs may originate by-products that can be more or equally toxic. Depending on the pH, ozone will oxidize the organic molecules directly (more acidic pHs) or indirectly (more alkaline pHs). The latter occurs when molecular ozone is decomposed into $HO^{\bullet}$ or other reactive species such as $O_2^{\bullet-}$, $HO_2^{\bullet}$, and $O_3^{\bullet-}$ radicals [60]. Considering the treatments and the pH of the SW under study (8.5), the indirect mechanism is the main expected degradation pathway. O_3/H_2O_2 (or peroxone) may accelerate the formation of reactive species, for instance by the reaction of the H_2O_2 conjugate base (H_2O^-) with O_3 generating HO• [41, 60]. However, O_3 alone was enough to remove (> 85%) 20 MPs under study, and the removal of PFOS was below 50% even when H_2O_2 was added. Therefore, SW polluted with ng L^{-1} levels of MPs can be treated with single ozonation. In fact, even O_3-resistant compounds (*e.g.*, atrazine > simazine > metoprolol > isoproturon > clarithromycin), with kinetic rate constants with O_3 (k_{O3}) lower than 10^5 L mol^{-1} s^{-1}, can be removed by ozonation because the other highly reactive species referred above are generated with this process, in particular at more alkaline pH values such as 8.5 [22, 61]. The recalcitrance of PFOS towards ozonation is due to its strong carbon–fluorine bonds (Table D3.1, Appendix D) that make this compound a very stable and resistant pollutant. It was demonstrated that ozonation at pH 11 can increase the removal of PFOS (43-85%: 4 h treatment), as well as the application of O_3/H_2O_2 at the same high pH (>99%: 4 h treatment) [62]; however, an undesirable pH increase from 8.5 to 11 is needed in both cases. In fact, a recent review on advanced oxidation/reduction processes for the treatment of PFOS [63] identifies this substance as recalcitrant to O_3 (removals < 2% in SW) and describes reductive photochemical/ photocatalytic processes (mainly mediated by hydrated electrons in absence of O_2) as more efficient to eliminate PFOS.

Beyond the chemical properties of the compounds, the water matrix can also affect the degradation of the MPs due to the presence of scavengers. However, the high efficiencies obtained suggest that the matrix does not have a noticeable impact in this case, most likely due to low DOC content of SW (3.5 mg L^{-1}, Table D3.2, Appendix D). The efficiency of O_3-based processes has been

extensively reported in the literature for WW treatment; however, there are much less studies for SW, in particular when considering multi-class MPs and treatment under more realistic conditions [23, 24, 64]. One of the major drawbacks of the ozonation treatment of real matrices is the common incomplete mineralization of the organic compounds. In this context, the formation of bromate by the reaction of bromide present in natural water with O_3 and OH• represents a concern, a limit of 10 µg BrO_3^- L^{-1} being defined for water intended for human consumption [24]. This anion was never detected in the present study after O_3 treatment (< 0.076 mg L^{-1}, Table D3.2, Appendix D); however, the high limits of detection hampered to conclude about the acceptability of bromate concentration after ozonation. Nonetheless, depending on each water supply and characteristics (mainly in terms of bromide concentration), as well as treatment train in the plant, there are different approaches that can minimise the formation of bromate during ozonation, namely lowering the pH, adding ammonia, lowering the ozone exposure, and using chlorine-ammonia or peroxone processes (depending largely on the dose (and point) of H_2O_2 addition in comparison with that of O_3) [65-67]. Therefore, single ozonation was selected to be applied in the next set of experiments, for water disinfection and removal of MPs under mild operating conditions.

5.3.3. Water disinfection and degradation of MPs: testing mild operating conditions

This set of experiments was performed with a single passage of the liquid into the column, *i.e.,* mimicking a more realistic ozonation bubble column, instead of recirculating a certain fraction of the liquid (Fig. 21). Adjustments on the HRT and O_3 dose were also performed in relation to the first set of experiments (*Section 5.3.2*), to implement mild operating conditions. Thus, a higher liquid flow rate of 33 mL min^{-1} was selected, resulting in an HRT of 10 min (corresponding to a t_s of 25 min), which is an O_3 contact time usually applied at full-scale DWTPs [11]. The aim was to find a low O_3 concentration that guarantees the removal of DW EU targeted bacteria, while attaining high removals of MPs (> 85%). Since the MPs represent only a small part of the total DOC content

of the SW matrix, and numerous water matrix constituents might act as scavengers of highly reactive species [68], the O_3 dose should be selected considering the DOC content of SW as expressed by the specific ozone dose parameter (g O_3 g^{-1} DOC). In this way, the impact of the matrix is taken into account on the process efficiency, allowing more reliable comparisons between experiments using different SW collected at a different time and/or location [24, 68-70].

5.3.3.1 Water disinfection

The ozonation efficiency regarding the inactivation of the microbial groups listed in national and European legislation [9, 45] was investigated for an HRT of 10 min and O_3 concentrations of 1.5 mg L^{-1} (~ 0.3 g O_3 g^{-1} DOC) and 3 mg L^{-1} (~ 0.7 g O_3 g^{-1} DOC). Information found in the literature describes O_3 doses applied in DWTPs between 1.5 and 3 mg L^{-1} for disinfection purposes [71, 72] and, for this reason, those were selected in the present work. The number of colony forming units (log CFU 100 mL^{-1}) of total heterotrophs (at 37 and 22 ºC), *E. coli* and enterococci were determined (i) before, (ii) immediately after treatment, and (iii) after 3-days storage (regrowth) in the dark and at room temperature (Fig. 24). In contrast to the experiments dealing only with the removal of MPs, biological assays were not undertaken with spiked bacteria in water since the preliminary analyses showed the presence of the target bacteria within quantifiable levels.

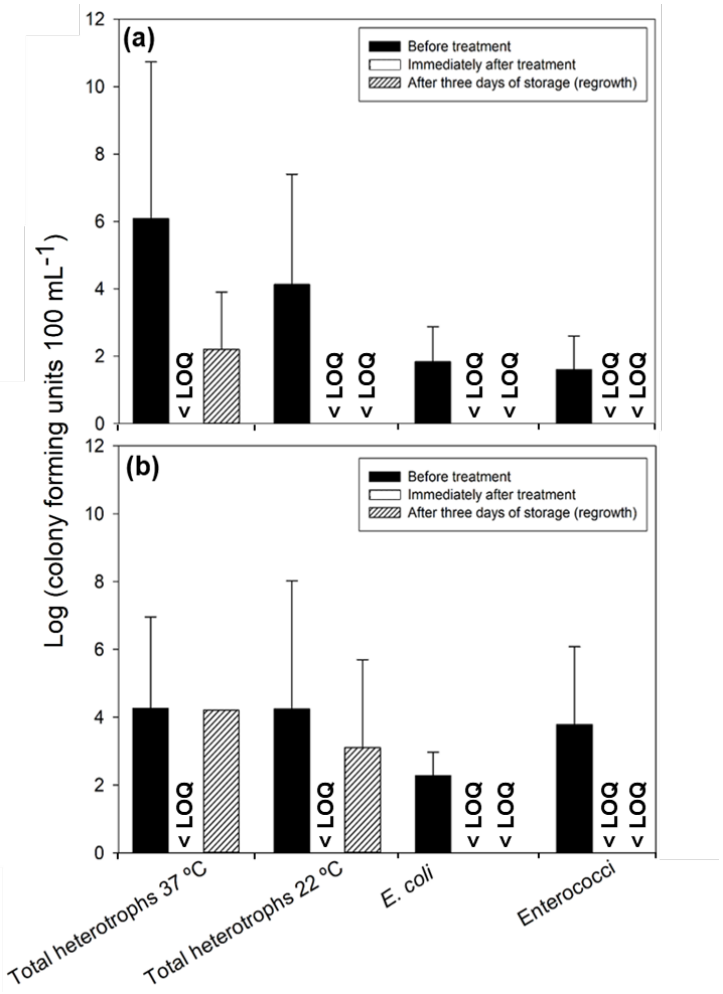

Figure 24. Microbial analyses (total heterotrophs at 37 and 22 ºC, *E. coli* and enterococci) in SW (i) before, (ii) immediately after ozonation treatment operating in continuous mode (HRT: 10 min), and (iii) after 3-days storage (regrowth) in the dark and at room temperature (LOQ: 0.5 CFU 100 mL^{-1}). Applied O_3 doses: **(a)** 3 mg O_3 L^{-1} and **(b)** 1.5 mg O_3 L^{-1}.

Inactivation of all targeted bacteria to values lower than the LOQ (0.5 CFU 100 mL^{-1}) was observed immediately after treatment for both dissolved O_3 concentrations tested (Fig. 24). In the case of the higher O_3 dose (Fig. 24a), regrowth did not occur for total heterotrophs at 22 ºC, *E. coli* and enterococci. In contrast, regrowth was observed after 3-days storage for total heterotrophs at

37 ºC, quantified at approximately 10^2 CFU 100 mL^{-1} (*i.e.,* 1 CFU mL^{-1}), but this value is below the maximum recommendable of 20 CFU mL^{-1} for the quality of water intended for human consumption [9, 45]. These results proved the effectiveness of ozonation to inactivate these regulated microbial groups using 3 mgO_3 L^{-1} and 10 min of HRT.

When half of the dissolved O_3 concentration (1.5 mg L^{-1}) was used (Fig. 24b), all microbial groups were again inactivated immediately after treatment. Total heterotrophs reactivated after 3-days storage, but in this case at both incubation temperatures (22 and 37 ºC). Moreover, although the quantification of total heterotrophs incubated at 22 ºC did not exceed the limit recommended (100 CFU mL^{-1}) [9, 45], the same did not happen when incubated at 37 ºC, resulting in values around 10^2 CFU mL^{-1}, *i.e.,* five times higher than those advisable (20 CFU mL^{-1}) [9, 45]. Although the addition of H_2O_2 together with low O_3 doses (1.5 mg L^{-1}) could be an interesting option in comparison with the application of O_3 alone at higher concentration (3 mg L^{-1}), studies have described O_3 alone as a preferential bactericide to peroxone due to the higher ozone residuals produced [73-76]. Moreover, in one of our previous studies dealing with ozonation for the treatment of urban WW [41], significant differences in terms of *E. coli* inactivation were not observed when H_2O_2 was added to ozonation, these results being in agreement with those obtained for the degradation of MPs in the same study, and also with those herein reported. Indeed, the microbial inactivation immediately after treatment does not represent a definitive removal since the available sources of carbon resulting from the partial oxidation of organic matter can still promote their reactivation, as already verified for total heterotrophs in a previous work [37]. Total heterotrophs cover a great diversity of microorganisms, which may justify their predominance after storage as already demonstrated in our previous study [7]. Thus, this group of bacteria proved to be a determinant indicator to select the best ozone dose for water disinfection. In the present study, an O_3 concentration of 3 mg L^{-1} ensured the water disinfection and microbiological quality of water after treatment and storage for at least 3 days (2–6 log reduction). These conditions correspond to a cumulative disinfectant over time (*Ct*) value of 30 mg O_3 min L^{-1} (*i.e.,* 3 mg O_3 L^{-1} × HRT of 10min). This parameter has been widely used for water disinfection [77, 78].

5.3.3.2 Degradation of organic MPs

The degradation of the selected MPs spiked in SW samples was performed under mild operating conditions (Fig. 25), *i.e.,* employing HRT of 10 min and a dissolved O_3 concentration of 3 mg L^{-1}, corresponding to specific ozone doses between 0.7–1.3 g O_3 g^{-1} DOC, depending on the DOC of collected SW. The idea was to remove these MPs while ensuring water disinfection, *i.e.,* bacteria inactivation as defined in *Section 5.3.3.1*. Once again, removals higher than 85% were registered for all target MPs, except for the recalcitrant PFOS (Fig. 25). Thus, these milder operating conditions (HRT of 10 min and 3 mg O_3 L^{-1}) can be employed for both water disinfection and removal of organic MPs. It should be mentioned that 1.5 mg O_3 L^{-1} also resulted in similar removals of organic MPs (results not shown); however, as described above, disinfection was not guaranteed.

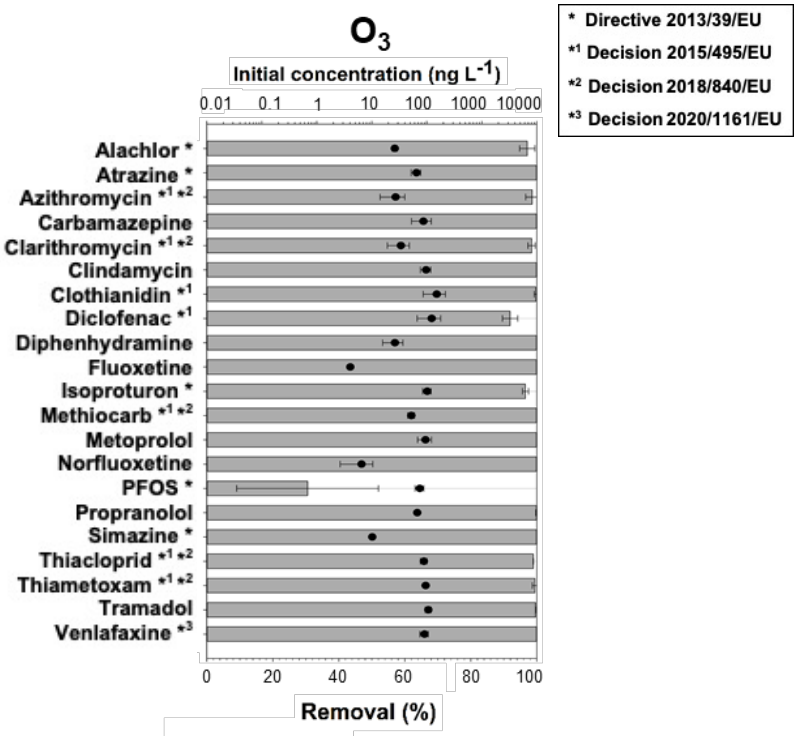

Figure 25. Removal of organic MPs by continuous ozonation without loop. Experimental conditions: spiked MPs in SW (100 ng L^{-1} of each MP), HRT: 10 min, t_s: 25 min, O_3: 3 mg O_3 L^{-1} in liquid phase, and pH: 8.1.

It is important to refer that the specific O_3 dose normalized with respect to DOC is a good parameter suggested in the literature to decide about the proper conditions to achieve the degradation of MPs by ozonation. However, as demonstrated in the present work, the O_3 dose must be also selected by taking into account the microbiological constitution of the SW that will be treated, *i.e.,* applying the minimum dissolved O_3 concentration able to inactivate bacteria to levels complying the regulation for water consumption [45]. Thus, the O_3 dose must be selected considering both DOC and microbial inactivation (rather than MPs only). Although ozonation has been applied in DWTPs for disinfection, and some studies have been published exclusively dealing with the removal of MPs in these facilities, this is the first report dealing with single ozonation for the simultaneous removal of both chemical and biological contaminants from SW. Moreover, bacteria regrowth in the treated water was investigated to assess the efficiency of the treatment process if storage, before its use, is envisaged. This work was conducted having as target the compliance with national and EU regulations on water for human consumption. In this context, further studies are needed to evaluate the simultaneous removal of MPs and biological contaminants by single ozonation at full-scale DWTPs, while performing toxicity bioassays (*e.g.*, mutagenicity, genotoxicity, and oxidative stress) to assess if complementary treatments are needed.

5.4. Conclusions

Five PSs of the EU Directive 2013/39 (alachlor, atrazine, isoproturon, PFOS, simazine), 5 CECs listed in EU Decisions (azithromycin, clarithromycin, diclofenac, thiacloprid and venlafaxine) and other 5 MPs of particular interest in the context of water policy (carbamazepine, clindamycin, diphenhydramine, fluoxetine, tramadol) were detected at low concentrations (< 20 ng L^{-1}) in SW supplying a DWTP in Northern Portugal. Among the studied treatment technologies (O_3, O_3/UVA-LEDs and O_3/H_2O_2), single ozonation was considered the best solution, because it is the simplest option and it was effective for removing (> 85%) most of the MPs (except PFOS) from spiked SW without the generation of bromates. Regardless of the O_3-based process applied, PFOS was always the most recalcitrant compound, with lower removals (< 50%). Moreover, single ozonation

using a low amount of O_3 (3 mg L^{-1}) with HRT of 10 min proved to be efficient for both removal of MPs (> 85%, except PFOS) and disinfection. *E. coli*, enterococci, and total heterotrophic bacteria complied the established limits for potable water immediately after treatment and after 3 days of storage, showing bacteria inactivation and regrowth prevention. Lower O_3 doses are not recommended because reactivation of total heterotrophs at 37 ºC after 3 days of storage of treated water showed bacterial values (10^2 CFU mL^{-1}) above those recommended for the quality of water intended for human consumption (20 CFU mL^{-1}).

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Chapter

6

Antibiotics removal from aquaculture effluents by ozonation: chemical and toxicity descriptors

Antibiotics are often applied in aquaculture to prevent fish diseases and they can cause disturbances on receiving waters, when not properly eliminated from the aquaculture effluents. In the previous chapter, continuous-flow mode ozonation proved to be a very efficient technology to eliminate a large number of MPs from SW. Therefore, studies with O_3 technology were extended towards the elimination of a specific group of antibiotics widely used in aquaculture, namely, florfenicol (FF), oxytetracycline (OTC), sulfadimethoxine (SDM), sulfamethoxazole (SMX), and trimethoprim (TMP). Moreover, the toxicity potential of the O_3 treatment was assessed by zebrafish bioassays to predict eventual risks of its application.

First, batch experiments were performed using ultrapure water (UPW) and aquaculture effluents spiked with a mixture of the target antibiotics at relatively high concentrations (10 mg L^{-1} each). OTC, SMX and TMP were fully removed (< 30 min) regardless of the tested conditions, mainly by O_3 direct attack. In contrast, FF was only removed in the presence of hydroxyl radicals (HO•), with the removal extent being highly dependent on the matrix under treatment. SDM degradation was also influenced by the water matrix, but its removal was always higher than 68%. Then, continuous-flow experiments were conducted applying more environmentally relevant antibiotic concentrations (100 ng L^{-1} each) and milder operation conditions, more specifically those that led to excellent MPs removals in *Chapter 5* (*i.e.*, 1.5 mg O_3 L^{-1} and an HRT of 10 min). In this case, all tested antibiotics were highly removed from aquaculture effluents (> 98%), except FF (68%). Although by-products were detected in treated samples, zebrafish (*Danio rerio*) embryotoxicity tests did not show increased toxicity by applying this ozonation treatment. Ozonation is thus a possible solution to remove antibiotics from aquaculture effluents. Nonetheless, full-scale studies in aquaculture farms are needed, and generation of $HO^{\bullet}$ may be favoured to oxidize the FF antibiotic readily.

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6.1. Introduction

In the last few years, the global fish consumption has increased more than that of any other animal protein foods (*e.g.*, meat, dairy, and milk), and aquaculture is one of the fastest-growing food-producing sectors [1]. Intensive aquaculture typically involves many cultured organisms in confined areas, which can promote the dissemination of parasites and bacterial infections and, consequently, considerable economic losses for the sector [2, 3]. In this context, to tackle infections and their undesirable effects, antibiotics such as tetracyclines, trimethoprim (TMP), sulfonamides, quinolones, β-lactams, fluoroquinolones, and phenicols have been widely supplied in aquaculture [4].

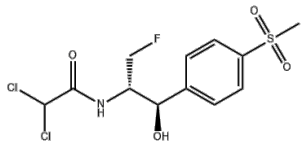
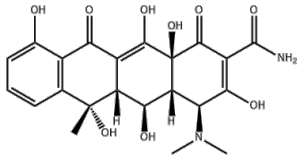
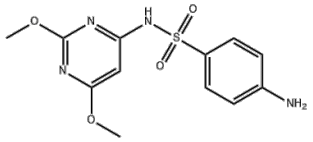
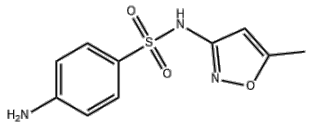
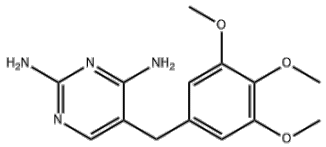
The excessive application of antibiotics in aquaculture can bring serious threats to the environment and human health, such as their spread in the different environmental compartments and the possible proliferation of ARB & ARGs, which is one of the main challenges that humans face today [5-7]. Indeed, high levels of ARB have been described in, and around, aquaculture farms [8]. Antibiotics are generally added directly to the water through food pellets, and thus, both the unconsumed fraction of antibiotics and the excreta of farming organisms end up in outlet waters [9-11]. Moreover, water treatment equipment installed in aquaculture farms is not commonly adequate for removing antibiotics, which are highly stable [12, 13].

One alternative process that has been used to improve water quality in aquaculture is ozonation. High removal rates of pathogens, organic matter, solids, colour, and inorganic species can be achieved with this treatment [14-16]. However, specific information regarding antibiotics removal in aquaculture is still very scarce – only 2 studies were found in the Scopus database using the keywords “ozone/ozonation”, “antibiotics” and “aquaculture” [8, 17]. In these 2 studies, O_3 significantly removed the fishery antibiotics amoxicillin [8], oxytetracycline (OTC) [8] and oxolinic acid [8, 17], but florfenicol (FF) was very refractory to the treatment [8, 17]. One of the main drawbacks of ozonation is the formation of oxidation by-products that might have toxicological effects similar or even higher than those of parent compounds [18]. For instance, bromate ion is a known ozonation by-product of toxicological concern originated from O_3 reaction with bromide

naturally found in water matrices [13, 19]. Thus, both physico-chemical and toxicological criteria are crucial to conclude about ozonation efficiency and quality of treated water [18, 20]. Specifically for aquaculture, guaranteeing the quality of ozonated effluents is essential when these effluents are discharged into the environment or recirculated to feed the aquaculture facilities. Despite that, studies involving aquaculture effluents are only focused on antibiotics degradation without assessment of toxicity and/or general quality of the treated effluent [8, 17], as is also the case of many other studies dealing with ozonation for the removal of antibiotics from different water matrices.

In this work, the possibility of applying ozonation to aquaculture effluents aiming at the removal of different antibiotics commonly used in these farms (FF, OTC, sulfadimethoxine (SDM), sulfamethoxazole (SMX), and TMP; Table 6) was investigated. Firstly, the degradation of target compounds was studied in batch mode, where different parameters such as the role of $HO^{\bullet}$ and water matrix were evaluated. Afterwards, the removal of target antibiotics was also assessed in a continuous-flow mode for which more realistic conditions were applied (environmentally relevant antibiotic concentrations, low O_3 concentration and short HRT). The potential toxicity effects of the continuous ozonated effluents were then estimated using zebrafish embryo assays.

Table 6. Target antibiotics and their properties: class, molecular structure, and reaction rate kinetic constants with O_3 (k_{O3}) and $HO^{\bullet}$ ($k_{HO\bullet}$) determined for each compound individually in UPW/deionized water.

Antibiotic Class	Compound	Molecular Structure	k_{O3} ($M^{-1}\,s^{-1}$)	$k_{HO\bullet}$ ($10^{9}\,M^{-1}\,s^{-1}$)
Phenicols	Florfenicol (FF)		< 10 [8]	-
Tetracyclines	Oxytetracycline (OTC)		7×10^{5} [21]***	5.6 [22]**,+
Sulfonamides	Sulfadimethoxine (SDM)		2.7×10^{6} [23]*,+	6.1 [24]
	Sulfamethoxazole (SMX)		2.5×10^{6} [25]*,+	5.5 [25]*,+
Others	Trimethoprim (TMP)		2.7×10^{5} [26]+	6.9 [26]+

* 20 ºC; + pH 7; ** 22 ºC; *** pH 9, 20-21 ºC;

6.2. Materials and methods

6.2.1. Chemicals and materials

All reference antibiotic standards (FF, OTC, SDM, SMX, and TMP), as well as the internal standard SDM-*d6*, were purchased from Sigma-Aldrich (Steinheim, Germany). Individual stock solutions of

approximately 1000 mg L^{-1} were prepared by dissolving the appropriate mass of each antibiotic or internal standard in methanol, which were then diluted to 10 and 1 mg L^{-1} for mass spectrometry analysis optimization. A working solution containing all antibiotic standards (2.5 mg L^{-1}) was also prepared by dilution in methanol, which was used to spike the aqueous matrix for the ozonation experiments. Likewise, a working solution of SDM-*d6* (5 mg L^{-1}) was prepared to be added to each sample before the SPE procedure. Methanol and acetonitrile MS grade were purchased from VWR International (Oregon, USA), whereas formic acid and sulphuric acid were supplied by Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-$Na_2 \cdot 2H_2O$) was acquired from PanReac (Castellar del Vallès, Barcelona) and ammonium formate (> 97% purity) was supplied by Honeywell Fluka (North Carolina, USA). 3,4-dichloroaniline (98%) was obtained from Alfa Aesar (Kandel, Germany). UPW (resistivity > 18.2 MΩ cm at 25 °C) was supplied by a Milli-Q water system from Millipore (Massachusetts, USA). Dechlorinated water used in zebrafish embryo assays was obtained by a reverse osmosis system (LSRO-101-A Lan Shan, Changhua County, Taiwan).

6.2.2. Aquaculture water samples

Aquaculture effluents were collected in a trout farm located in Portugal. After sampling, effluents were stored in pre-rinsed amber glass bottles, transported at 4 °C to the laboratory and kept refrigerated until use. Moreover, collected effluents were characterised in terms of (i) general physico-chemical parameters (*i.e.,* pH, DOC, chemical (COD) and biochemical (BOD) oxygen demand, total suspended (TSS) and dissolved (TDS) solids, and several inorganic ions: ammonium, bromate, bromide, calcium, chloride, magnesium, nitrate, nitrite, phosphate, potassium, sodium, and sulphate) (Table D4.1, Appendix D, Supplementary Material), (ii) concentrations of the selected antibiotics (Table D4.2, Appendix D, Supplementary Material), and (iii) toxicity, before and after continuous-flow ozonation.

6.2.3. Ozonation experiments

6.2.3.1. Batch mode ozonation experiments

Batch experiments were performed in a lab-scale glass reactor filled with 500 mL of solutions (UPW or aquaculture effluents) spiked with the target antibiotics at 10 mg L^{-1} each. For spiking, 5 mg of each target compound were directly added to the matrices and subjected to sonication for 10 min. For $HO^{\bullet}$ scavenging experiments, apart from the antibiotics, a known amount of methanol (commonly used as radical scavenger) [26-28] was added (< 1%) to UPW. The reaction medium was always magnetically stirred at 400 rpm, approximately at 18 ºC. O_3 was produced from pure oxygen in a BMT 802X O_3 generator (BMT Messtechnik, Germany) at a constant flow rate (150 $Ncm^3\ min^{-1}$) and inlet concentration (~ 50 g Nm^{-3}), and the O_3 concentration in the gas phase was monitored with a BMT 964 ozone analyser (BMT Messtechnik, Germany). The O_3 leaving the reactor was removed using gas washing bottles filled with potassium iodide solution. 1 mL aliquots were collected periodically from the reactor (*i.e.,* 0, 1, 2, 5, 10, 12, 15, 17, 20, 30, 40, 45, and 60 min) for analysis of antibiotics concentrations. The collected samples were placed immediately in the fridge until analysis.

6.2.3.2. Continuous-flow mode ozonation experiments

Experiments with continuous-flow ozonation were performed in a bubble column reactor (3.0 cm internal diameter × 70 cm height) at a temperature of approximately 17 °C, applying conditions selected based on our previous study dealing with ozonation for the removal of other organic micropollutants from SW [18] and information regarding ozonation at full-scale aquaculture systems for water disinfection [29-31]. Briefly, for spiking the aquaculture effluents (100 ng L^{-1}), 400 µL of a methanolic solution containing all target antibiotics (2.5 mg L^{-1}) was added to an empty 10 L ISO bottle blue cap and the residual solvent was evaporated using a nitrogen flow. Afterwards, a 10 L aliquot of the aquaculture effluent was added into the bottle and stirred for 2 min. The spiked

effluent was continuously pumped at the bottom of the reactor with a peristaltic pump (Watson-Marlow, UK) and constantly collected at its top after the respective treatment (*i.e.*, one single passage in the column). The reactor was packed with glass Raschig rings (3 mm diameter × 3 mm length, resulting in a filling volume of 355 mL after packing), in this way promoting the gas-liquid O_3 mass transfer during the treatment [32]. The BMT 802X O_3 generator (BMT Messtechnik, Germany) was used to produce O_3 from pure oxygen at a constant flow rate (300 Ncm^3 min^{-1} in the gas phase) and inlet concentration (~ 20 g Nm^{-3}), which was fed to the reactor through a ceramic diffuser. O_3 in the gas phase was monitored by a BMT 964 ozone analyser (BMT Messtechnik, Germany), while dissolved O_3 was measured by a Q45H/64 probe (Analytical Technology, USA). The O_3 leaving the reactor was eliminated as described in the previous section. In a typical run, the reactor was filled with distilled water and when the spiked effluent starts to be pumped (t_0), a dilution effect is observed until the steady state (t_s), which was determined using sodium chloride as tracer [33]. At this point (*i.e.*, when the ts is achieved), a first sample of 1 L was collected (blank, t_1), and then the treatment began by bubbling O_3 to the spiked water fed into the reactor. After that, another period equivalent to that needed to achieve ts was considered before the next sampling of the ozonated effluent (t_2). Three 1 L samples were withdrawn for antibiotic concentrations analysis in each experiment (t_{2a}, t_{2b}, t_{2c}), whereas the remaining volume of treated water was stored in the fridge for toxicity tests and other physico-chemical analyses. The experiments were performed in triplicate, and the removal efficiency of each antibiotic was estimated considering its average concentration abatement between t_1 (without O_3) and $t_{2(a, b, c)}$ (with O_3) for the three independent ozonation experiments.

6.2.4. Quantification of antibiotics concentration

The selected antibiotics (FF, OTC, SDM, SMX and TMP) were analysed using liquid LC–MS/MS. Regarding continuous O_3 experiments (*i.e.*, with lower antibiotics concentration), SPE was needed prior to chromatographic analyses. For that, a SPE protocol was adapted from two previous studies

on the determination of veterinary antibiotics in aqueous matrices [34, 35]. In summary, samples of 1 L were filtered through 1.2 µm glass-fiber filters (47 mm GF/C, Whatman™, UK) and acidified to pH ~ 3 using sulphuric acid. Moreover, SDM-*d6* (20 µL) was added as internal standard and EDTA-$Na_2 \cdot 2H_2O$ (0.2 g) as complexing agent. The samples were then extracted by Oasis® HLB cartridges (150 mg, 6 mL) using an extraction manifold, both acquired from Waters (Milford, USA). After the total sample volume was loaded onto the cartridges (without preconditioning), they were rinsed with UPW (10 mL) and dried using a vacuum pump (ILMVAC GmbH, Germany) for 60 min. The dried cartridges were eluted with 6 mL of MeOH (3×2 mL), which were then evaporated in a Labconco® CentriVap (Kansas City, USA). Finally, the residues were re-suspended in 250 µL of a methanol/UPW mixture (40:60, v/v). The average recovery during SPE, accuracy, precision, and matrix effect for each target analyte are described in Table D4.3 (Appendix D, Supplementary Material).

A Shimadzu Corporation apparatus (Tokyo, Japan) with LC (UHPLC, Nexera) and triple quadrupole tandem mass (MS/MS) detection (LCMS-8040) was used for chromatographic analyses. Separation was achieved using a Kinetex ™ 1.7 µm F5 100 Å column (2.1 × 100 mm) supplied by Phenomenex, Inc. (California, USA), applying a mobile phase composed of water and methanol, both containing 0.1% of formic acid, at a flow rate of 0.2 mL min^{-1}. The chromatographic run was operated at gradient mode consisting of 27.5% of methanol during 4 min, a linear gradient during 6.5 min up to 50%, after which the initial conditions were set again in 0.5 min to condition the column during 5 min, totalizing a 16 min run. Column oven and autosampler temperatures were set at 25 °C and 4 °C, respectively. The volume of injection was 5 µL. The mass spectrometer was operated using ESI in PI or NI modes, and multiple reaction monitoring (MRM) was used to quantify the target compounds (selected reaction monitoring instrument parameters can be consulted in Table D4.4, Appendix D, Supplementary Material). Capillary voltage, drying gas flow rate, nebulizing gas flow rate, desolvation and source temperature of the mass spectrometer were 4.5 kV, 15 dm^3 min^{-1}, 3 dm^3 min^{-1}, 250 °C and 400 °C, respectively. The analysis was based on a triplicate matrix-matched calibrations curve, by adding selected amounts of each antibiotic standard solution and a constant amount of internal standard solution to 1 L of matrix. Retention time, range,

linearity, instrumental limits, and MDLs and MQLs of the target antibiotics are available in Table D4.5 (Appendix D, Supplementary Material).

6.2.5. Physico-chemical parameters determinations

A TOC-L analyser (Shimadzu Scientific Instruments, Japan) was used to determine the DOC content, following the procedure 5310B of the Standard Methods for Examination of Water and Wastewater [36]. COD, BOD, and TSS were measured following procedures 5220D, 5210B and 2540D, respectively [36]. The concentrations of cations (ammonium, calcium, magnesium, potassium, and sodium) and anions (bromide, chloride, nitrate, nitrite, phosphate, and sulphate) in solution were determined by ionic chromatography using Metrosep C4 Cationic Exchange and Metrosep A Supp 7 Anionic Exchange columns, respectively (250 mm × 4.0 mm) in a Metrohm 881 Compact IC Pro equipment. Bromate ions were determined by LC-MS/MS, using a Waters™ ACQUITY UPLC® BEH Amide 1.7 µm column (2.1 × 100 mm, Milford, USA), with a mobile phase of acetonitrile/10 mM ammonium formate (80/20), operating at isocratic mode. The pH values were measured using a pH meter pHenomenal® pH 1100 L (VWR, Oregon, USA).

6.2.6. Zebrafish embryotoxicity tests

Zebrafish (*Danio rerio*) breeders were maintained under standard culture conditions at CIIMAR (Matosinhos, Portugal) certified facilities for aquatic organisms. For reproduction, males and females were placed in a maternity inside a 30 L tank the day before reproduction. After reproduction, shortly after the beginning of light period, embryos (0-1 hpf (h post fertilisation)) were collected, cleaned, counted, and assessed for fertilization success (> 90%). The assays were carried out in 24 well plates (VWR, Oregon, USA). Ten embryos were placed in each well in a final volume of 2 mL of test solution. A total of 40 embryos (10 embryos/well) were exposed per test condition in each assay. Each assay plate was loaded with the test solutions for 24 h before the

embryo assays were done, to minimise contaminant losses from the test solutions to the media by adsorption to the plates. During the assays, the test solutions were completely renewed every day. Test solutions included: (i) aquaculture effluents as collected; (ii) aquaculture effluents spiked with FF, OTC, SDM, SMX and TMP (100 ng L^{-1} of each antibiotic); (iii) spiked aquaculture effluents after continuous ozonation treatment; (iv) dechlorinated water (negative control); and (v) 3,4-dichloroaniline solution at 4 mg L^{-1} (positive control). Control groups were used for quality control of the assay. Embryonic development was evaluated at 24, 48, 72, 96, 120 144 and 168 hpf using an inverted microscope (Nikon Eclipse TS100, Amsterdam, Netherlands). Mortality (24, 48, 72, 96, 120 144 and 168 hpf), hatching (24, 48, 72, 96, 120 hpf), malformation in the somites (24, 48, 72, 96 hpf), tail detachment (24, 48, 72, 96 hpf), yolk sac (24, 48, 72, 96, 120, 144 and 168 hpf), otoliths (48, 72, 96, 120, 144 and 168 hpf), eyes (48, 72, 96, 120, 144 and 168 hpf), heartbeat (48, 72, 96, 120, 144 and 168 hpf), blood circulation (48, 72, 96, 120, 144 and 168 hpf), skeleton (72, 96, 120, 144 and 168 hpf) and side-wise position (144 and 168 hpf) were the endpoints observed during the assays. Three independent repetitions of the assays were carried out to assess the potential embryotoxicity of the aquaculture effluent as collected and after treatment with continuous-flow ozonation, following the experiments described above (*Section 6.2.3.2*).

6.2.7. Statistical analysis

Removal of antibiotics by ozonation was compared by single-factor analysis of variance (ANOVA), followed by post-hoc Tukey's test using the software SPSS (Version 25.0 for Windows). The significance level was set to 0.05. In toxicity assays, mortality and hatching data are presented as cumulative frequency. The data were analysed using the Pearson Chi-square test, taking the duration of the exposure and the experimental conditions as factors. When significant differences were identified, pairwise comparisons were done using either the Chi-square test or the Fisher Exact test, with a Bonferroni correction, to identify their origin. The median time to hatching (HT_{50}) was estimated using the Probit method [37]. The statistical analyses were performed in SPSS 27, against a significance level of 0.05.

6.3. Results and discussion

6.3.1. Removal of antibiotics

6.3.1.1. Batch mode ozonation experiments

Fig. 26A shows the normalized concentrations of the target antibiotics spiked in UPW (10 mg L^{-1} of each antibiotic) in the ozonation experiments (60 min; ~ 50 g Nm^{-3}; pH 6). Among the five tested antibiotics (FF, OTC, SDM, SMX and TMP), only SDM was not completely removed in 60 min (a_{SDM}*, $p < 0.05$), *i.e.*, SDM was the only compound with a concentration higher than the detection limit after 60 min of ozonation. OTC and SMX were the compounds with faster degradation, showing removals of 85% and 74%, respectively, in 10 min of contact time with O_3. These antibiotics and TMP were not detected in any of the 30 min treated samples. Regarding FF, a slower degradation was observed, but it was fully removed after 60 min. In the case of SDM, a fast removal is depicted in the first 10 min of reaction, followed by a slower removal after that period, a maximum removal of 82% being achieved in 60 min of ozonation.

It is well known that organic contaminants can be removed in ozonation by direct and indirect modes, depending on whether direct attack of O_3 or reaction with generated radicals (in particular $HO^{\bullet}$) respectively occur [38]. As can be seen in Table 6, all the studied antibiotics have reaction rate kinetic constants above 10^5 M^{-1} $s^{-}1$ with O_3 (k_{O3}), except FF (< 10 M^{-1} s^{-1}). This observation agrees with the slower degradation observed for FF in Fig. 26A. The k_{O3} are higher for the other antibiotics because the amino groups are expected to activate the aromatic rings and double bonds of the chemical structures [22, 39]. In fact, sulfonamides, tetracyclines, and TMP are antibiotics for which ozonation have demonstrated high removal efficiencies, mainly by direct oxidation [23, 40-43], whereas poor removals have been reported for FF [7, 16, 44, 45]. Thus, the degradation of FF observed in Fig. 26A (closed circles) seems to be related to an indirect oxidation pathway by the reaction with $HO^{\bullet}$.

To infer about the role of $HO^{\bullet}$ (and other possible radicals) in the degradation of these antibiotics, methanol was added to UPW in the second set of experiments (Fig. 26B). The removal of FF was negligible (5%), indicating that the indirect oxidation pathway is crucial for its removal, as confirmed by the significant differences of ANOVA analysis between UPW with and without addition of scavenger (b_{FF}, Fig. 26B). It is known that $HO^{\bullet}$ is generated after a series of reactions which starts by the primary reaction between O_3 and hydroxide ions present in water (Eqs. C1-14, Appendix C), meaning that the formation of $HO^{\bullet}$ can be accelerated by increasing the pH [46]. In the present study, a pH of 6 was sufficient to remove FF in 60 min of ozonation (Fig. 26A). Unlike FF, OTC, SMX, and TMP were removed in less time in the presence of methanol, but the differences were not significant (Fig. 26A vs 26B, $p < 0.05$). In any case, the radical scavenger consumes $HO^{\bullet}$ and, without regenerating the $O_2^{\bullet-}$, the O_3 decomposition may be retarded (Eqs. C4 and C7 (Appendix C), respectively), thus O_3 being available for direct attack [47]. ANOVA analysis also suggested that $HO^{\bullet}$ does not affect SDM degradation (a_{SDM}, Fig. 26B). Thus, these results confirmed that OTC, SMX, TMP, and SDM are preferentially removed by direct oxidation.

A set of additional experiments without methanol was performed with FF alone and adding each one of the other antibiotics to FF in UPW (Fig. D4.1a, Appendix D, Supplementary Material). Interestingly, these other antibiotics affected the FF degradation when simultaneously present in the solution, perhaps due to the readily O_3 consumption by direct attack of either parent (OTC, SMX, TMP, and SDM) or its intermediate compounds. Complementary experiments with SDM also suggested that the co-existence of the other 4 antibiotics is affecting its own removal (Fig. D4.1b, Appendix D, Supplementary Material), perhaps by generating by-products that can react with SDM. Beyond the chemical properties of the compounds, the water matrix can also affect the degradation of antibiotics. As shown in Fig 26C, the matrix under analysis (*i.e.*, aquaculture effluents) considerable impacted SDM removal (b_{SDM}). These results are in accordance with a recent study on ozonation of SDM [48], where the important role of the water matrix in its degradation was also highlighted. Furthermore, FF degradation was also affected by water matrix (c_{FF}), following the tendency of the experiments carried out in the presence of methanol (Fig. 26C). Despite the pH of this matrix (pH of 8) being more favourable to the decomposition of O_3 into $HO^{\bullet}$, the matrix

constituents may have acted as inhibitors of the formation of $HO^{\bullet}$ or instead, they might have consumed the generated radicals. The complexity and diversity of water matrices turn difficult to predict their impact as initiator, promoter, or inhibitor during ozonation [49, 50]. As such, the fraction of O_3 and $HO^{\bullet}$ (and other possible reactive radials) which effectively oxidizes the antibiotics is highly dependent on the action of all co-existing substances in the water matrix [51]. Thus, the collected aquaculture effluent (with physico-chemical characterization shown in Table D4.1, Appendix D, Supplementary Material) probably suppressed the effect of $HO^{\bullet}$ on FF removal, which did not exceeded 40%. In contrast, OTC, SMX, TMP, and SDM were efficiently eliminated from the aquaculture effluent in 60 min (to levels below MDLs).

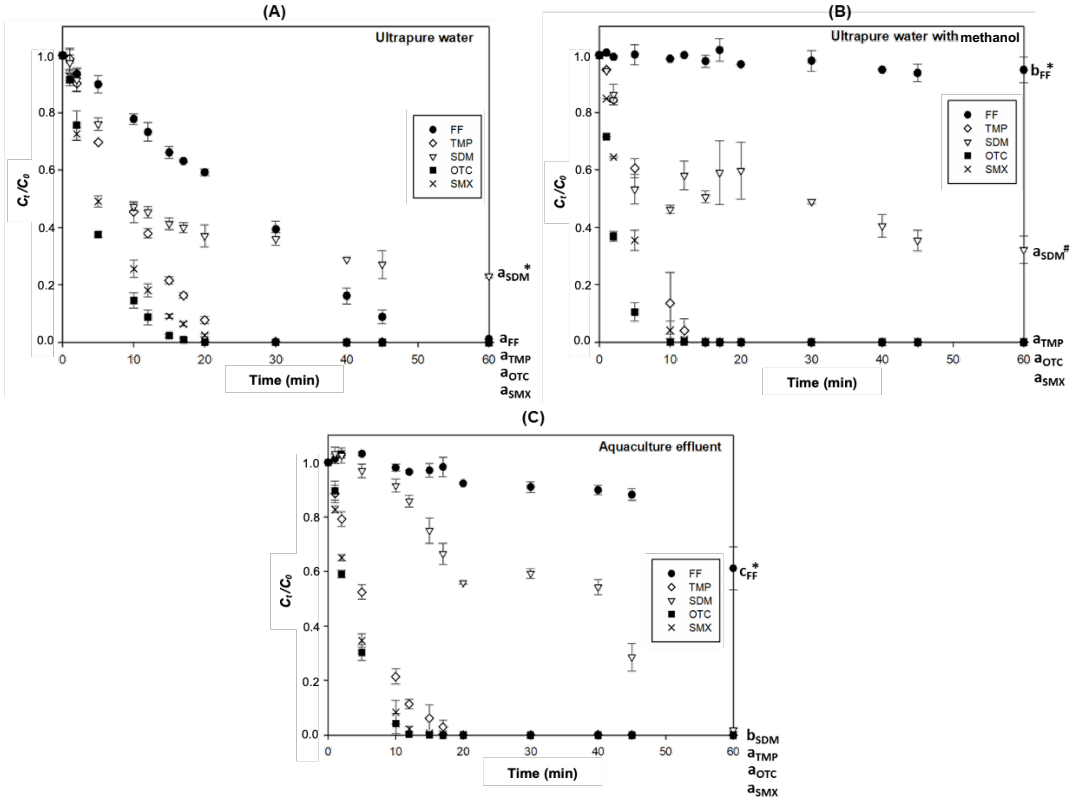


Figure 26. Degradation of FF, TMP, SDM, OTC and SMX in batch ozonation treating UPW (A), UPW with methanol as HO• scavenger (B) and aquaculture effluent (C), spiked with the mixture of five antibiotics. Experimental conditions: [FF, OTC, SDM, SMX, or TMP]$_0$ = 10 mg L^{-1}, T =18 ºC, $[O_3]_{gas\ phase}$ ~ 50 g Nm^{-3}, pH = 6 in (A) and (B), 8 in (C). The results correspond to the mean removals of three replicates, with the respective standard deviation. For each compound, letters a, b and c represent significant different removals between the tested matrices, considering samples collected after 60 min of ozonation - assessed by One-Way ANOVA followed by Tukey *post hoc test* $p < 0.05$. $^{*,\#}$ represents significant different removals among samples for each compound in the same matrix.

6.3.1.2. Continuous-flow mode ozonation experiments

The degradation of antibiotics in continuous-flow ozonation was investigated for an HRT of 10 min (corresponding to a t_s of 33 min), applying an O_3 concentration of 1.5 mg L^{-1}. As referred above,

these selected conditions were based on the information available in the literature regarding ozonation at full-scale aquaculture systems for disinfection [29-31] and our previous study focused on ozonation for the removal of several other organic micropollutants from SW [18]. For these experiments, the aquaculture effluent was spiked with 100 ng L^{-1} of each antibiotic (FF, OTC, SDM, SMX and TMP), *i.e.*, the spiked level was considerably lower than in batch experiments (hundreds of ng L^{-1} instead of tens of mg L^{-1}), mimicking concentrations frequently detected in aquaculture effluents [34, 52, 53]. In Fig. 27 (closed circles, top axis), it is possible to verify some deviations between the predicted and achieved concentrations of these spiked compounds (*i.e.*, ranging from 80 to 320 ng L^{-1}), which can be, for instance, related to the occurrence of the antibiotics in the collected effluent (Table D4.2, Appendix D, Supplementary Material), matrix effects or other possible interferences.

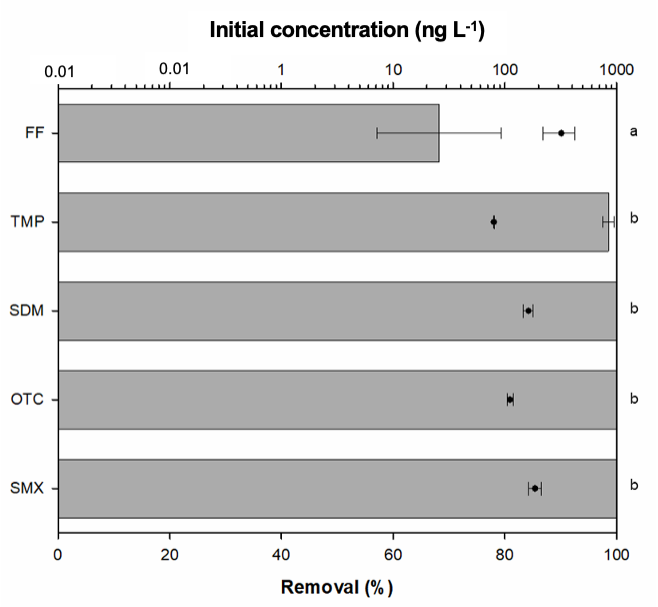


Figure 27. Removal percentages of FF, TMP, SDM, OTC and SMX (grey bars, average of three independent experiments) in continuous-flow ozonation of spiked aquaculture effluent (100 ng L^{-1} of each antibiotic). Antibiotic initial concentrations after spiking (closed circles) are also shown for comparison purposes (top axis). Experimental conditions: T = 17 ºC, HRT = 10 min, $[O_3]_{gas\ phase}$ ~ 20 g Nm^{-3}, $[O_3]_{liquid\ phase}$ = 1.5 mg L^{-1}, pH = 8. For each compound, letters a and b represent significant differences among samples assessed by One-Way ANOVA followed by Tukey *post hoc test* $p < 0.05$.

Removals above 98% were observed for TMP, SDM, OTC, SMX, whereas FF did not surpass 68% (Fig. 2, grey bars, $p < 0.05$). Therefore, in these experiments, FF was the only compound recalcitrant to O_3, its degradation being highly influenced by the presence of HO•, as confirmed in the previous sub-section. The action of HO• will depend on the composition of the matrix to be treated, and thus, it is very difficult to predict the removal extent of FF. The degradation of FF in aquaculture waters has been also investigated by applying UV and UV-photocatalysis, removals in the ranges of 4-85% and 98-100% being respectively registered [4]. Thus, combining UV or H_2O_2 with ozonation can be an interesting alternative in future works to enhance the production of HO• and, consequently, the more efficient degradation of FF; however, the respective costs should also be considered when selecting one of these alternatives [55].

6.3.2. Influence of continuous ozonation in the general quality of aquaculture effluent

Among the target ions (ammonium, bromate, bromide, calcium, chloride, magnesium, nitrate, nitrite, phosphate, potassium, sodium, and sulphate), nitrite and ammonium were never detected, while magnesium was always below the method quantification limit (0.4 mg L^{-1}) (Table D4.1, Appendix D, Supplementary Material). Before treatment, calcium, chloride, nitrate, phosphate, potassium, sodium, and sulphate ions were detected at concentrations of respectively 0.2, 6.3, 3.8, 3.2, 1.3, 6.3 and 1.1 mg L^{-1} (Table D4.1, Appendix D, Supplementary Material); values which were similar after ozonation, except a slight difference for potassium ion (0.3 mg L^{-1}). Moreover, bromide ions were oxidized to bromates after reaction with O_3 (Fig. D4.2, Appendix D, Supplementary Material). Likewise, if nitrites were detected in the aquaculture effluent, it would also be expectable their oxidation to nitrates [56, 57]. As expected, results of ions analysis suggested that apart from bromides and bromates, ozonation had a low impact on the ionic composition of the spiked aquaculture effluent. The aquaculture effluent had a very low organic load. COD was lower than the MDL (3.5 mg L^{-1}), and DOC was quantified at approximately 2 mg L^{-1} (Table D4.1, Appendix D, Supplementary Material). Still, DOC slightly decreased (~ 4%) after ozonation. Typically, O_3 is

a powerful oxidant of aromatic compounds. Still, it reacts slowly with low-molecular-weight carboxylic acids that are often formed from these parent compounds, which can lead to the accumulation of several by-products that cannot be easily oxidized by molecular O_3 [58]. In any case, the theoretical DOC due to the five MPs corresponds to less than 0.3% of the initial DOC content, since they are present at very low concentrations (Fig. 27). Regarding the particulate matter, O_3 eliminated approximately 60% of TSS.

6.3.3. Toxicity evaluation before and after continuous ozonation

Bromates (6 ug L^{-1}) and antibiotics by-products (Figs. D4.2 and D4.3, Appendix D, Supplementary Material) were detected by LC-MS/MS in the samples collected from the continuous ozonation reactor, highlighting the importance of toxicological evaluation. Zebrafish is a very popular model vertebrate used to evaluate the toxicity of water and it was used here to verify the potential risks of removing antibiotics from aquaculture effluents with O_3. The assays carried out in this work fulfilled all the validation criteria of the OECD guidelines 212 and 236 [59, 60]. Namely, at the end of the exposure period, the negative control group had a low mortality (< 10%) and a hatching success above 80%, while embryos exposed to 3,4-dichloroaniline (positive control) registered a mortality above 30% (Fig. 28). These results allowed to completely validate the applied methodology. Results for mortality and hatching obtained for the treated and non-treated aquaculture effluent are presented in Fig. 28.

Focusing on the mortality (Fig. 28a), it was possible to verify that no significant mortality was elicited either by the aquaculture effluent, the spiked effluent or the ozonated effluent. For the negative control and the tested effluent (squares and triangles, Fig. 28a), mortality occurred mostly during the first 48 h of exposure, which is in accordance with the expected outcome of these types of toxicological assays. The first 48 h of zebrafish development are critical to development. Not only

all the major gastrulation and segmentation processes occur in this period, but also defence mechanisms against external contamination are not fully maturated at this time frame [61-63].

Hatching (Fig. 28b) occurred mainly between 48 hpf and 72hpf, as described in the literature [61]. However, significant differences among experimental conditions were found in the time to hatching, compared to the controls (Chi-square = 66.0, degrees of freedom df = 6, $p < 0.00001$). The subsequent pairwise comparisons showed that hatching was significantly anticipated in the spiked aquaculture effluent (Chi-square = 24.8, df = 2, $p < 0.00001$) and the treated aquaculture effluent (Chi-square = 39.4, df = 2, $p < 0.00001$). Table 7 shows the median time to hatching (HT_{50}) obtained for each experimental condition. According to these results, embryos exposed to the spiked effluent took 10 h less than the control to hatch, while those exposed to the treated effluent took 13 h less.

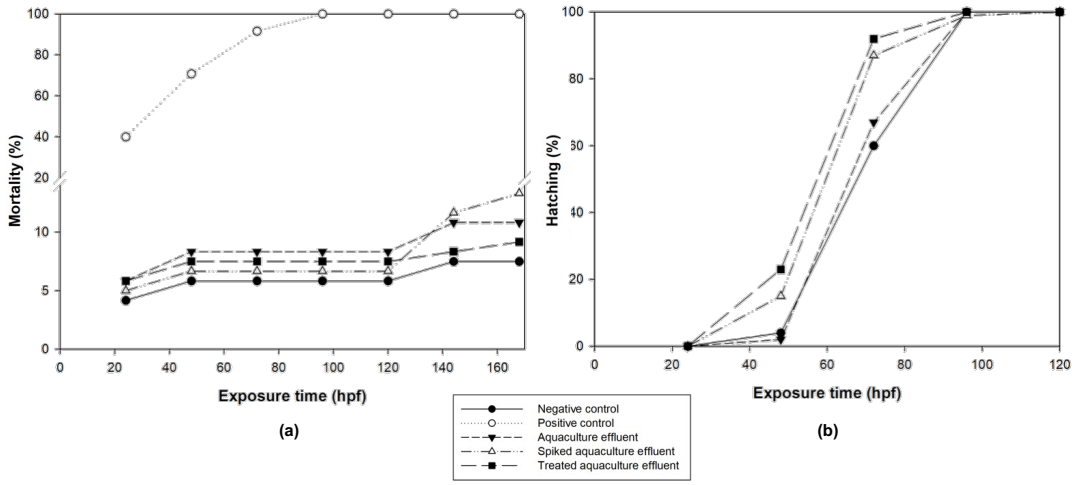


Figure 28 Mortality **(a)** and hatching **(b)** recorded for embryos exposed to the aquaculture effluent and the same effluent treated with continuous ozonation. Data are presented as cumulative frequency.

Table 7. Median time to hatching (HT_{50}) of embryos exposed to the tested experimental conditions.

Experimental condition	HT_{50} (hours) (95% Confidence Interval)
Negative control	68 (66, 71)
Aquaculture effluent	68 (65, 70)
Spiked aquaculture effluent	58 (56, 60)
Treated aquaculture effluent	55 (53, 57)

The implications of premature hatching are yet to be fully understood. It has been observed previously that premature hatching in zebrafish may occur upon exposure to ionic stress [64], hypoxia [65] or toxic substances such as tributyltin [66]. The work of Ord [64], seems to support that premature hatching may be an adaptive response allowing for animals to escape unfavourable conditions. In contrast, the investigation of Leite-Ferreira, *et al.* [67] suggests differential sensitivity to alcohol of premature and late hatching zebrafish larvae, with stronger responses of premature larvae to high exposure concentrations, compared to late hatching ones. Further investigations will help understanding the implications of such anticipation in relation to treatment benefits to water quality.

Statistically significant differences among test conditions were observed for the rate of malformations (Chi-square = 72.9, df = 3, $p < 0.00001$) (Fig. 29). The pairwise comparisons indicated a significantly higher proportion of abnormal embryos at 168 hpf in the aquaculture effluent (Fisher Exact test, $p < 0.00001$, compared to control embryos) and the spiked aquaculture effluent (Fisher Exact test, $p = 0.0005$, compared to controls) (Fig. 29d). Yolk sac malformations, side-wide position and skeletal deformities were the most prevalent abnormalities found, all interfering with larvae swimming, and thus their ability to feed and escape predators (Fig. 29a,b,c). Though less prevalent, alterations in the blood circulation and heartbeat could also be observed (data not shown). Nevertheless, in the group exposed to the effluent treated with continuous ozonation the rate of malformations was very low, comparable to the control group (Fisher Exact test, $p = 0.672$). Overall, the results suggest that the ozonation treatment would effectively reduce the toxicity of the aquaculture effluent, improving water quality for the development of the embryos.

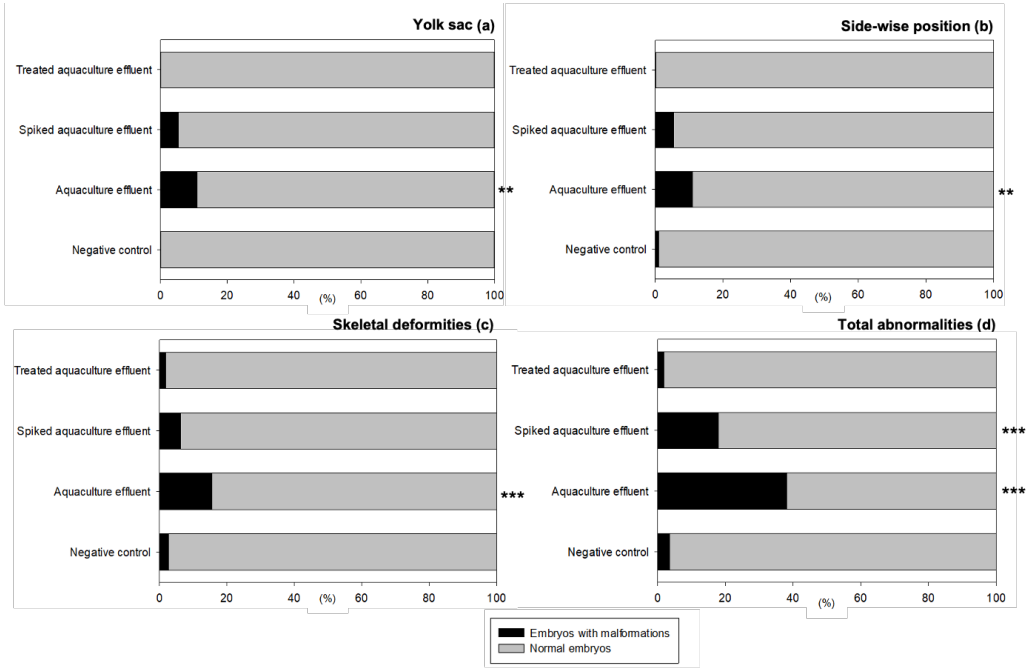


Figure 29. Rate of embryonic malformations detected at 168 hpf in embryos exposed to the different experimental conditions. Statistically significant differences relative to the control group are indicated by the stars according to the results of the Fisher Exact test (** $p < 0.01$, *** $p < 0.001$).

Therefore, the by-products generated during ozonation were not harmful to zebrafish. Bromate was detected at 6 ug L^{-1} in treated effluent samples; however, in marine aquaculture, O_3 may result in bromate concentrations considerable higher because of the large amount of bromide in seawater (~ 60 mg L^{-1}) [12, 16]. Investigation on toxicity effects of bromate ions in zebrafish are still scarce, but studies for sodium and potassium bromate have reported embryotoxic effects only at very high concentrations (> 1 g L^{-1}) [68, 69]. It is important to refer that zebrafish toxicity tests for ozonated aquaculture effluents were performed in this study for the first time, with encouraging results.

6.4. Conclusions

Batch ozonation experiments (antibiotics at 10 mg L^{-1} each; 60 min of treatment; 50 g NO_3 m^{-3}) showed that ozonation was an efficient solution for removing TMP, OTC and SMX from aqueous matrices (< 30 min); direct oxidation being their main degradation pathway. As such, both $HO^{\bullet}$ and the type of water matrix did not considerably affect the degradation of these three antibiotics. In contrast, FF was only removed in the presence of $HO^{\bullet}$, whereas SDM was fully eliminated in aquaculture effluent. The recalcitrance of FF to O_3 direct oxidation has already been reported; however, the results of this work showed that it can be successfully removed by the indirect reaction pathway (*i.e.*, through $HO^{\bullet}$). Additionally, in continuous-flow ozonation experiments (antibiotics at 100 ng L^{-1} each; 10 min of treatment; 20 g NO_3 m^{-3}), all antibiotics were successfully removed from aquaculture effluent (> 98%), except FF (68%). For FF, its removal should be enhanced by promoting the generation of $HO^{\bullet}$. Complementary analysis showed that bromates (6 µg L^{-1}) and other reaction by-products were formed in the medium during ozonation treatment. However, endpoints of the zebrafish assays (mortality, hatching and malformations) did not reveal toxicity extent after O_3 treatment. Molecular, biochemical, and genetic biomarkers (*e.g.*, genotoxicity, oxidative stress, endocrine disruption, and neurotoxicity) can also be used to assess the possible toxicity effects on zebrafish, and thus, more specific analysis may be addressed in the future.

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Chapter

7

Final Remarks

Considering the significant growth of the aquaculture sector in the last years, as well as its potential impact on the water quality and food safety, specifically by the presence of MPs, this PhD project intended to establish feasible alternatives for removing MPs from aquaculture waters. Thus, CWs and AOTs (singly applied or eventually combined) were the technologies selected for this purpose (Fig. 30) and this chapter intends to rationalize the findings in a broader perspective. A literature survey showed that despite their very distinct nature, CWs and AOTs have demonstrated promising results regarding MPs elimination, but information for aquaculture waters is still scarce (*Chapter 2*). Furthermore, there are few data on the CWs performance for a considerable number of PSs and CECs defined in the EU legislation, even less when they occur simultaneously in the aquatic medium to be treated. Therefore, studies should be extended to other pollutants, ensuring that they are tested at realistic concentrations in the environmental matrix. On the contrary, AOTs have been explored for a wide range of MPs, but applying operating conditions that are still far from full-scale feasibility. For this reason, they must be optimized in order to establish technologies that meet actual requirements.

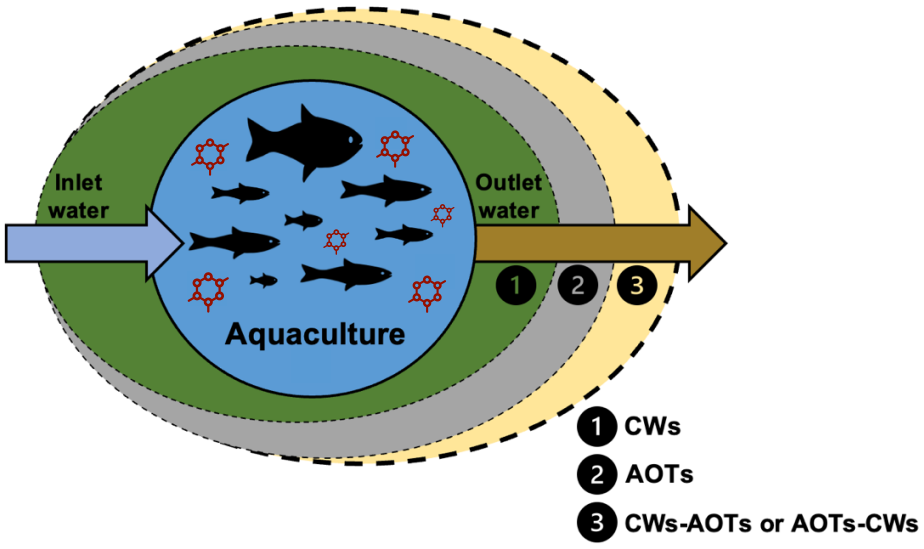


Figure 30. Schematic diagram of the main purpose of this PhD, which was to apply: (1) CWs, (2) AOTs or (3) CWs-AOTs or AOTs-CWs to remove MPs from aquaculture waters.

In this PhD work, excellent removal efficiencies for most target organic MPs in spiked and non-spiked aquaculture effluents (including EU PSs and CECs) were achieved by VSSF-CWs planted with *Phragmites australis.* In addition, the ozonation technology, which was studied as close as possible to realistic scenarios, also provided high removals for most investigated MPs. Therefore, both biological (CWs) and chemical (AOTs) treatments were effective when applied alone to remove several MPs relevant in the context of water quality/water policy, representing interesting solutions to be implemented in aquaculture farms. As shown in Figure 30, combined CWs-AOTs or AOTs-CWs may also be an option to remove MPs from aquaculture waters, namely for those not totally removed by one of the technologies. Combining CWs and AOTs can benefit from the advantages of both technologies, *i.e*. the low cost of CWs and the high treatment efficiency of AOTs. Thus, from the literature survey performed, it was concluded that CWs might function as: (i) pre-treatment of AOTs, that is, CWs removing the majority of MPs and the most recalcitrant ones being eliminated by AOTs; or (ii) as post-treatment of AOTs, that is, CWs treating a more biodegradable effluent and removing possible by-products generated by AOTs. Although this option can be more expensive than CWs or AOTs applied alone, it can be especially advantageous either if the previous technologies are inefficient in removing some MPs (or its by-products) or if the single treatment increases the toxicity of water. Notwithstanding, the results of this PhD end up supporting the single application of the studied technologies because both are efficient to remove a wide range of organic MPs, the main specific target of this PhD project. Moreover, the acquired know-how in the field of aquaculture production by performing the several searches, participating in conferences, and resulting from the direct contact with farmers, allowed me to recognize that simple, economic, and efficient solutions will be valorised, mainly those which can be easily integrated in the industries, without creating disturbances in the normal function of the systems. Therefore, CWs or AOTs should be selected according to their specific characteristics and respective compatibility with the type of aquaculture farm for which an upgrade in terms of MPs elimination is required. In Figure 31, the main strengths of CWs (1) and AOTs (2) and their respective application according to the type of aquaculture are represented.

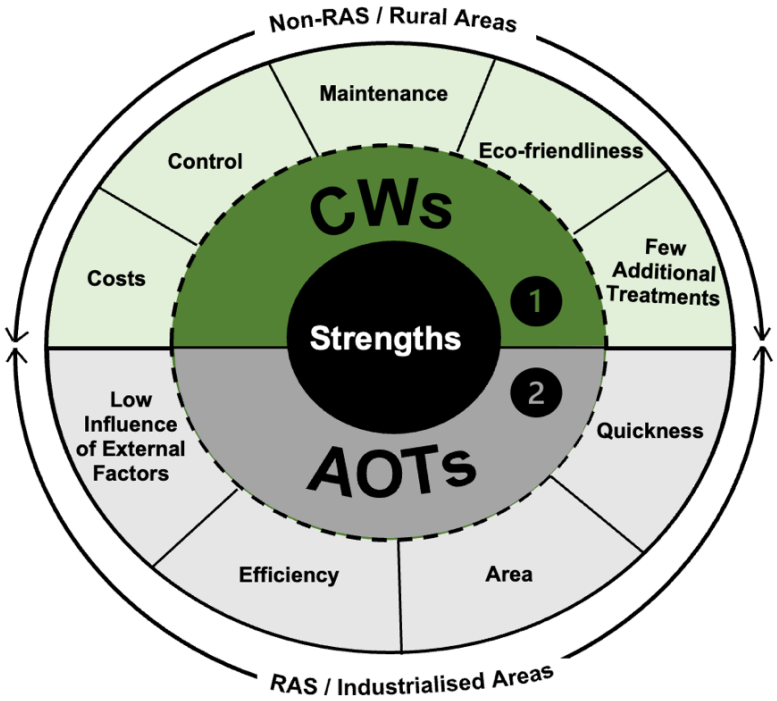


Figure 31. Main strengths of CWs and AOTs, and suitable application according to the type of aquaculture (*i.e.*, non-RAS in rural areas and RAS in industrialised areas).

As a low-cost, ecological, and biological solution, CWs are an interesting option for the removal of organic MPs in non-RAS, especially when these farms have spacious outdoor areas that allow their implementation, and as a consequence, the creation of additional ecosystems and habitats is possible. CWs are affected by external factors such as temperature, humidity, and sunlight exposure; thus, long acclimatisation periods may occur. Furthermore, a slower treatment is also expected because CWs performance is dependent on the natural processes occurring in the system. For this reason, CWs seem to be a more compatible treatment for non-RAS, since these types of facilities are easily implemented in rural areas (such as inland flow-through systems) and do not require advanced or rapid treatment technologies. In contrast, AOTs are more compatible with RAS because, in general, these farms are more industrialized and modernised (often installed distant from natural water sources), with optimised profits and strict requirements, which demand efficient equipment to treat water before reuse. Beyond the capacity to remove organic MPs, CWs

and AOTs can also eliminate other relevant pollutants, such as microorganisms, ions, and particulate matter. Therefore, research on these treatments should be reinforced to maximise the performance and reduce the costs of treatment of aquaculture waters. In this way, sustainable aquaculture can be favoured, and environmental pollution can be prevented.

Chapter

8

Conclusions and Future work

The occurrence of MPs in aquatic systems is a hot topic for the scientific community because of their possible long-term effects in ecosystems and human health. In this context, this PhD work was based on the search for treatment solutions able to deal with the occurrence of organic MPs in aquaculture waters, contributing to an environmentally safer production in this sector. After a 4-years work on this subject, the most relevant conclusions are summarized below, taking into consideration the main findings of the studies presented in the previous chapters.

In order to build knowledge under the investigated themes, an extensive search was developed to study the application of CWs and AOTs to remove organic MPs from aqueous matrices, namely in aquaculture facilities (*Chapter 2*). Results of this survey showed that there is a large number of organic MPs defined in EU legislation lacking information regarding their elimination in CW systems. Moreover, for those MPs that were studied, the analysis was ambiguous because a wide range of removal percentages was observed for each MP. This may be ascribed to the very different CWs configurations and operational conditions reported, resulting in distinct performances of CWs. Nevertheless, the results found were promising, being important to extend the research to more organic MPs, especially occurring as mixtures. On the other hand, information on AOTs has grown in recent years; UV and O_3-based technologies being the most investigated processes to remove organic MPs. Most studies are still conducted at lab/pilot scale, looking to the influence of several parameters that can affect the elimination of MPs. Thus, research at more realistic conditions should be encouraged to bring more representative scenarios for discussion. In relation to the specific application of CWs and AOTs in aquaculture, information is still limited to few studies, with more knowledge and recommendations being needed to accomplish sustainable full-scale applications.

The confirmed lack of data on CWs performance for a large number of PS and CECs (and respective mixture of them), together with the poor information about their application in aquaculture farms triggered the study of CWs microcosms to remove organic MPs from freshwater aquaculture effluents (*Chapter 3*). The applied conditions in CWs were defined according to information available in the literature, aiming at favouring the removal of a maximum number of organic compounds. VSSF-CWs planted with *Phragmites australis* operating at batch mode

(through an automatic recirculation system with an HRT of 7 days) achieved removals higher than 87% for 34 of the 35 analysed compounds (initial concentrations below 800 ng L^{-1}), the only exception being PFOS in the first week of non-spiked experiments. Experiments were conducted during 4 cycles of 1 week, and the efficiency of CWs was not affected by the continuous feeding for 1-month. Despite this, some MPs have registered slightly lower removals in the first week, suggesting an adaptation period of the CWs systems. Beyond the MPs elimination, CWs also registered high removals of nutrients. Thus, CWs were proven to be an interesting solution to simultaneously remove various MPs from aquaculture effluents.

In a subsequent stage, AOTs were studied to remove organic MPs with the aim of approaching the investigated processes to real scenarios, a gap that has been identified in the field of MPs degradation by AOTs. However, an unexpected analytical interference in the LC-MS/MS analysis of MPs was observed after adding Na_2SO_3 to quench H_2O_2 applied in the UV/H_2O_2 AOTs experiments, which was the basis for a comprehensive detailed analysis of that effect never reported before (*Chapter 4*). The results of this study pointed out that Na_2SO_3 used as quencher of residual H_2O_2 (or O_3) should be replaced by catalase. The addition of Na_2SO_3 to treated water samples resulted in a significant signal suppression on the analytical signal of those MPs monitored under PI mode and with a higher pK_a (> 5.9), which does not occur when catalase was used. Moreover, matrix-matched validation and internal calibration proved to be crucial tools to avoid bias conclusions from the elimination results. The removal of MPs by UV/H_2O_2 did not exceed 21%; thus, the following studies on AOTs (O_3, O_3/UVA-LEDs, and O_3/H_2O_2) were conducted in a continuous-flow reactor for removing a mixture of 21 organic MPs at environmental realistic concentrations (several pharmaceuticals, pesticides, and the industrial compound PFOS), and microorganisms from SW - a conventional type of water used in aquaculture (*Chapter 5*). Regardless of the applied treatment, organic MPs (21 in total) were always removed above 85%, except PFOS (< 50%), and single ozonation was selected as the best “cost-benefit” process. The concentration of O_3 and the HRT in the continuous-flow reactor were afterwards adjusted envisaging more representative conditions of full-scale applications. Results showed that complete removal of most MPs simultaneously with water disinfection could be achieved with a concentration

of 3 mg O_3 L^{-1} and an HRT of 10 min, also preventing a possible bacteria regrowth after 3 days storage of treated water. This study was the first one with SW to assess the capability of single ozonation to simultaneously remove MPs and bacteria (*E. coli*, enterococci and total heterotrophs), showing very encouraging results that reinforce the need for investigation at full scale. In the following studies, continuous-flow ozonation (1.5 mg O_3 L^{-1}; 10 min) was applied for degradation of 5 antibiotics commonly used in aquaculture industry, including toxicological evaluation (*Chapter 6*). The results showed that all target antibiotics (initial concentrations between 80 and 320 ng L^{-1}) were successfully removed from aquaculture effluents in the steady state (> 98%), except FF (68%). Contrary to the other target MPs, FF was mainly oxidised by indirect way, and thus, $HO^{\bullet}$ and water matrix had a great influence on its removal extent. Although complementary analysis suggested the formation of oxidation by-products during the O_3 treatment, the toxicity assays with zebrafish embryos did not show increased toxicity after treatment. In this sense, it was also shown that ozonation could be a good alternative to remove MPs in aquaculture farms, also acting as disinfectant when needed, for instance in RAS - recirculating aquaculture systems.

With all this background, specific considerations about selecting the best treatment solution (AOTs, CWs or CWs-AOTs/AOTs-CWs) were proposed according to the type of aquaculture (*Chapter 7*). Since both CWs and AOTs registered high eliminations of organic MPs, the option of a combined system becomes less attractive for the specific main target herein considered: removal of MPs. In fact, there are some advantages of combining them, but the good results of each technology applied alone might not justify the high investment required. In this sense, as conclusion, CWs have more potential to be implemented in aquacultures without water recirculation since these types of facilities are generally implemented in rural areas (such as inland flow-through systems) and do not require advanced or rapid treatment technologies. In contrast, ozonation is more compatible with RAS that often involves more industrialised and modernised facilities, which demand efficient equipment to treat water before reuse/disposal.

Finally, it is undeniable that this PhD work provided new insights on the application of CWs and AOTs for organic MPs removal, particularly from the point of view of their potential implementation

in aquaculture. However, some questions must be addressed in the future to extend knowledge on this subject, namely by performing:

- Monitoring programs in different aquaculture farms to evaluate the occurrence of organic MPs in their influents and effluents. Moreover, the sampling campaigns should include antibiotics (typically applied in this industry) and more compounds from distinct classes. It would also be interesting to ascertain about the presence of these MPs in fish muscle, being needed to evaluate the best analytical methodologies to be applied;
- CWs studies focused on the processes involved in the MPs removal to surpass the loophole of CWs being recognised as "black-box" systems where information about removal mechanisms is practically unknown. This will be useful to create more general criteria of CWs configuration maximizing MPs elimination;
- Investigations focused on the formation of transformation products by CWs and AOTs to predict the potential risks of both treatments. If relevant, removal of by-products from one treatment can be studied by applying the other (*i.e.*, AOTs to remove by-products of CWs and *vice-versa*);
- Develop collaborative works with aquaculture industries to test CWs or AOTs (depending on the farming type) at pilot/full scale and determine capital and operating costs. By doing this, it is possible to analyse the performance of the technologies and optimize costs and specific parameters, aiming at creating a solution able to protect the environment and to satisfy the requirements of the farming entities.

In conclusion, efforts must be aligned to define global methodologies and strategies that contribute to a more sustainable food industry sector. In this way, we can contribute to environmental pollution mitigation, food safety, and human health protection.

Appendixes

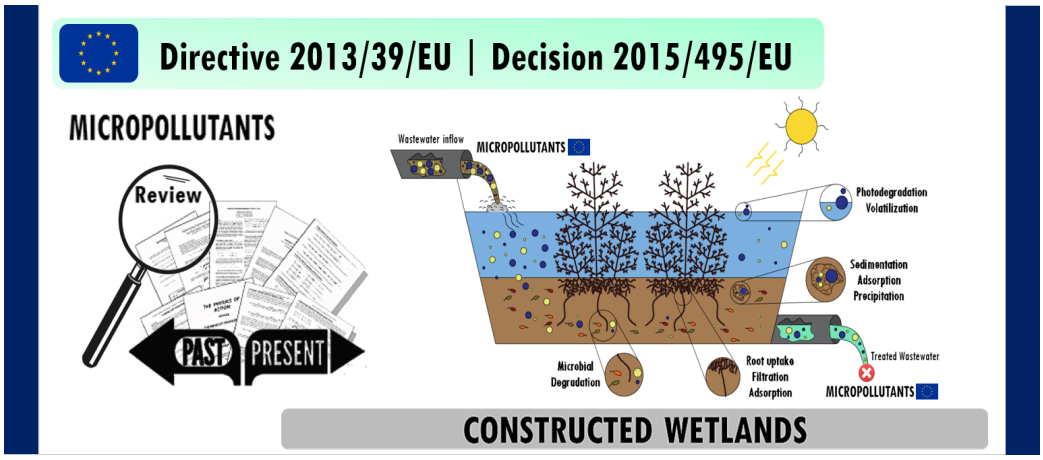
Appendix A

Published articles

A1.

"A review on the application of constructed wetlands for the removal of priority substances and contaminants of emerging concern listed in EU legislation"

Gorito, A.M., Ribeiro, A.R., Almeida, C.M.R, Silva, A.M.T., published in the *Environmental Pollution Journal*, 227 (2017) 428-443.



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A review on the application of constructed wetlands for the removal of priority substances and contaminants of emerging concern listed in recently launched EU legislation☆

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ABSTRACT

The presence of organic pollutants in the aquatic environment, usually found at trace concentrations (i.e., between ng L^{-1} and μg L^{-1} or even lower, known as micropollutants), has been highlighted in recent decades as a worldwide environmental concern due to their difficult elimination by conventional water and wastewater treatment processes. The relevant information on constructed wetlands (CWs) and their application for the removal of a specific group of pollutants, 41 organic priority substances/classes of substances (PSs) and 8 certain other substances with environmental quality standards (EQS) listed in Directive 2013/39/EU as well as 17 contaminants of emerging concern (CECs) of the Watch List of Decision 2015/495/EU, is herein reviewed. Studies were found for 24 PSs and 2 other substances with EQS: octylphenol, nonylphenol, perfluorooctane sulfonic acid, di(2-ethylhexyl)phthalate, trichloromethane, dichloromethane, 1,2-dichloroethane, pentachlorobenzene, benzene, polychlorinated dibenzo-p-dioxins, naphthalene, fluoranthene, trifluralin, alachlor, isoproturon, diuron, tributyltin compounds, simazine, atrazine, chlorpyrifos (chlorpyrifos-ethyl), chlorfenvinphos, hexachlorobenzene, pentachlorophenol, endosulfan, dichlorodiphenyltrichloroethane (or DDT) and dieldrin. A few reports were also published for 8 CECs: imidacloprid, erythromycin, clarithromycin, azithromycin, diclofenac, estrone, 17-beta-estradiol and 17-alpha-ethinylestradiol. No references were found for the other 17 PSs, 6 certain other substances with EQS and 9 CECs listed in EU legislation.

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1. Introduction

Fresh water is one of the most important resources on Earth, but is likely to become scarce in the next decades. The continuous world population growth and intensive urbanisation led to an increase of water usage for domestic, agricultural and industrial purposes, resulting in a great production of wastewater, which in some cases is directly released into the environment (Agarwal, 2005). Water quality is intrinsically related to preservation of ecosystems, food safety, human health, poverty reduction, and other associated factors, such as economic growth and social development. Therefore, maintaining global water quality is a requirement for sustainable progress, in order to satisfy ecosystem requirements and to guarantee the overall well-being of humans (UNESCO, 2015).

Over the last few decades, the occurrence of pollutants in the aquatic environment and their potential adverse effects to wildlife and human health have become worldwide issues of increasing concern. Organic pollutants can be anthropogenic or natural substances, frequently present in water at trace concentrations,

☆ This paper has been recommended for acceptance by Dr. Harmon Sarah Michele.

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ranging from ng L^{-1} to several μg L^{-1} or even lower, at pg L^{-1}, known as micropollutants (Escher and Leusch, 2012). Although some pollutants persist in the environment, others are considered "pseudo-persistent" due to their continuous introduction into the environment which is not surpassed by their elimination rate. The "low concentration" and diversity of several pollutants complicate the analytical procedures for their detection (Coquery et al., 2005) and create problems for conventional water and wastewater treatment systems (Luo et al., 2014), which are not specifically designated to eliminate many of them.

1.1. European policy: priority substances/classes and contaminants of emerging concern

The contaminants can be classified either as priority substances/classes (PSs), "presenting a significant risk to or via the aquatic environment" - Water Framework Directive (WFD) (DIRECTIVE 2000/60/EC, 2000), or as contaminants of emerging concern (CECs), depending on the existence or absence of regulation, respectively. Most PSs are organic contaminants, but some toxic metals and organometallic compounds have been also identified as PSs. CECs include substances that have been recently detected in natural streams (often due to improved analytical detection capacity) and/or pose risks (normally not fully understood yet) to human health and/or ecosystems (Workgroup, 2008). CECs comprise several types of compounds such as pharmaceuticals and personal care products (PPCPs), steroids and hormones, pesticides, industrial and household chemicals, metals, surfactants, industrial additives, among others.

The European WFD Directive is probably the most significant mark in the European Union (EU) legislation on water, intending to intensify the monitoring of pollutants in ecosystems and enhance the control of contaminants release. In 2008, 33 PSs were identified (DIRECTIVE 2008/105/EC, 2008) as well as environmental quality standards (EQS) defined, and 13 other pollutants/group of pollutants suggested for possible identification as PSs, based on available data of acute and chronic effects to aquatic environment and human health. More recently, the Directive was again updated (DIRECTIVE 2013/39/EU, 2013), in which (i) EQS were revised; (ii) new PSs were identified; (iii) EQS for the newly identified substances were defined; and (iv) biota EQS were defined for some existing and newly identified PSs. Directive 2013/39/EU includes a list of 45 PSs (41 organic substances/classes and 4 metals, namely cadmium, lead, mercury and nickel) and also certain other pollutants ($n = 8$) with defined EQS to be considered (Ribeiro et al., 2015). This update reinforces the preventive action (highlighting the polluter pays principle), the identification of pollution causes, the need to deal with emissions of pollutants at the source and the need to develop innovative water/wastewater treatment technologies. Moreover, Directive 2013/39/EU calls the attention to the significance of monitoring CECs for which legislation still does not exist. In this context, a list of 10 substances/groups of substances (a total of 17 compounds) was later established in an entitled Watch List (DECISION 2015/495/EU, 2015), and these compounds must be monitored in surface water within the EU (Barbosa et al., 2016a). Thus, the concern of the EC about these substances is clear.

1.2. Pollution sources, environmental fate and effects of organic pollutants

Organic pollutants reach the environment through diverse sources (Burkhardt, 2011; Ribeiro et al., 2015). Industrial contamination encompasses the steps of manufacturing, processing and distribution of several compounds. Considering that industrial effluents can contain various contaminants, including those herein reviewed, industries are one of the possible sources of these pollutants. On the other hand, hospital effluents and domestic wastewaters can also be significant pathways of the above mentioned pollutants (Barbosa et al., 2016a). Pharmaceuticals can be completely or partially metabolised in the human body, therefore the unchanged parent drugs and the produced metabolites are excreted via urine and faeces, reaching the WWTPs through their effluents (Ribeiro et al., 2016). Other products commonly used in home and hospitals (e.g., soaps, shampoo, toothpaste and disinfectants) can contain several compounds which have the same destination. Agriculture is another important source of pollutants, mainly due to the pesticides used to improve the productivity (e.g., controlling the pests and vectors), which can be found in waterways as result of agricultural runoff (Moore et al., 2002). Moreover, veterinary drugs and food additives used in livestock farming are excreted by animals and may reach the aquatic systems via runoff. A fraction of these pollutants can also infiltrate the soil after manure application and pollute the groundwater system. Leaching from dumping sites and sewage treatment facilities, as well as environmental disasters, are other possible sources (Burkhardt, 2011; Ribeiro et al., 2015).

Despite all existing pollution routes, one of the most significant for the aquatic environment is the discharge of effluents from industrial, hospital and urban WWTPs (Burkhardt, 2011; Fatta-Kassinos et al., 2015; Luo et al., 2014; Michael et al., 2013). Although many substances are efficiently eliminated in WWTPs, the removal of organic pollutants, and in particular micropollutants, is often insufficient. Conventional WWTPs are not specifically designed to eliminate this type of pollutants and therefore, many of these compounds are able to pass through wastewater treatment processes. Consequently, their introduction into the receiving surface water constitutes a threat not only to wildlife, but also to human health. Once surface water is widely used as raw water to produce drinking water, and the drinking water treatment plants (DWTPs) are not able for their complete removal, tap water containing micropollutants can be ingested by humans, exposing them to such substances and their possible effects (Barbosa et al., 2016b).

The environmental fate of organic pollutants is basically determined by the properties of the compound (chemical structure, octanol partition coefficient, acidity constant, etc.) and by the conditions of the surrounding environment. Depending on these aspects, processes such as sorption, volatilization, dispersion, hydrolysis, oxidation, isomerisation, photodegradation and biodegradation can occur (Burkhardt, 2011). As the environmental compartments are constantly interconnected, pollutants are spread by surface water, groundwater, soil and air. Furthermore, pollutants may also bioaccumulate in plants and other organisms (Rozas et al., 2016).

The occurrence of pollutants in the aquatic environment has been frequently linked to ecotoxicological adverse effects, which are still not totally understood. Although the acute toxic effects are unlikely to occur due to their quite low concentrations in water, the precautionary principle should prevail due to the possible chronic and long-term exposure which are more difficult to evaluate (Tijani et al., 2015). Some reports have shown that exposure to certain pollutants, even at very low concentrations, can originate impacts on biological systems, mostly in fish and aquatic species (Raghav, et al. 2013). Moreover, accumulation of some pollutants, such as antibiotics, can potentially promote the development and accumulation of antibiotic resistant bacteria and/or resistance genes (ARB&ARG) in the environment (Fatta-Kassinos et al., 2015; Michael et al., 2013; Rizzo et al., 2013). Some researchers found higher levels of ARG downstream of WWTPs compared to upstream, suggesting that WWTPs effluents can be a significant

source of antibiotic resistance spreading into the environment (Raghav et al., 2013; Rizzo et al., 2013). Besides the development of resistance mechanisms by bacteria and other chronic toxic effects, another issue of high environmental concern is the endocrine disrupting effects caused by some compounds, which may interfere with organisms endocrine system and produce adverse developmental, reproductive, neurological and immune effects in both humans and wildlife (UNESCO, 2015). It is important to mention that according to some authors, DWTPs might additionally increase the endocrine disrupting effect of treated water through the chlorination step, by generating chlorinated by-products (Rozas et al., 2016).

1.3. Treatment of water containing organic pollutants

Considering the possible effects that pollutants can cause to both the environment and human health, their release into the environment must be minimized. As abovementioned, since conventional WWTPs are not specifically designed to eliminate various pollutants, and in particular micropollutants, they are one of the pathways of these compounds into the environment. Thus, alternative wastewater treatment technologies are urgently required to transform organic pollutants into less toxic compounds or ideally to mineralize them. Technologies already applied at full-scale include membrane bioreactors (MBR), adsorption by activated carbon (AC) and membrane processes (e.g., microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO)), and some chemical oxidation technologies (COTs) and advanced oxidation processes (AOPs), such as ozonation and UV/H_2O_2.

MBRs are slightly more efficient than conventional activated sludge for the removal of various organic pollutants, as they combine aerobic biodegradation and membrane separation (Oulton et al., 2010). Besides the general longer sludge retention time and higher biomass concentration of MBRs (Verlicchi et al., 2012), the other important variance of MBRs is the separation stage between the liquid phase and the sludge. This final step uses MF or UF membranes, reducing the chemical oxygen demand (COD) and promoting the retention of suspended solids and pathogens (Petrovic et al., 2009). Powdered activated carbon (PAC) and granular activated carbon (GAC) have a great potential as adsorption processes for wastewater treatment; however, the performance of AC might be compromised by the competition of natural organic matter (NOM) for binding sites. Membrane technologies, mainly used for microorganisms and salts removal from water/wastewater, comprise moderately low-pressure systems (e.g., MF and UF) and high-pressure systems (e.g. NF and RO). The latter are more suitable for removal of organic micropollutants (Coday, et al., 2014; Oulton et al., 2010; Peters, 2010). The major shortcoming of membrane processes is the generation of large volumes of a concentrate that have to be handled (Bagastyo et al., 2011; Justo et al., 2014). COTs and AOPs can be used as post-treatment, ideally yielding the complete mineralization of organic pollutants by producing highly reactive oxidizing species (Hoigné, 1997). One of their limitations is the typical presence of radical scavengers in the wastewater.

Constructed wetlands (CWs), often including plants, have also been applied to wastewater treatment, due to their recognized advantages (eco-friendliness, simplicity and low operation/maintenance costs). CWs are able to efficiently remove total suspended solids, organic matter, nutrients (e.g., ammonia and phosphorus), and metals. CWs have also been investigated to remove organic pollutants, demonstrating auspicious results for the removal of some of these compounds (Anderson et al., 2013; Carranza-Diaz et al., 2014; Carvalho et al., 2013; Matamoros et al., 2008; Park et al., 2009; Zhang et al., 2014); however, additional studies are needed in this field of research.

2. Constructed wetlands

2.1. Mechanisms, configurations and operating conditions

Since natural wetland systems are able to remove pollutants present in the water that flows through, improving water quality, artificial wetlands have been constructed to replicate the process (United_States_Environmental_Protection_Agency-EPA, 2004). CWs are currently used to treat wastewater effluents, being most commonly applied to the treatment of secondary domestic sewage effluents and as polishing step (Davis, 1995).

CWs are complex systems containing water, substrate, plants in most of cases, and native microorganisms. Physical, chemical and biological processes (Fig. 1), such as volatilization, sorption and sedimentation, photodegradation, plant uptake and microbial degradation, may occur simultaneously, contributing to eliminate several types of compounds (Zhang et al., 2014). The occurrence of these processes depends on various factors (Zhang et al., 2014). For instance, volatilization is closely related to the vapour pressure of the compounds, and thus pollutants with higher vapour pressure values are more likely to volatilize, presenting shorter half-lives (Suthersan, 2002). The physical-chemical properties of the compounds also affect their sorption in soil, mineral surfaces and biofilms. Sorption (Kd) and organic carbon (Koc) partition coefficients are appropriate settings; however, they are difficult to predict since a variety of mechanisms for sorption of compounds onto both organic and inorganic matrices can occur. The octanol-water coefficient (K_{ow}) is an alternative and it was adopted as the standard measure of the compound hydrophobicity. Generally, hydrophobic compounds (high K_{ow}) can be easily adsorbed onto soil, whereas the hydrophilic ones (low K_{ow}) are mostly removed by other processes (Zhang et al., 2014). Sorption is a mechanism still dependent on other factors, such as the sorbent characteristics, pH, temperature, ionic strength, and presence of co-solutes (Stefanakis et al., 2014). In turn, photodegradation is influenced by the seasonal variation of light intensity and the specific absorbance spectrum of each compound (Zhang et al., 2014). Similarly to other processes, plant uptake is influenced by the physical-chemical characteristics of the pollutants, namely the K_{ow}, pKa and their concentration. In general, compounds with a log K_{ow} between 0.5 and 3 are uptaken easily (Stottmeister et al., 2003). Despite the capacity of plants to detoxify organic pollutants, they play a secondary role in CWs, in comparison to the microorganisms. The nature and extent of microbial degradation is also strongly related to the physical-chemical properties of the contaminant (Zhang et al., 2014) and the metabolism depends on the presence of oxygen and the availability of other electron acceptors (Stottmeister et al., 2003).

CWs design and operational factors, such as wetland configuration, soil/substrate matrix, depth of the substrate, plant species, operating mode (batch or continuous) and hydraulic residence time (HRT), can also affect the removal of pollutants (Zhang et al., 2014). CWs configuration strongly influences the physical-chemical conditions (e.g. redox potential and insolation) of the system (Zhang et al., 2014). For instance, CWs can be classified according to: (i) hydrology (water surface flow and subsurface flow); (ii) plants growth form (free-floating, floating leaved, submerged or emergent); and (iii) flow path (horizontal and vertical). Thus, CWs are usually distinguished in surface flow constructed wetland (SF-CWs), horizontal subsurface flow constructed wetlands (HSSF-CWs) and vertical subsurface flow constructed wetlands (VSSF-CWs), as shown in Fig. 2 where different plants growth forms are represented in the case of SF-CWs (Vymazal, 2007, 2011). Various types of CWs may also be combined (hybrid systems) to achieve higher removal efficiencies (Ebie et al., 2013). Thereby, the configuration should be defined according to the characteristics of the

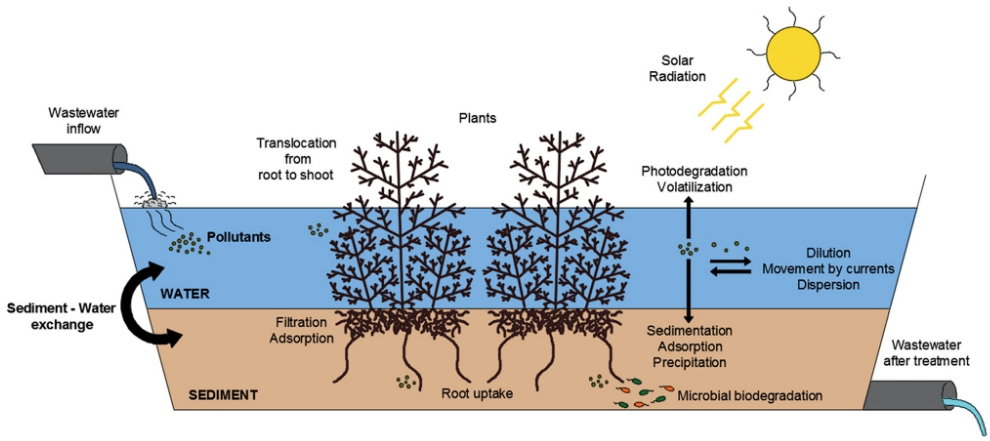

Fig. 1. General mechanisms for the removal of pollutants in CWs - adapted with permission from Zhang et al., 2014. Removal of pharmaceuticals and personal care products in aquatic plant-based systems: a review. Environ Pollut 184, 620–639. Copyright (2014) Elsevier (Zhang et al., 2014).

contaminants to be removed. For example, SF-CWs show better performance for compounds susceptible to photodegradation because water can be directly exposed to sunlight, when the vegetation is regularly harvested, whereas SSF-CWs have a higher potential to eliminate biodegradable compounds, because their substrate promotes higher adsorption and interactions between wastewater, soil, plants and microorganisms. When compared to HSSF-CWs, VSSF-CWs have an enhanced microbial degradation as result of a higher oxygenation originated by the wastewater drainage in the different soil layers (Zhang et al., 2014).

The soil/substrate matrix is also important because it not only supports the growth of plants and microorganisms, but also promotes a series of chemical and physical processes. For example, the sorption by the solid matrix can play an important role in the retention of contaminants, the selection of a material with high sorption capacity being essential to enhance the CWs performance. In addition, substrate depth also affects pollutants removal rates. The selection of the plants species is another fundamental parameter for the operation of CWs, because plants have a variable capacity for nutrient uptake and soil oxygenation, also interfering with the functioning and structure of microbial communities involved in the removal of contaminants (Zhang et al., 2014). Their roots, for example, provide the surface area that is fundamental for the growth of microorganisms. Furthermore, organic compounds can be released as root exudates and, together with dead plant material, can function as an additional electron donor in the microbial anaerobic processes, such as denitrification and sulphate reduction. On the other hand, the plants may also liberate oxygen from their roots, which are able to increase the aerobic microbial degradation. Meanwhile, the root exudates, which contain various compounds, might stimulate the activity of microbes (Chen et al., 2012b). The most popular species existing in CWs are *Typha* ssp. and *Phragmites* ssp. (Li et al., 2014).

Regarding the operating mode, although some conflicting data are available in the literature, batch mode generally provides higher treatment performances than continuous feeding for the reduction of conventional parameters, namely COD, biochemical oxygen demand (BOD_5), total suspended solids (TSS), nitrogen species and phosphorus (Zhang et al., 2012d; Ni et al., 2013). This fact may be explained by the entrainment of air into the micropores of the soil matrix during draining and filling operations, which can promote a micro-aerobic environment, increasing microbiological activity and, as consequence, a higher degradation of pollutants. In a study dealing with planted and unplanted SSF-CWs, no significant differences between batch and continuous flow modes were found for COD removal, whereas phosphorus removal efficiency was higher in batch operating mode and ammonium removal efficiency was enhanced only in batch-loaded planted SSF-CWs (Zhang et al., 2012d). SSF-CWs implementing plants, relatively long HRT and operating in batch mode, were found as a good approach for removal of COD, nitrogen and phosphorus (Ni et al., 2013).

Finally, the HRT also influences contaminants removal efficiencies. Higher HRT allows a longer interaction of contaminants with the wetland system, while lower HRT reduces the contact time between the wastewater, the rhizosphere and the microorganisms. HRT is variable and depends on the type of CWs application; however, it has been reported that the most efficient pollutant removal in CW systems can be achieved in the range of 4–15 d (Zhang et al., 2014).

2.2. Literature survey on organic PSs and CECs removal by CWs

Since the occurrence of PSs and CECs in aquatic systems is nowadays a recognized environmental issue and considering CWs as a promising wastewater treatment solution to eliminate some organic pollutants from the water cycle (Luo et al., 2014; Zhang et al., 2014), a literature survey was performed to gather relevant information on the applicability of CWs for the removal of these pollutants from water/wastewater. The survey was focused on the application of CWs for the treatment of water/wastewater containing organic PSs and certain other substances with EQS defined in the Directive 2013/39/EU as well as CECs of the Watch List of Commission Decision 2015/495/EU. The search was done in *Scopus* database (accessed until June 2016), using as keywords "constructed wetland" and the "name of each PS/substance with EQS or CEC", respectively listed in Tables 1 and 2, including those referred in the footnotes of these tables. The survey was performed by choosing "Article title, Abstract, Keywords" in the search field type. All types of articles were considered, when dealing with the removal of at least one of the target compounds. Studies on this

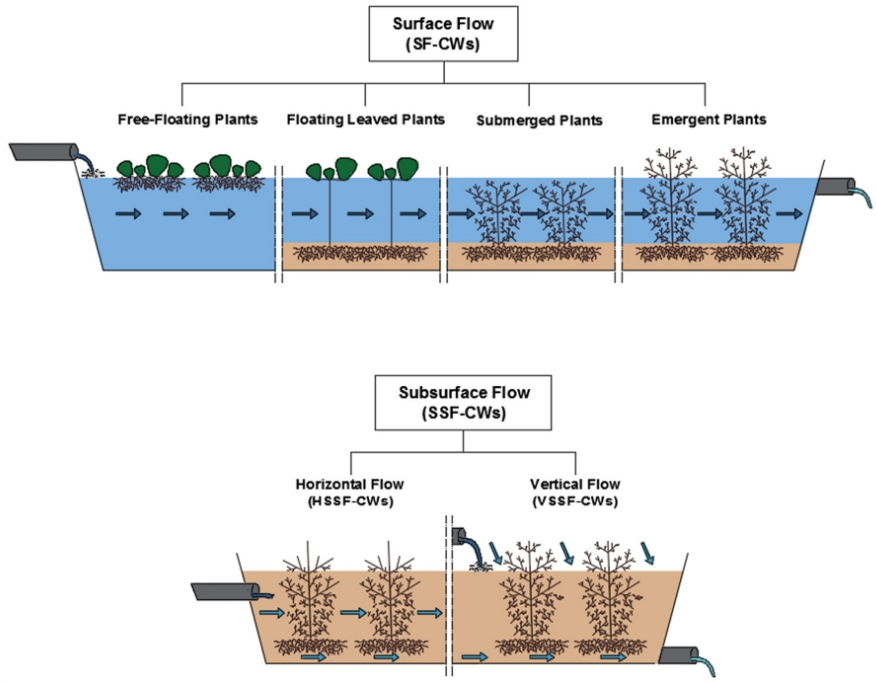

Fig. 2. CWs configurations, according to hydrology (surface or subsurface flow), plants growth forms for surface flow (free-floating, floating leaved, submerged or emergent) and flow path for subsurface flow (horizontal or vertical) - adapted with permissions from: Vymazal, 2007. Removal of nutrients in various types of constructed wetlands. Sci Tot Environ 380(1–3), 48–65. Copyright (2007) Elsevier (Vymazal, 2007); and Vymazal, 2011. Constructed Wetlands for Wastewater Treatment: Five Decades. Environ Sci Technol 45(1), 65–69. Copyright (2011) American Chemical Society (Vymazal, 2011).

subject were only found for 24 of the 41 PSs, 2 of the 8 other substances with EQS mentioned in the Directive 2013/39/EU, and for 8 CECs of the 17 defined in the Watch List. The nominal and relative frequencies of studies dealing with PSs/substances with EQS and CECs are described in Figs. 3 and 5, respectively. The data shown for the concentrations and removals of these pollutants by CWs (i.e., Figs. 4 and 6) was based on the information that was possible to be obtained from the studies listed in Tables 1 and 2 (i.e., a few of which with abstract only available - (Gao et al., 2014; Wen et al., 2013b; Yang et al., 2015)).

2.2.1. Organic PSs defined in Directive 2013/39/EU

The number of studies (n) published so far about removal by CWs of organic PSs that have been recently defined in the Directive 2013/39/EU (Table 1) is shown in Fig. 3, including the distribution by different classes. It is notorious that a quite significant number of studies has been performed for some compounds (e.g., benzene and atrazine, followed by nonylphenol and chlorpyrifos). Pesticides, including triazine, organophosphorus, organochlorine, phenylurea, and other classes of pesticides, namely trifluralin and alachlor, are the class of PSs most studied. There are also several studies dealing with the removal of solvents by CWs, among them the most representative compound being benzene (n = 25), followed by 1,2-dichloroethane (n = 2), dichloromethane (n = 1), trichloromethane (n = 1) and pentachlorobenzene (n = 1). Industrial compounds represent 18.0% of the works testing CWs for their removal, in the following decreasing order of number of studies: nonylphenol > di(2-ethylhexyl)phthalate (DEHP) > octylphenol ≈ perfluorooctane sulfonic acid and its derivatives (PFOS). Polycyclic aromatic hydrocarbons (PAHs), dioxins and dioxin-like compounds, as well as organometallic compounds, are the PSs least reported.

The inlet concentrations of organic PSs in CWs (when it was possible to obtain this information), shown in Fig. 4a and b for non-spiked and spiked wastewater effluents, respectively, were very variable. The removal of benzene, nonylphenol and chlorpyrifos was studied at the highest initial concentrations, each PS at different concentration levels, up to tens of mg L^{-1}, at few mg L^{-1} and at μg L^{-1} levels, respectively. The pesticides isoproturon and diuron were studied up to 88 and 45 μg L^{-1}, respectively, whereas concentrations of only a few μg L^{-1} were found in non-spiked CWs inlets for trifluralin, alachlor, atrazine, simazine, pentachlorophenol and endosulfan, as well as for the industrial compounds octylphenol, PFOS and DEHP, and for the solvents trichloromethane and dichloromethane. Comparing with non-spiked CWs inlets (Fig. 4a), spiked CWs inlet concentrations (Fig. 4b) were quite higher and did not really match non-spiked ones. For instance, nonylphenol is one of the PSs which was found at the highest range of concentrations in the non-spiked CWs inlets but it was studied at the lowest concentration in spiked matrices. Chlorpyrifos and isoproturon, also found at high concentrations in non-spiked CWs inlet solutions, were not considered in any spiked experiment. Simazine, which is normally found in real wastewater at lower concentrations than the other triazine pesticide, atrazine, has been studied at

Table 1
Studies on the applicability of CWs for the removal of organic PSs (and certain other substances with EQS marked with [a]) defined in Directive 2013/39/EU. No studies were found for the substances/classes listed in footnote.

Class	Name of PS - (n = no. of studies)	References
Industrial compounds	Octylphenol - **(3)**	(Hsieh et al., 2015; Hsieh et al., 2013; Sacco et al., 2006)
	Nonylphenol - **(12)**	(Belmont et al., 2006; Belmont and Metcalfe, 2003; Braun et al., 2003; Carranza-Diaz et al., 2014; Gebremariam and Beutel, 2010; Hsieh et al., 2015; Hsieh et al., 2013; Olujimi et al., 2013; Papaevangelou et al., 2016; Qiang et al., 2013; Sima et al., 2013; Toro-Velez et al., 2016)
	Perfluorooctane sulfonic acid and its derivatives (PFOS) – **(3)**	(Chen et al., 2012a; Ololade et al., 2016; Plumlee et al., 2008)
	Di(2-ethylhexyl)phthalate (DEHP) – **(4)**	(Ahn et al., 2004; Wen et al., 2013a; Wen et al., 2016; Xiaoyan et al., 2015)
Solvents	Trichloromethane – **(1)**	(Keefe et al., 2004)
	Dichloromethane – **(1)**	(Keefe et al., 2004)
	1,2-dichloroethane – **(2)**	(Kassenga et al., 2004; Kassenga and Pardue, 2006)
	Pentachlorobenzene – **(1)**	(Matamoros et al., 2007b)
	Benzene – **(25)**	(Braeckevelt et al., 2007; Brovelli et al., 2011; Chen et al., 2012b; Chen et al., 2014b; De Biase et al., 2013; De Biase et al., 2011; Eke and Scholz, 2008; Fester, 2013; Ho et al., 2012; Mothes et al., 2010; Ranieri et al., 2013; Ranieri et al., 2014; Ranieri et al., 2015; Reiche et al., 2010; San Miguel et al., 2013; Schurig et al., 2015; Seeger et al., 2013; Seeger et al., 2011; Tang et al., 2009a,b; Tang et al., 2010; Van Afferden et al., 2011; Voyevoda et al., 2012; Wei et al., 2015; Xu et al., 2014)
Dioxin-like compounds	Polychlorinated dibenzo-p-dioxins (dioxin and dioxin-like compounds) – **(1)**	(Avila et al., 2014a)
PAHs (listed separately)	Naphthalene – **(1)**	(Poerschmann and Schultze-Nobre, 2014)
	Fluoranthene – **(1)**	(Cottin and Merlin, 2008)
Others: dinitroaniline pesticide	Trifluralin – **(2)**	(Miller et al., 2002a; Warsaw et al., 2012)
chloroacetanilide pesticide	Alachlor – **(2)**	(Matamoros et al., 2007b; Miller et al., 2002a)
Phenylurea	Isoproturon – **(5)**	(Passeport et al., 2013; Passeport et al., 2010; Vallee et al., 2016; Vallée et al., 2014; Vallee et al., 2015)
	Diuron – **(4)**	(Bois et al., 2011; Matamoros et al., 2007b; Page et al., 2010; Rose et al., 2006)
Organotins	Tributyltin compounds – **(2)**	(Anderson et al., 2002; Matamoros et al., 2009)
Triazines	Simazine – **(6)**	(George et al., 2003; Matamoros et al., 2007b; Page et al., 2010; Page et al., 2011; Stearman et al., 2003; Wilson et al., 2011)
	Atrazine – **(25)**	(Alvord and Kadlec, 1995; Anderson et al., 2013; Andersona, et al., 2002; Bouldin et al., 2005; Douglass et al., 2014, 2015; Friesen-Pankratz et al., 2003; Kadlec and Hey, 1994; Lin et al., 2008; Lizotte et al., 2014; Locke et al., 2011; McKinlay and Kasperek, 1999; Miller et al., 2002b; Moore et al., 1999; Page et al., 2010; Page et al., 2011; Passeport et al., 2013; Reinhold et al., 2010; Runes et al., 2001a; Runes et al., 2001b; Runesa et al., 2003; Sharif et al., 2014; Spongberg and Martin-Hayden, 1997; Wanko et al., 2009; Weaver, et al., 2004)
Organophosphorus	Chlorpyrifos (chlorpyrifos-ethyl) – **(11)**	(Agudelo et al., 2012; Agudelo et al., 2011; Agudelo et al., 2010; Budd et al., 2009; Budd et al., 2011; Gao et al., 2014; Gebremariam and Beutel, 2010; Moore et al., 2002; Rogers and Stringfellow, 2009; Schulz and Peall, 2001; Sherrard et al., 2004)
	Chlorfenvinphos – **(1)**	(Blankenberg et al., 2007)
Organochlorine	Hexachlorobenzene – **(2)**	(Zhou et al., 2013; Zhou et al., 2012)
	Pentachlorophenol – **(2)**	(Choudhary et al., 2013; Matamoros et al., 2007b)
	Endosulfan – **(5)**	(Matamoros et al., 2007b; Rose et al., 2008; Rose et al., 2006; Schulz and Peall, 2001; Zhao et al., 2014)
	[a] Dichlorodiphenyltrichloroethane (DDT) – **(1)**	(Peters et al., 2001)
	[a] Dieldrin – **(1)**	(Peters et al., 2001)

No studies were found for the other 17 organic PSs (some are classes of substances) defined in Directive 2013/39/EU, namely: trichlorobenzenes, chloroalkanes, dioxin and dioxin-like compounds (polychlorinated biphenyls and polychlorinated dibenzofurans), anthracene, polycyclic aromatic hydrocarbons (indeno(1,2,3-cd)pyrene, benzo(k) fluoranthene, benzo(g,h,i)perylene, benzo(k)fluoranthene, benzo(b)fluoranthene, benzo(a)pyrene), hexabromocyclododecanes (γ-hexabromocyclododecane, β-hexabromocyclododecane, α-hexabromocyclododecane, 1,2,5,6,9,10-hexabromocyclododecane, 1,3,5,7,9,11-hexabromocyclododecane), brominated diphenylethers (heptabromodiphenylether, hexabromodiphenylether, pentabromodiphenylether, tetrabromodiphenylether), bifenox, aclonifen, quinoxyfen, cypermethrin, terbutryn, cybutryne, dichlorvos, hexachlorobutadiene, hexachlorocyclohexane, heptachlor and its epoxide, dicofol.

[a] No studies were either found for 6 of the other substances with EQS defined in Directive 2013/39/EU, namely: trichloro-ethylene, tetrachloro-ethylene, carbon tetrachloride, endrin, isodrin and aldrin.

higher concentrations than atrazine in studies using spiked inlet solutions. In addition, benzene and the triazine pesticides (atrazine and simazine) were the compounds spiked at the highest concentrations, at 1 g L^{-1} and up to hundreds of mg L^{-1}, respectively. Overall, the removal of atrazine has been evaluated at the largest range of concentrations (Fig. 4b). The elimination of the pesticides alachlor and diuron, of the industrial compound PFOS and of the solvent pentachlorobenzene was assessed only in one study for each compound, by spiking (few mg L^{-1}) the inlet solution with one of these compounds. Only one study using spiked (few μg L^{-1}) sample was also available for nonylphenol. The pesticide chlorpyrifos was studied up to 3 μg L^{-1} and hexachlorobenxene up to 2.5 μg L^{-1}. It is important to stress that the concentrations of the pollutants can affect the removal efficiency in processes involving biodegradation and adsorption. In the case of biodegradation, high concentrations can lead to destabilization of the microbial community, whereas a saturation of the binding sites can occur in the case of adsorption (Hussain et al., 2015).

The removal percentages obtained in CWs to eliminate organic PSs in both non-spiked and spiked water/wastewater are shown in Fig. 4c. The industrial compounds octylphenol, nonylphenol and PFOS were removed at poor to high extent, whereas DEHP was eliminated between 19 and 49%. The pesticides pentachlorophenol and endosulfan were highly removed by CWs (>80%), and

Table 2
Studies on the applicability of CWs for the removal of CECs defined in the Watch List of Commission Decision 2015/495/EU. No studies were found for the compounds listed in footnote.

Class	Name of CEC - (n = no. of studies)	References
Neonicotinoids	Imidacloprid – **(1)**	(Mahabali and Spanoghe, 2013)
Macrolide antibiotics	Erythromycin – **(5)**	(Berglund et al., 2014; Carlson et al., 2013; Du et al., 2014; Hijosa-Valsero et al., 2011a; Rühmland et al., 2015)
	Clarithromycin – **(4)**	(Berglund et al., 2014; Carlson et al., 2013; Hijosa-Valsero et al., 2011a; Rühmland et al., 2015)
	Azithromycin – **(1)**	(Berglund et al., 2014)
Anti-inflammatory	Diclofenac – **(28)**	(Avila et al., 2014b; Avila et al., 2010; Avila et al., 2013; Carranza-Diaz et al., 2014; Du et al., 2014; Hijosa-Valsero et al., 2011b; Hijosa-Valsero et al., 2010; Hijosa-Valsero et al., 2011c; Hijosa-Valsero et al., 2016; Hijosa-Valsero et al., 2011d; Li et al., 2014; Matamoros et al., 2007a; Matamoros et al., 2009; Matamoros et al., 2012; Matamoros and Bayona, 2006; Matamoros et al., 2008; Matamoros and Salvado, 2012; Nowrotek et al., 2016; Nowrotek et al., 2015; Reif et al., 2011; Reyes-Contreras et al., 2012; Rossi et al., 2013; Rühmland et al., 2015; Zhang et al., 2015; Zhang et al., 2012a; Zhang et al., 2012b; Zhang et al., 2012c)
Estrogens	Estrone (E1) – **(10)**	(Chapman, 2003; Chen et al., 2014a; Gray and Sedlak, 2005; Herrera-Melián et al., 2014; Peterson and Lanning, 2009; Qiang et al., 2013; Shappell et al., 2007; Song et al., 2009; Vymazal et al., 2015; Yang et al., 2015)
	17-beta-estradiol (E2) – **(10)**	(Cai et al., 2012; Chen et al., 2014a; Gray and Sedlak, 2005; Herrera-Melián et al., 2014; Hsieh et al., 2015; Peterson and Lanning, 2009; Qiang et al., 2013; Shappell et al., 2007; Song et al., 2009; Vymazal et al., 2015)
	17-alpha-ethinylestradiol (EE2) – **(5)**	(Avila et al., 2014b; Hsieh et al., 2015; Kumar et al., 2011; Song et al., 2009; Vymazal et al., 2015)

No studies were found for the other 9 CECs, namely: triallate, oxadiazon, acetamiprid, clothianidin, thiamethoxam, thiacloprid, methiocarb, 2-ethylhexyl-4-methoxycinnamate and 2,6-di-*tert*-butyl-4-methylphenol.

chlorpyrifos was eliminated by more than 50%, but the concentration of isoproturon and hexachlorobenzene was only moderately reduced (from 30 to 75%, depending on the compound). Whereas trifluralin was not removed in CWs, the performance observed for the elimination of alachlor and diuron varied up to 80 and 55%, respectively. The triazine pesticides had different behaviours, simazine was removed between 20 and 96%, whereas atrazine presented very dissimilar removals (either inefficient removal or removed to levels below the analytical detection limits).

2.2.2. CECs in the Watch List of Commission Decision 2015/495/EU

The studies on the removal of CECs by CWs (Table 2) are represented in Fig. 5. The most studied substances were: diclofenac (with almost half of the studies), followed by the macrolide antibiotics (erythromycin, clarithromycin and azithromycin), 17-beta-estradiol and estrone (respectively E2 and E1) with the same % of studies and then the synthetic hormone 17-alpha-ethinylestradiol (EE2). It is interesting to note that diclofenac, E2 and EE2 suggested in Directive 39/2013/EU (i.e. earlier in 2013) as compounds to be included in the first Watch List, now represent together almost 70% of the published studies on CWs, in contrast with the other compounds that were only identified in 2015, pointing a possible impact of the EU legislation on the research done. The data collected show also that the three macrolide antibiotics, erythromycin, clarithromycin and azithromycin, are still poorly studied (15.6% for the three drugs) in comparison to diclofenac and the three estrogens, despite their known potential negative impact on the environment. Neonicotinoids (imidacloprid) are only reported in one study. It seems that the other CECs included in the Watch List were not considered in any study, namely the pesticides triallate, oxadiazon, acetamiprid, clothianidin, thiamethoxam, thiacloprid and methiocarb, the UV filter 2-ethylhexyl 4-methoxycinnamate (EHMC) and the antioxidant 2,6-di-*tert*-butyl-4-methylphenol commonly used as food additive.

The CWs inlet concentrations of the CECs included in the Watch List are summarized in Fig. 6a and b, respectively for non-spiked and spiked wastewater. In both cases, diclofenac was the compound found at the highest range of concentrations. Diclofenac removal was evaluated in spiked inlet solutions with concentrations of the same order of magnitude than those found in realistic samples, i.e., up to 37 μg L^{-1} (Fig. 6a), but higher concentrations were also tested in spiked water/wastewater, i.e., up to 5000 μg L^{-1} (Fig. 6b).

The macrolide antibiotics clarithromycin and erythromycin were found in the CWs inlets and their elimination assessed at concentrations lower than 0.3 μg L^{-1}; however, the experiments performed with spiked water/wastewater employed higher concentrations (0.4 μg L^{-1} and 2 μg L^{-1}, respectively). These experiments also included azithromycin (ca. 0.3 μg L^{-1}). The three estrogens, E1, E2 and EE2, were studied in non-spiked CWs inlets at concentrations lower than 0.2 μg L^{-1}, but only E2 was reported in spiked experiments, using concentrations quite higher (2250 μg L^{-1}). Overall, CECs listed in the Watch List were found at lower concentrations in the realistic samples when compared with PSs (Section 2.2.1). The spiked experiments were also designed using inlet solutions with lower concentrations of CECs than those of PSs.

Regarding the performance of CWs to remove CECs, Fig. 6c shows very wide ranges for the removal of diclofenac, the antibiotics erythromycin and clarithromycin, and the estrogens E1 and E2. The three estrogens were eliminated up to 100%, with a minimal removal of 17% for EE2. The spiked concentration of the neonicotinoid pesticide imidacloprid was reduced by $\geq$ 70%, whereas azithromycin was removed to levels below the analytical detection limits.

2.3. Influence of CWs configuration and operating parameters

As referred above, CWs are complex systems where physical, chemical and biological processes may occur simultaneously, and these processes depend among other factors, on the design and operating parameters of the CWs. Thus, distinct conditions tested can have as consequence variable efficiencies. By the analysis of Figs. 4c and 6c, it is possible to verify that the studied PSs and CECs can be highly removed by CWs in most cases, exceptions being found for DEHP, trifluralin, isoproturon and diuron, with removals always lower than 60%. However, it is important to mention that the comparison between different studies and the respective removal efficiency achieved for different compounds is merely indicative since different CWs systems and operating conditions were tested in most of these studies. Moreover, the characteristics of the inlet solution may also influence the treatment occurring in

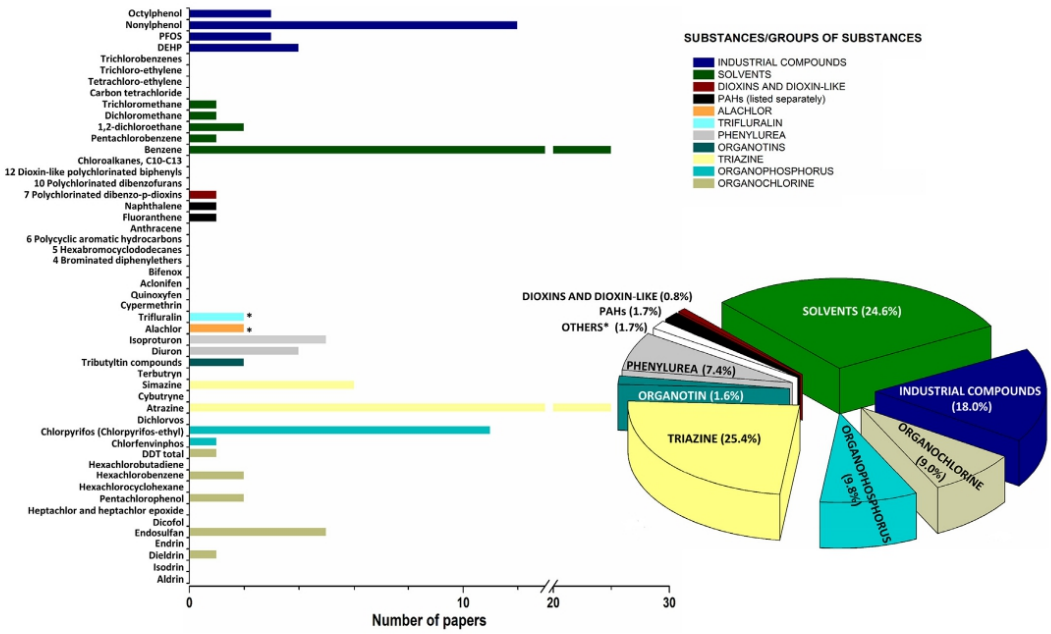

Fig. 3. Number and percentage of studies published dealing with removal by CWs of organic PSs and substances with EQS listed in Directive 2013/39/EU, based on the *Scopus* database search employing as keywords "constructed wetland" and the "name of each PS/substance with EQS" (accessed until June 2016). *Others: trifluralin and alachlor.

CWs, which can also explain the different results for the same contaminant.

In any case, among the different parameters, wetland configuration can have a drastic effect on pollutants removal. For instance, nonylphenol removal rates may vary between negative and 88% due to the CWs configuration (Papaevangelou et al., 2016). The highest removal was observed in VSSF-CWs where aerobic conditions prevail and both planted and unplanted HSSF-CW systems had, in most cases, higher concentrations of nonylphenol in the outlet than in the inlet. This fact might be explained by the biodegradation of its precursors nonylphenol ethoxylates under anaerobic conditions, that leads to the formation of the parent compounds. However, in another study, a significant influence of the season in the removal of nonylphenol was observed. Results demonstrate very different performances in winter and summer for the same subsurface CW system, respectively with removals of 25 and 80%. The lower removal observed in winter may be due the colder temperature, which can inhibit microbial activity (Qiang et al., 2013). Wetland configuration also affected DEHP removal, which varied between 19 and 49%, being the highest removal also observed in VSSF-CWs (Xiaoyan et al., 2015). In this configuration, the unsaturated media promotes higher oxygen diffusion inside the matrix pores, increasing the oxygen transfer capacity and, as consequence, the DEHP biodegradation and removal (Xiaoyan et al., 2015). The different CW designs also influenced erythromycin and clarithromycin removals. In a study analyzing the efficiency of SF-CWs and HSSF-CWs (with or without vegetation), erythromycin was only eliminated in a planted HSSF-CW system (64%), whereas clarithromycin was partially removed in a free water SF-CW (50%). For erythromycin, the results emphasize the general better performance of SSF-CWs when compared with SF-CWs. In the case of clarithromycin, the SF-CW system was better, maybe because photodegradation is the main elimination pathway in this case (Hijosa-Valsero et al., 2011a). In another study, the removal efficiency of clarithromycin varied in a VSSF-CW system, depending on the season (0–17% in the summer and 89% in the winter), whereas the elimination of erythromycin was higher than 86% in all sampling campaigns (Rühmland et al., 2015). However, authors did not provide an explanation for these different behaviours.

The HRT is another operating parameter that can affect the removal of pollutants. For instance, in a study including the two natural estrogens E2 and E1, no degradation occurred at HRT = 27.5 h, whereas the removals significantly increased (up to 59% and 85%, respectively) with a HRT of 137.5 h (Chen et al., 2014a), probably due to a higher time of contact between the pollutant and CW substrate.

In general, plants have a positive impact on the removal of the studied pollutants, namely due to the stimulation of the microbial communities present in the CWs systems, as mentioned previously in section 2.1. Benzene removal was often higher in the presence of plants than in systems without them (Chen et al., 2012b), whereas erythromycin was only removed in HSSF-planted systems (Hijosa-Valsero et al., 2011a). The selection of plant species is also important. For instance, nonylphenol removal in a VSSF-CW planted with *Typha latifolia*, showed the highest removal capacity (87.5%) whereas the VSSF-CW unit planted with *Phragmites australis* showed a negative removal for this compound, probably due to the presence of its precursor in the inlet solution (Papaevangelou et al., 2016). Plant species also affected PFOS removal, with *Hygrophila pogonocalyx Hayata* showing higher PFOS uptake in comparison with other three aquatic plants, due to a strong adsorption of PFOS and the plant characteristics, such as its small-size root biomass,

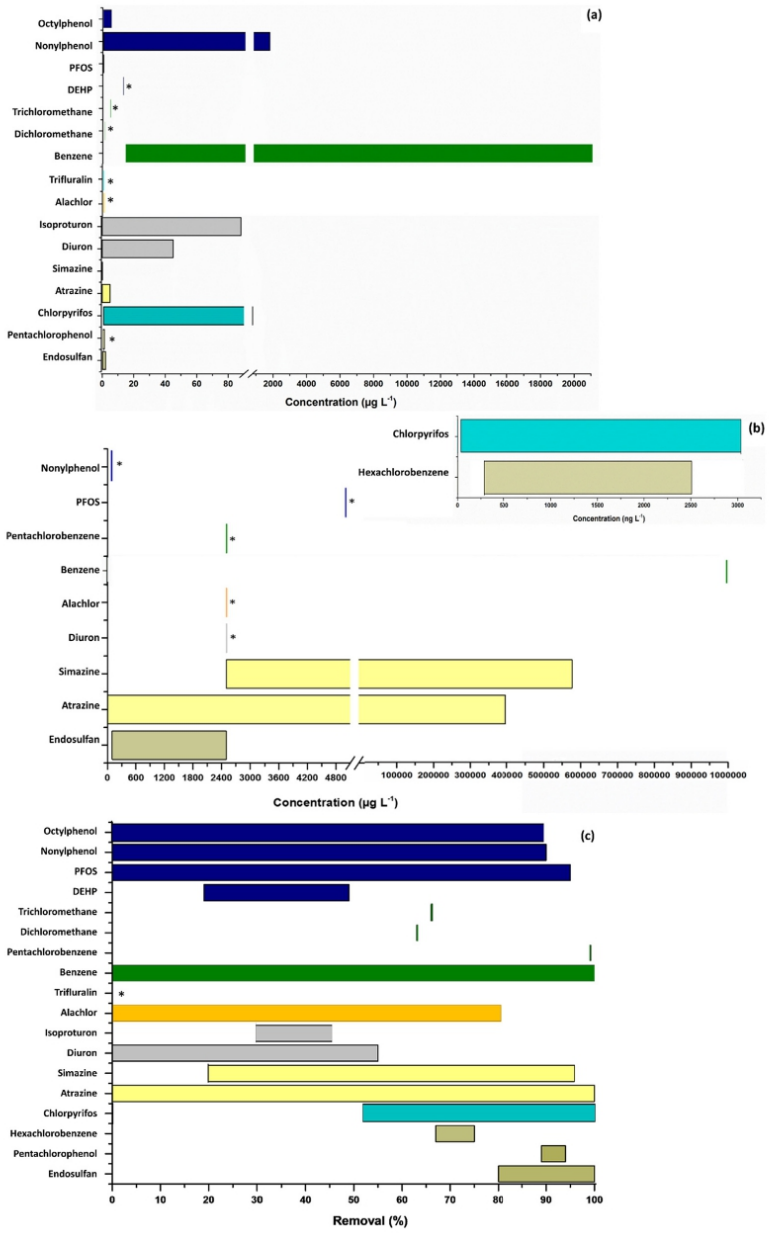

Fig. 4. Concentration range of **(a)** 16 organic PSs in non-spiked wastewater and **(b)** 11 organic PSs in spiked water/wastewater used as inlet solution for the removal of PSs by CWs (* only one initial concentration was found). **(c)** Removals (%) achieved for 18 PSs in CWs. The compound marked with * was not removed.

larger root surface area, faster root growth and shallower root distribution comparatively to the other tested plants (Chen et al., 2012a). Erythromycin was only removed in *Phragmites australis* planted CWs (removal up to 64%) whereas no removal of this CEC was observed in *Thypha angustifolia* planted CWs (Hijosa-Valsero et al., 2011a). So, plants characteristics can have a clear effect on

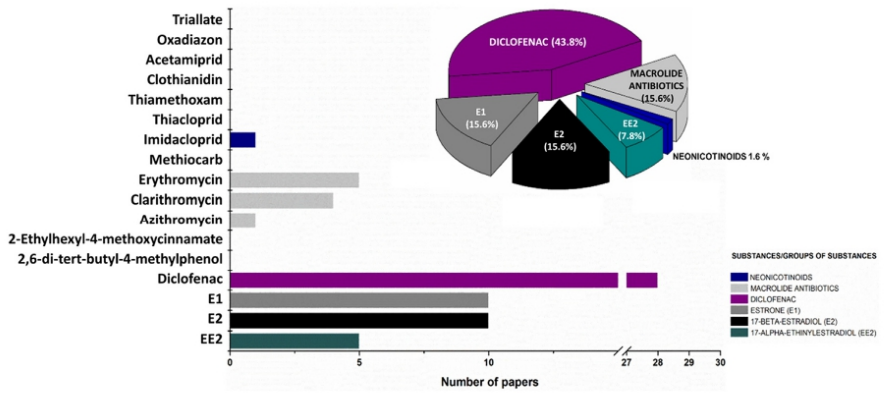

Fig. 5. Number and percentage of studies published dealing with removal by CWs of CECs listed in Watch List of Decision 2015/495/EU, based on the *Scopus* database search employing as keywords "constructed wetland" and the "name of each CEC" (accessed until June 2016).

CWs removal processes.

Substrate type and its depth can also affect pollutants removal due to its sorption characteristics. As example, gravel appeared to be the most efficient filter material, in comparison to vesuvianite or zeolite (both with higher porosity), to remove DEHP from wastewater (a PS with a high K_{ow}), probably because that material favoured the retention of organic matter and the growth of biofilm on it, contributing for a higher microbial activity which biodegraded more efficiently the compound (Xiaoyan et al., 2015). Different depth VSSF-CWs (7.5, 30, 60 cm) were tested to remove E1, E2 and EE2, which were more efficiently eliminated with a depth of 7.5 cm (Song et al., 2009). This result could be related to the highest root density and the higher penetration of oxygen. Removal of atrazine from a wastewater was also very variable (inefficient removal or elimination to levels below the analytical detection limits) and very dependent upon the specific CWs substrates (Anderson et al., 2013).

So, results obtained so far clearly indicate that the CWs parameters must be properly optimized for an efficient removal of pollutants. A clear example is diclofenac, the most studied CEC (Fig. 5) and the most studied among all pollutants considered in this review (Tables 1 and 2). Fig. 6c shows that diclofenac removal varies between 0 and more than 96% (but often up to 60%), the removal depending on wetland configuration, substrate type, HRT and plant species (Hijosa-Valsero et al., 2016; Reyes-Contreras et al., 2012).

Reported studies indicate that CWs are still viewed as a "black box" where only influent and effluent concentrations are measured to evaluate their performance, without details about the processes occurring, that affect the fate or transformation pathways of the contaminants. For instance, during degradation, pollutants can originate other compounds (transformation products) that can be more toxic than their parent compounds. In this regard, it is imperative to understand the basic elimination and transformation processes that drive the removal of pollutants in such "black box", to optimize the CWs design and operational parameters and consequently get better treatment efficiencies for a higher number of pollutants (Zhang et al., 2014).

As shown above, some PSs and CECs can be eliminated to levels below the analytical detection limits when using CWs. However, due to the refractory nature of other PSs and CECs, CWs seem to be limited in providing their adequate elimination from contaminated water/wastewater. Combining or integrating CWs with other existent technologies, such as membrane processes, COTs and AOPs, with proved efficiency on the removal of PSs (Ribeiro et al., 2015) and CECs (Barbosa et al., 2016b), could be a good solution. It can maximize the individual advantages of these processes, such as the low cost of CWs and the high efficiency (but high cost, due to the need of external energy and chemicals) of other separation and oxidation processes. Most of the studies reporting the combination of CWs with other processes are focused on pollutants in general, few considering the substances listed in EU legislation. As example, a report (Araña et al., 2008) employed heterogeneous TiO_2 photocatalysis as pre-treatment of a wetland to treat two fungicides and two commercial mixtures of pesticides, one containing oxadiazon which is included in the Watch List. This compound was the only one refractory to TiO_2 photocatalysis and seemed to inhibit the biological process. In another work (Rossi et al., 2013), a sand filter/CW was combined with UV degradation to enhance adsorption and the elimination of recalcitrant compounds, including diclofenac, from the effluents of conventional WWTPs. In this study, diclofenac was photodegraded to below the analytical detection limit in less than 1 min.

The combination of CWs with other treatment technologies was recently reviewed targeting organic and nutrient removals, the elimination of persistent organics and metals in general (Liu et al., 2015). Considering the advantages, disadvantages and respective operating cost of different alternatives (activated sludge, anaerobic reactor, membrane bioreactor, TiO_2-based photocatalysis, electrolysis, RO), the authors also concluded that more research is required to propose optimal combinations for each type of wastewater.

3. Conclusions

Removal of pollutants by CWs is a recent area of study, which needs more research to get the feasibility in terms of full-scale application. Despite the recognized potential of CWs to remove pollutants from contaminated water/wastewater, studies dealing with pollutants defined in recently launched EU legislation are still limited to a few of these pollutants. In fact, only 24 out of a total of 41 organic PSs plus 2 of 8 pollutants with defined EQS to be considered, and 8 CECs out of 17, were studied.

Regarding PSs, benzene and atrazine are those receiving higher attention in literature regarding their removal by CWs, followed by

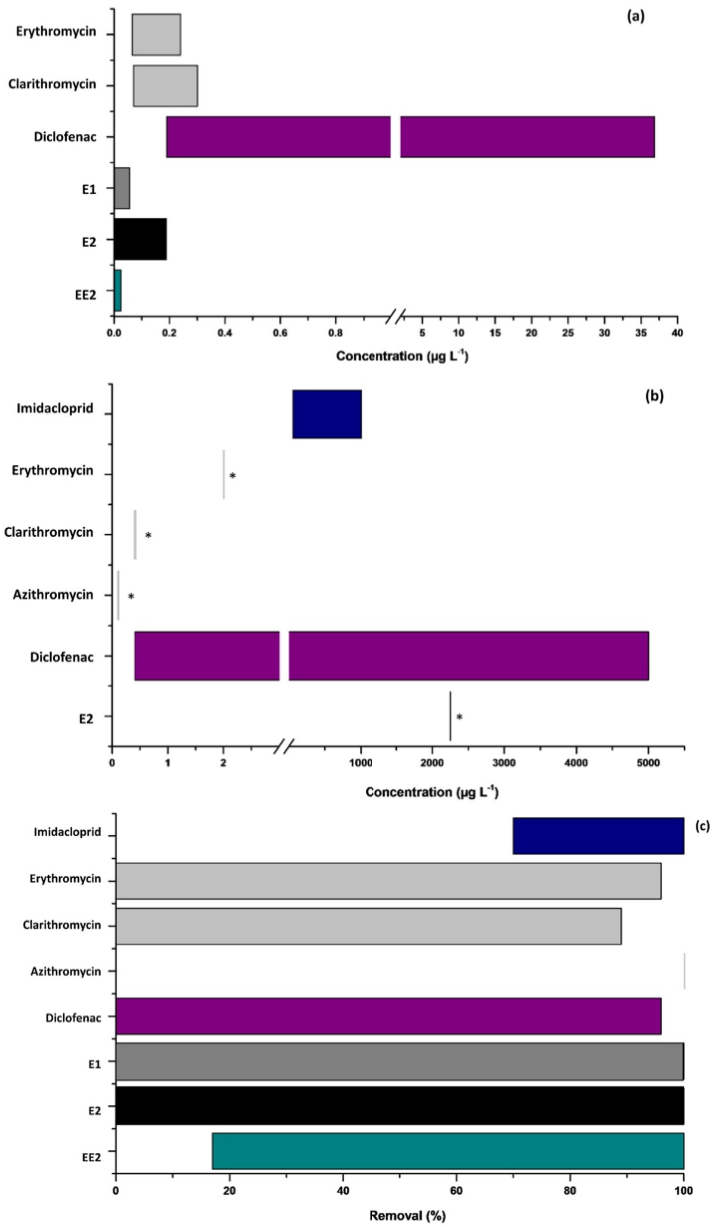

Fig. 6. Concentration range of CECs in (**a**) non-spiked and (**b**) spiked water/wastewater used as inlet solution for the removal of CECs by CWs (* only one spiked concentration was found). (**c**) Removals (%) achieved for 8 CECs in CWs.

nonylphenol and chlorpyrifos. Although several reports deal with the removal of solvents (24.6% of total studies), pesticides are the most studied group of substances, including triazine (25.4%), organophosphorus (9.8%), organochlorine (9.0%), phenylurea (7.4%) and other classes of pesticides. Diclofenac is the CEC that has been most investigated (appearing in more than 40% of the publications with CECs), followed by the macrolide antibiotics and the estrogens E1 and E2. Thus, studies related to the application of CWs for the

removal of many other organic PSs, certain other substances with EQS and some CECs are still missing, as well as reports dealing with mixtures of several of these pollutants. These studies must be preferentially performed at realistic concentrations. In fact, the concentrations of PSs, certain other substances with EQS, and CECs, were below 2000 μg L^{-1} in studies with non-spiked water/wastewater, with exceptions found for benzene. Spiked experiments were usually done for one pollutant or a set of some pollutants with concentrations that were several orders of magnitude higher than those actually found in water/wastewater.

Despite the need to extend the studies for other organic pollutants defined in EU legislation, studies regarding their removal in CWs must imperatively contemplate the understanding of the processes responsible for it. Only with that information one can optimize CWs design and operating parameters, consequently getting better treatment efficiency and fully supporting CWs utility. Tasks that are suggested to be addressed in the future, extending the research in this area still poorly explored, are: (i) seasonal sampling campaigns to evaluate the most frequent PSs, certain other substances with EQS and CECs in each type of water/wastewater; (ii) CWs experiments using different operating conditions (configuration, light penetration, residence time), to assess their effects and achieve the best performance of CWs; (iii) CWs experiments with inlet solutions spiked with more PSs, certain other substances with EQS and CECs at realistic levels to evaluate CWs removal of a wider set of pollutants; (iv) to study the transformation products of pollutants during CWs treatment, evaluating their possible elimination together with the parent compound; (v) evaluation of adequate approaches that can be coupled to CWs to eliminate more recalcitrant compounds.

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"Advanced oxidation technologies and constructed wetlands in aquaculture farms: What do we know so far about micropollutant removal?"

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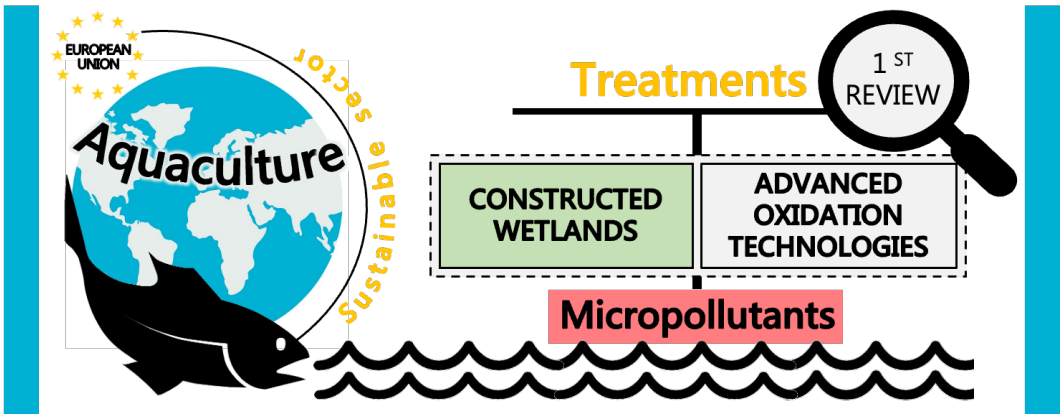

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Advanced oxidation technologies and constructed wetlands in aquaculture farms: What do we know so far about micropollutant removal?

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ABSTRACT

Aquaculture is the fastest growing animal food-producing sector. Water is the central resource for aquaculture, and it is essential that its quality be preserved. Micropollutants (MPs) can reach aquaculture through anthropogenic addition or inlet water, and may cause harmful effects such as endocrine disruption and antibiotic resistance, adversely affecting the fish species being farmed. Furthermore, the discharge of aquaculture effluents into the environment may contribute to the deterioration of water courses. In this sense, the implementation of environmentally responsible measures in aquaculture farms is imperative for the protection of ecosystems and human health. The European Commission (EC) has recently launched a guiding document promoting ecological aquaculture practices; however, options for water treatment are still lacking. Conventional processes are not designed to deal with MPs; this review article consolidates relevant information on the application of advanced oxidation technologies (AOTs) and constructed wetlands (CWs) as potential strategies in this regard. Although 161 studies on the application of AOTs or CWs in aquaculture have already been published, only 34 focused on MPs (28 on AOTs and 6 on CWs), whereas the others reported the removal of contaminants such as bacteria, organic matter, solids and inorganic ions. No study coupling both treatments has been reported to date for the removal of MPs from aquaculture waters. AOTs and CWs are prospective alternatives for the treatment of aquacultural aqueous matrices. However, the type of aquaculture activity and the specifications of these available technologies should be considered while selecting the most suitable treatment option.

1. Introduction

Ensuring the quality and safety of water is among the foremost environmental concerns of this century. Micropollutants (MPs), pollutants that exist in very small traces in water (usually ranging between ng L^{-1} and μg L^{-1} levels), have been attracting the attention of the scientific community because of their frequent occurrence in the aquatic environment even after passing through conventional water and wastewater treatment systems (Barbosa et al., 2016; Luo et al., 2014). MPs include diverse classes of substances such as pharmaceuticals and personal care products, hormones, industrial compounds, X-ray contrast media and pesticides. A large number of sources thus contribute to the introduction of MPs into the environment. Aquaculture farms are also considered to be a possible source of MPs in the aquatic environment (Barbosa et al., 2016; Gorito et al., 2021). The artificial food and medication applied in this type of food industry can introduce MPs in aquaculture effluents. Furthermore, the water collected for use in these activities can also contain MPs. Therefore, MPs are present in both aquaculture influents and effluents as well as in the resulting food products, posing risks to wildlife and human health alike (Muñoz et al., 2010).

At present, aquaculture provides nearly half of the total fish consumed worldwide, and a production increase superior than 60 % is expected during the next years (FAO, 2018). In this context, it is important to establish an environmentally responsible aquaculture sector. Appropriate treatment processes capable of removing MPs and other relevant pollutants need to be adopted in order to preserve the environment (Gorito et al., 2018; Huang et al., 2019). The removal of

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Abbreviations

Advanced oxidation technologies AOTs
Antibiotic resistant bacteria ARB
Antibiotic resistance genes ARGs
Constructed wetlands CWs
Contaminants of emerging concern CECs
Environmental quality standards EQS
European Commission EC
European Union EU
Hydrogen peroxide H_2O_2
Micropollutants MPs
Non-recirculated aquaculture systems Non-RAS
Ozone O_3
Priority substances PSs
Recirculating aquaculture systems RAS
Taste and odour T&O
Titanium dioxide TiO_2

MPs from aquaculture waters is a crucial issue but is poorly investigated. Thus, given the potential of advanced oxidation technologies (AOTs) and constructed wetlands (CWs) to remove various types of contaminants from aquatic environments, the present review gathers relevant information on the topic, providing a detailed analysis of data reported in the literature concerning AOTs and CWs to remove MPs from aquaculture waters (influents and effluents). The current and future perspectives on the application of these treatments in the aquaculture sector are also reviewed and discussed.

1.1. Characterization of aquaculture farms

The practice of aquaculture consists of farming aquatic organisms (finfish, shellfish and/or plants) in fresh, salty or brackish water, using specific techniques to increase their production beyond the natural capacity of the environment (Muñoz et al., 2010). Owing to the diversity of aquaculture farms, they are usually characterised according to their: (i) intensity (extensive, semi-intensive or intensive); (ii) water exchange (open, semi-closed or closed); (iii) culture structure (e. g. cages, raceways or concrete/fiberglass tanks) and (iv) farming methods (mono- or poly-culture).

Based on their intensity regime, aquaculture farms can be classified as extensive, semi-intensive or intensive, depending on the production levels of the organisms and the resources needed to support them (European Commission, 2015; Oddsson, 2020). Industrial-scale aquaculture farms often work under an intensive regime because they involve a large number of produced organisms and the control of several parameters through external intervention, namely the water quality and flow, fish breeding and growth, and food supplementation (Muñoz et al., 2010).

Farms can be classified as open, semi-closed or closed systems depending on the water exchange category utilized (Colt, 1991; Tidwell, 2012). Open systems are based on the use of the natural environment as the farm itself; although food can be supplied, the water quality is maintained by natural flows and processes. Thus, the operational costs are very low, but the production rates are highly dependent on natural conditions (Lawson, 1995). Semi-closed or non-recirculated aquaculture systems (non-RAS) are still influenced by the natural environment, but the production units are broadly human-made. In such cases, a natural source of water is used, and external inputs supplement natural processes and maximise production (Tidwell, 2012). Static ponds and flow-through systems are the main types of non-RAS, typically used to produce channel catfish, trout and salmon (Colt, 1991). In non-RAS, high yields are obtained without a considerable cost increase; however, water quality issues and related diseases in the organisms are likely to occur (Tidwell, 2012). In contrast, closed or recirculating aquaculture systems (RAS) follow a regime where the water is reused, and thus, a high degree of external intervention is required to monitor all processes. Physical, chemical, and microbiological parameters are carefully controlled, and several types of equipment are necessary to guarantee the quality of recirculated water (e.g. removal of solids, nutrients, and pathogens) (Badiola et al., 2012). Therefore, capital costs are high, and specialised operators as well as careful management are required (Badiola et al., 2012; Schneider et al., 2006).

There are many types of aquaculture structures, and their selection depends on the category of the farm in question. Cages, raceways and concrete/fiberglass tanks are typically employed in open, semi-closed and closed systems, respectively (Wahid et al., 2017). Moreover, the farming method depends on the number of the species produced, specifically monoculture for one species, or polyculture if two or more species are involved (Iliyasu et al., 2016). Although uncommon, it is possible to take advantage of different systems by combining some of their aspects to produce hybrid systems (such as aquaponics, in-pond raceways, and portioned aquaculture systems), creating a more profitable process (Tidwell, 2012).

As expected, the large variety of aquaculture farms results in distinct environmental impacts, as well as different levels of contamination and required control measures. Therefore, adequate ecological prevention actions for each type of system in the sector should be encouraged towards establishing sustainability as well as more universal food safety standards (Lazard et al., 2010).

1.2. Occurrence rates and treatment options for MPs in aquaculture water

Aquaculture water is a heterogeneous system that varies in the composition and content of each specific substance. Nitrogen and phosphorus are the main focus of most studies on the environmental impacts of aquaculture effluents, as they are excreted by aquatic species and contribute to eutrophication (Buhmann and Papenbrock, 2013; Simões et al., 2008). More recently, MPs have also gained attention in this domain (Bennett et al., 2018; Gorito et al., 2018; Muñoz et al., 2010; Rodriguez-Gonzalez et al., 2019). MPs are mainly introduced in aquaculture waters by the addition of certain substances during production (Gorito et al., 2018). Veterinary drugs, disinfectants, and antifoulants are frequently used to improve the survival rates of cultured organisms and to control pathogens (European Commission, 2016). Tetracyclines, trimethoprim, sulfonamides and phenicols are some of the most commonly used medicines (Leal et al., 2016), whereas dichlorvos and diuron are examples of other chemicals that are used to disinfect water and prevent the biofouling of immersed surfaces and equipment (Claude and McNevin, 2014; Guardiola et al., 2012). These substances and their respective by-products and metabolites can be considered MPs when the outlet water is discharged into the environment without proper treatment. Moreover, water reservoirs that supply water to aquaculture farms are threatened by the presence of MPs. Many studies have reported the occurrence of MPs in surface and ground waters (Barbosa et al., 2016; Kim and Zoh, 2016; Luo et al., 2014; Sousa et al., 2017), highlighting the high probability of the presence of MPs in these aquatic compartments and, consequently, in the aquaculture waters that they provide.

Aquaculture facilities are generally interconnected with surrounding water bodies; thus, MPs not ingested/absorbed or excreted by farmed organisms and their related by-products and metabolites can spread to different environments (Pereira et al., 2013). Moreover, within farms, MPs can also be bioaccumulated by organisms, which are subsequently consumed by humans (Guardiola et al., 2012). Therefore, several pathways can contribute to the dissemination and/or accumulation of these substances. Although acute toxic effects are unlikely to occur given the low concentrations of MPs in water, chronic and long-term exposure may have several impacts on fauna, flora and even human health (Tijani et al., 2016). These effects are very difficult to correlate with specific

MPs, as residual concentrations of these substances usually occur as mixtures. Many MPs are associated with endocrine disruption, and diverse reproductive, neurological and immune effects may occur as well (Tijani et al., 2016). The proliferation of antibiotic-resistant bacteria and/or their related resistance genes (ARB & ARGs) due to the persistence of antibiotics in aquatic systems has also been observed (Cabello, 2006).

1.2.1. Environmental policy

Sustainability is a common goal for aquaculture production, but the means to achieve it depends on the traditions and values of each region. Since these traditions and values are not uniform across all jurisdictions, there are different types of aquaculture governance (FAO, 2014). In European Union (EU) countries, the Water Framework Directive (WFD, 2000) and the Marine Strategy Framework Directive (MSFD, 2008) are the two main documents that guide the practices for good water quality in aquaculture farms, envisaging a healthy ecological and chemical status for both fresh and marine waters. With respect to MPs, the EU recognises concerns about their occurrence in aquaculture activities, namely cleaning agents and medicines, which are often used to improve the survival, performance and quality of fish (European Commission, 2016). A recent European Commision (EC) guiding document has provided best practice guidelines and suggestions to national authorities and aquaculture producers, aiming to minimise the possible undesirable effects of these substances in the environment. Some recommendations include enforcing limits on the administration of veterinary medicines, prediction models to assess environmental impacts, monitoring results published on open access websites and applying alternative cleaning techniques over the use of antifoulants (European Commission, 2016). MPs are not yet regulated; however, the Water Framework Directive recognised the need to define and prioritise substances with significant risks for aquatic environments (WFD, 2000). Accordingly, a list of 33 priority substances (PSs) and 8 "certain other pollutants" was ratified by the Directive, 2008/107/CE. This standardised the Environmental Quality Standards (EQS) for each compound, according to their concentration in water, sediment or biota that should not be exceeded for the protection of the environment and of human health (Directive, 2008). This document was subsequently updated by the Directive 2013/39/EU that revised some EQS of PSs already identified, and included additional PSs and their respective EQS, totalling an inventory of 45 PSs (41 organic substances and 4 metals) and 8 "certain other pollutants" with EQS to be considered (Directive, 2013). Directive 2013/39/EU has also recommended the monitoring of other substances recently detected in water bodies, referred to as contaminants of emerging concern (CECs), for which the environmental risks are not fully understood (Directive, 2013). In this context, the first watch list of CECs for Union-wide monitoring was published by Directive 2015/495/EU (Decision, 2015), which was revised by Directive 2018/840/EU (Decision, 2018), and more recently, by Directive 2020/1161/EU, including 19 CECs that should be monitored in order to gather information for further possible prioritisation exercises (Decision, 2020). In addition to the monitoring of PSs and CECs (in both fresh and marine waters), EU water policies have also highlighted the need to develop new water treatment solutions that can remove them (Directive, 2013).

1.2.2. Water treatment options

Water treatment options suitable for MP removal must be considered in aquaculture farms because of the possible adverse effects of these substances on the aquaculture farmed organisms, surrounding ecosystems and human health. Since conventional water/wastewater treatment processes are not specifically designed to remove MPs, alternative technologies are required. Although concerns about MP removal are mainly centred on aquaculture-produced wastewaters, the treatment of influent waters should not be disregarded. Since the removal of MPs can be set as a milestone for both types of water matrices, this review includes all possible approaches, analysed according to each specific study.

Adsorption on activated carbon and membrane processes (including microfiltration, ultrafiltration, nanofiltration and reverse osmosis) are some examples of alternative technologies already applied to remove MPs at full scale, namely in drinking water and wastewater treatment plants (Boehler et al., 2012; Radjenović et al., 2008; Sui et al., 2010; Ternes et al., 2002). Powdered and granular activated carbon systems are highly effective at adsorbing MPs from water; however, their performance might be compromised by the competition between MPs and natural organic matter for binding sites (Haist-Gulde et al., 1995). Furthermore, activated carbon must be incinerated or regenerated when its adsorption capacity is achieved, which consumes high amounts of energy and generates a stream that itself must be treated as hazardous waste (Yu et al., 2018). Among the membrane processes, high-pressure systems (including nanofiltration and reverse osmosis) are more suitable for the removal of MPs (Kimura et al., 2003; Oulton et al., 2010; Yangali-Quintanilla et al., 2010). Nevertheless, such processes, which show better performance with low-molecular-weight and neutrally charged MPs, also form a concentrate containing all retained compounds, which requires further treatment and disposal (Yu et al., 2018). Due to this, AOTs such as ozonation and UV-based processes are gaining attention in this domain, as they can degrade organic MPs without necessitating their transfer to a different phase or their retention in a concentrate requiring additional treatment (Esplugas et al., 2007; Meijers et al., 1993; Nicole et al., 1990; Yang et al., 2014). Nonetheless, AOTs are typically costly options and could lead to the formation of intermediate products, which in some cases, are more toxic than the original compounds (Prieto-Rodríguez et al., 2013). Another interesting option is the application of CWs. Although CWs were originally applied to remove conventional pollutants from waste water in small communities or industries, they have demonstrated promising performance concerning the removal of organic MPs (Gorito et al., 2017; Matamoros, 2008; Zhang et al., 2014). When compared with AOTs, CWs involve lower costs of implementation and operation, but require more treatment time and space. Moreover, CWs are not as efficient as AOTs in removing MPs. Considering the potential of both technologies to remove organic MPs, studies aiming to understand the suitability of these processes for this purpose on aquaculture water are still in an early stage of investigation.

1.3. Aim and scope

This review summarises the relevant data on the application of AOTs and CWs to treat aquaculture waters, with particular attention on the removal of organic MPs. A comprehensive analysis of the available literature is provided after a brief description of AOTs and CW technologies (Sections 2.1 and 3.1, respectively). The search comprised all studies on AOTs and CWs treating aquaculture waters, retrieved from the *Scopus* database (n = 161, accessed before October 27, 2020), using keywords including the names of a broad range of "AOTs" (i.e., "advanced oxidation technologies/advanced oxidation processes", "UV", "ozone", "photocatalysis", "photocatalytic", "photocatalytic ozonation", "Fenton", "photo-Fenton", "electrochemical oxidation", "electrocatalysis", "sonolysis", "hydrodynamic cavitation", "catalytic wet air oxidation", "catalytic wet peroxide oxidation" or "catalytic wet oxidation") and "aquaculture" (n = 102), and "constructed wetlands" and "aquaculture" (n = 59). For both AOTs and CWs, the studies from the *Scopus* database were organized according to the goal of the treatment: (i) removal of organic MPs; (ii) removal of conventional pollutants; (iii) removal of bacteria; and (iv) possible combinations among (i), (ii) and/or (iii), with the studies dealing with MPs being the main focus of this review article. In this sense, investigations dealing with removal of MPs are carefully detailed and discussed in Sections 2.2.1 (AOTs) and 3.2.1 (CWs), whereas the elimination of other types of pollutants is briefly addressed in Sections 2.2.2 (AOTs) and 3.2.2 (CWs). After the literature overview, perspectives for the removal of MPs by AOTs and

CWs from aquaculture waters are discussed, considering the actual status and the development of proper criteria for their implementation.

2. Advanced oxidation technologies

2.1. Background and operating principles

AOTs are treatment technologies based on the production of highly reactive species that oxidize organic compounds (Kasprzyk-Hordern et al., 2003). In most cases, AOTs involve the generation of hydroxyl radicals ($HO^{\bullet}$), known as advanced oxidation processes (Hoigné, 1997), but other reactive species can also be involved, such as the oxide anion ($O_2^{\bullet-}$), hydroperoxyl ($HO_2^{\bullet}$), ozonide anion ($O_3^{\bullet-}$), sulphate ($SO_4^{\bullet-}$) and chlorine ($Cl^{\bullet}$) radicals (Fig. 1) (Fernández-Castro et al., 2015; Lado Ribeiro et al., 2019). As organic MPs are not efficiently eliminated by conventional water/wastewater treatment processes, AOTs have attracted significant interest as a suitable alternative to remove these contaminants from aqueous matrices (Broséus et al., 2009; Prieto-Rodríguez et al., 2013; Yao et al., 2018).

There are several categories of processes included in AOTs (Fig. 1), which are differentiated according to: (i) the generation of highly reactive species (chemical, photochemical, electrochemical, and sonochemical); and (ii) the number of phases involved in the oxidative reactions (homogeneous or heterogeneous processes) (Babuponnusami and Muthukumar 2014). Thus, chemical technologies involve the use of oxidants such as ozone (O_3) and hydrogen peroxide (H_2O_2), or catalytic mixtures as the Fenton reagent, whereas photochemical processes require radiation (such as UV/O_3, UV/H_2O_2, UV/Fenton). Typically, these reactions are conducted at near-ambient temperature and pressure, but more severe conditions can also be applied (e. g. wet air/-peroxide oxidation, supercritical water oxidation) (Ribeiro et al., 2015). Electrochemical and sonochemical processes involve more complex treatment using electrical currents (such as anodic oxidation, electro-Fenton, photo-electro-Fenton, and solar photo-electro-Fenton processes) and ultrasonic irradiation (including sonolysis, sonocatalysis, sonophotolysis, and sonophotocatalysis) to produce free radicals, respectively (Joseph et al., 2009; Moreira et al., 2017). Homogeneous processes occur in a single phase, whereas heterogeneous processes occur in two or more phases. Metal oxides (based on Ti, Cu, Zn, Mn, Fe, Co, and Bi), supported noble metals (such as Ru, Pt, Pd, Ir, and Rh) or even metal-free carbon materials, such as activated carbons, carbon xerogels, carbon nanotubes, carbon foams and fibers, and graphene derivatives are examples of catalysts that can be applied in heterogeneous AOTs (Ribeiro et al., 2015). In particular, heterogeneous photocatalysis is based on the activation of the catalyst by a radiation source with energy equal to or higher than its band gap, leading to the generation of electrons and holes, highly reactive species and subsequent degradation of organic MPs (Fujishima et al., 2000; Herrmann, 2005). Titanium dioxide (TiO_2) is the most widely used photocatalyst because of its outstanding activity, stability, low cost and relatively low toxicity (Frederichi et al., 2021; Hoffmann et al., 1995). To increase the treatment efficiency and high removal of MPs, heterogeneous photocatalysts have been combined with ozone in a process called photocatalytic ozonation (Bertagna Silva et al., 2019; Chávez et al., 2019; Moreira et al., 2016; Rajeswari and Kanmani, 2009; Solís et al., 2015). Overall, ozone-based technologies have been extensively studied to remove

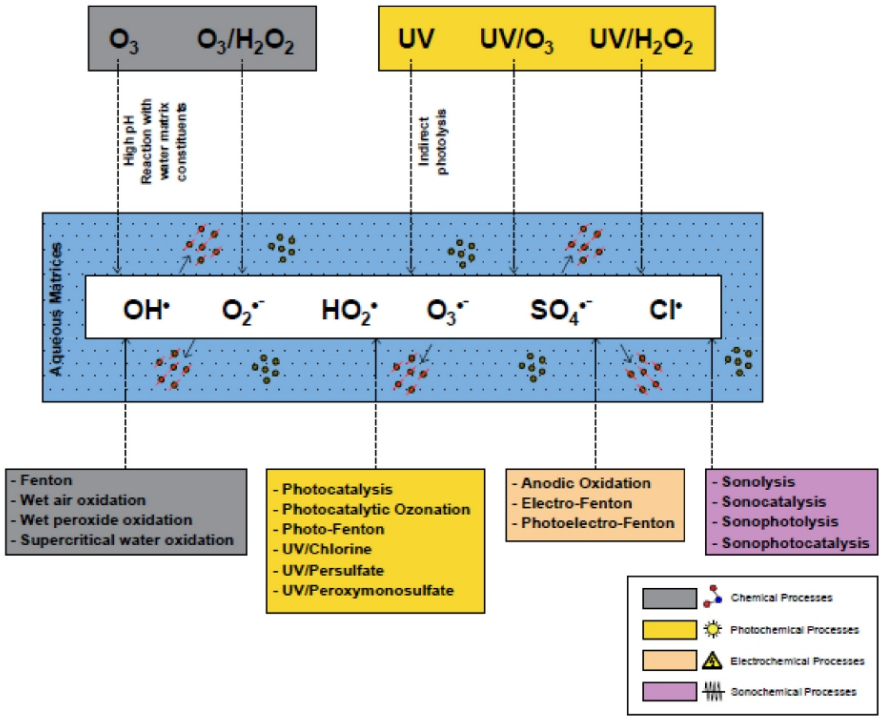

Fig. 1. AOTs general mechanism for removing organic MPs from aqueous matrices (chemical, photochemical, electrochemical and sonochemical processes).

organic MPs from water/wastewater (Bourgin et al., 2017; Broséus et al., 2009; El-taliawy et al., 2017; Iakovides et al., 2019; Lee et al., 2014); however, the choice of the most suitable AOT depends on several factors related to specific case of treatment.

AOTs are employed to transform organic MPs into less toxic compounds, ideally yielding CO_2, H_2O and inorganic ions as the final products. For this reason, they can be used as a polishing treatment before water discharge/reuse, or applied before biological processes to reduce water toxicity and enhance biodegradability (Barbosa et al., 2016). The application of AOTs to treat industrial water and wastewater contaminated with organic MPs is a hot topic of research in the scientific community; however, pilot and full-scale studies are limited (Liu et al., 2020; Mahy et al. 2019, 2020; Mousel et al., 2021). In the specific case of aquaculture facilities, the need for water disinfection in RAS systems has triggered interest in the use of AOTs (Malara et al., 2017; Sharrer and Summerfelt, 2007); recently, the potential for the application of AOTs for the removal of organic MPs has gained much attention (Nomura et al., 2020; Pereira et al., 2013).

2.2. Treatment of aquaculture waters by AOTs

A total of 102 studies from the database dealt with the application of AOTs in aquaculture farms (Fig. 2). UV (51 %) and ozone-based (31 %) processes were the most representative AOTs, followed by electro-oxidation (14 %), Fenton (3 %) and hydrodynamic cavitation (1 %) processes. Most reports (~70 %) were regarding the removal of bacteria and/or conventional pollutants from aquaculture effluents, mainly in RAS systems, to guarantee the quality of recirculating water and protect the farmed organisms. However, the removal of organic MPs already represents approximately 30 % of the total studies published so far (mostly published since 2018; Table 1), showing emerging concerns about the presence of these substances in aquaculture waters.

2.2.1. Removal of organic MPs

The collected information (Table 1) shows that most investigations have focused on heterogeneous photocatalysis to remove organic MPs from aquaculture effluents. TiO_2 is the most investigated photocatalyst among all the tested photocatalysts (TiO_2, porphyrin-TiO_2, Au-TiO_2, Zeolite-TiO_2, TiO_2-rGO, TiO_2-Fe^0, Pd/WO_3, Zeolite-$TiCl_4$/$FeCl_3$, CN-TiO-CNT, GO/CN-TiO-CNT, and CuO/ZnO), whereas UV or solar light is generally applied to activate photocatalyst. Experiments using TiO_2 nanotubes alone or combined with Au nanoparticles (Do et al., 2019), TiO_2 with silica zeolite (Nomura et al., 2020) and reduced graphene oxide (rGO) (Leal et al., 2020) are some examples of photocatalysts that have been developed to remove organic MPs from aquaculture waters. Alternatives to TiO_2-based photocatalysts have also been explored, in particular CuO/ZnO nanocomposites for the treatment of marine aquaculture effluents (Liu et al. 2019a, 2019b). A novel Pd/WO_3 catalyst was also investigated to remove organic MPs (Xue et al., 2016). All photocatalytic studies were conducted at bench scale, except one performed at full-scale RAS (Rodriguez-Gonzalez et al., 2019) and two performed in pilot plants involving solar compound parabolic collectors (Pereira et al., 2013) and packed-bed photocatalytic reactors (Pestana et al., 2014). Likewise, the other studied technologies were performed at the lab scale, except three studies on UV-and ozonation-based processes implemented in RAS (Good et al., 2017; Klausen and Grønborg, 2010;

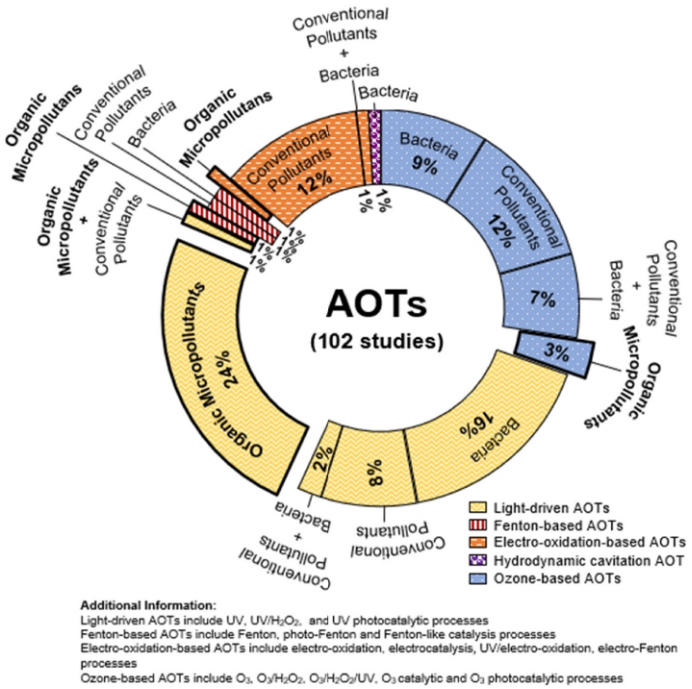

Fig. 2. Total number of studies dealing with the application of AOTs in aquaculture farms (102) and the distribution of studies regarding the type of contaminant studied (i.e., organic MPs, bacteria or conventional pollutants – e.g. organic matter, total suspended solids, and nutrients). The results were retrieved from the *Scopus* database using the keywords "*name of AOTs*" and "*aquaculture*" (accessed until October 2020).

Table 1
Studies on the applicability of AOTs for the removal of organic MPs from aquaculture effluents (only one study‡ refers to the treatment of both aquaculture influents and effluents).

Treatment technology	Target Micropollutant	Type of aquaculture (category of water)	Concentration (μg L^{-1})	Removal (%)	Ref.
Solar photolysis	Oxytetracycline	Non-RAS (Marine; synthetic and non-synthetic)	4000	20–75	Leal et al. (2016)
Solar photolysis	Oxytetracycline	Non-RAS (Marine and freshwater; synthetic)	20000–40000	25–78	Pereira et al. (2013)
	Oxolinic acid		20000–40000	3–18	
Solar TiO_2 photocatalysis	Oxytetracycline		20000–40000	18–100	
	Oxolinic acid		20000–40000	20–100	
Solar photolysis	Oxytetracycline	n.d. (n.d; synthetic)	13813	35–53	Gaeta et al. (2020)
	Oxolinic acid		7837	8–28	
Solar TiO_2 photocatalysis	Oxytetracycline		13813	37–66	
	Oxolinic acid		7837	47–94	
Solar Porphyrin-TiO_2 photocatalysis	Oxytetracycline		13813	18–60	
	Oxolinic acid		7837	10–69	
Solar Pd/WO_3 photocatalysis	Geosmin	n.d. (n.d; synthetic)	0.5–2	58–100	Xue et al. (2016)
UV photolysis	Florfenicol	RAS (Marine; non-synthetic)	0.2–1.8	4–45	Zhang et al. (2020a)
TiO_2 UV photocatalysis	Tetracycline	n.d. (n.d; synthetic)	20000–200000	58–100	Wang et al. (2020)
TiO_2 UV photocatalysis	Geosmin	RAS (Freshwater; synthetic and non-synthetic)	≤0.04	0–77 %	Rodriguez-Gonzalez et al. (2019)
	2-methylisoborneol		≤0.06	0–70 %	
TiO_2 UV photocatalysis	Geosmin	RAS (n.d; synthetic)	2	25–100	Lawton et al. (2003)
	2-methylisoborneol		2	75–100	
TiO_2 UV photocatalysis	Geosmin	RAS (Freshwater; synthetic and non-synthetic)	0.014–0.1	91–96	Pestana et al. (2014)
	2-methylisoborneol		0.019–0.1	84–96	
UV	Geosmin	RAS (Freshwater; synthetic and non-synthetic)	0.05	20–80	Pettit et al. (2014)
	2-methylisoborneol		0.05	8–77	
TiO_2 UV photocatalysis	Geosmin		0.05	17–90	
	2-methylisoborneol		0.05	10–62	
UV photolysis	Metronidazole	Non-RAS (Marine; synthetic and non-synthetic)	40000	4–47	Santana et al. (2017)
TiO_2 UV photocatalysis	Metronidazole		40000	20–100	
TiO_2/Au-TiO_2 UV/VIS photocatalysis	Amoxicillin	Non-RAS (Freshwater; synthetic)	0.5	*	Do et al. (2019)
	Ampicillin		0.5	*	
	Doxycycline		0.5	*	
	Oxytetracycline		0.5	*	
	Lincomycin		0.5	32–100	
	Vancomycin		0.5	*	
	Sulfamethazine		0.5	*	
	Sulfamethoxazole		0.5	*	
TiO_2-rGO UV photocatalysis	Formalin	Non-RAS (Marine and freshwater; non-synthetic)	40000	16–64	Leal et al. (2020)
UV	Ciprofloxacin	n.d. (n.d; synthetic)	30000	15	Diao et al. (2017)
TiO_2 photocatalysis	Ciprofloxacin		30000	13–60	
TiO_2-Fe^0 UV photocatalysis	Ciprofloxacin		30000	5–100	
Zeolite-TiO_2 UV photocatalysis	Sulfamonomethoxine	Non-RAS (Freshwater; non-synthetic)	100	96–100	Nomura et al. (2020)
Zeolite-TiO_2/USY UV photolocatalysis	Gesomin	RAS (Freshwater; non-synthetic)	0.1	98–99	Wee et al. (2015)
Zeolite-$TiCl_4$/$FeCl_3$ UV photocatalysis	Florfenicol	n.d. (Freshwater; synthetic)	10000–50000	98–100	Esmaili et al. (2017)
CN-TiO-CNT photocatalytic membranes	Sulfamethoxazole	n.d. (n.d; synthetic)	5000	24–30	Zhang et al. (2018)
	Bisphenol A		5000	30–36	
GO/CN-TiO-CNT photocatalytic membranes	Sulfamethoxazole		5000	54–78	
	Bisphenol A		5000	80–84	
CuO/ZnO UV photocatalysis	Oxytetracycline	Non-RAS (Marine; n.d.)	10000–30000	43–89	Liu et al. (2019a)
CuO/ZnO UV photocatalysis	Chlortetracycline	Non-RAS (Marine; n.d.)	10000–30000	17–89	Liu et al. (2019b)
UV/peroxydisulfate	Ofloxacin	Non-RAS (Marine; synthetic)	18000	17–100	Zhu et al. (2020)
UV/persulfate	Sulfamethoxazole	Non-RAS (Marine; synthetic)	25000	6–100	Zhang et al. (2020b)
UV	E1	RAS (Freshwater; synthetic and non-synthetic)	10	63–96	Bennett et al. (2018)
	E2		10	10–98	
	E3		10	24–95	
UV/H_2O_2	E1		10	65–96	
	E2		10	19–98	
	E3		10	22–96	
O_3	E2	RAS (Freshwater; non-synthetic)	0.475	~60	Good et al. (2017)
O_3*	Amoxicillin	Non-RAS (Marine; non-synthetic)	731	21–100	Choi et al. (2020)
	Florfenicol		716	0–5	
	Oxolinic acid		523	21–98	
	Oxytetracycline		921	17–78	
UV *	Amoxicillin		731	10–59	
	Florfenicol		716	36–85	
	Oxolinic acid		523	3–10	
	Oxytetracycline		921	5–25	
UV/H_2O_2	Geosmin	RAS (Marine; non-synthetic)	0.0042–0.0067	23–100	Klausen and Grønborg (2010)
	2-methylisoborneol		0.032–0.089	25–100	
UV/O_3	Geosmin		0.0042–0.0067	5–90	
	2-methylisoborneol		0.032–0.089	20–100	
	Rifampicin		30000–60000	53–99	Liu et al. (2021)

(continued on next page)

Table 1 (*continued*)

Treatment technology	Target Micropollutant	Type of aquaculture (category of water)	Concentration (μg L^{-1})	Removal (%)	Ref.
Fenton-oxidation with rGO@nFe/Pd nanocomposite		n.d (n.d; synthetic and non-synthetic)			
Electro-oxidation	Sulfadimidine	(n.d. Marine; synthetic)	2000	100	Lang et al. (2020)
	Norfloxacin		2000	100	

n.d.- not defined; [a]Removals for lincomycin. Most of the other target compounds showed maximum removal efficiencies close to 100 % for photocatalysis. E1: estrone, E2: 17-beta-oestradiol, E3: estriol.

Zhang et al., 2020a) that were conducted at pilot- and full-scale water treatment units.

Antibiotics are the most studied substances, with an emphasis on tetracyclines and sulfonamides classes. Tetracyclines, sulfonamides, trimethoprim, quinolones, β-lactams, fluoroquinolones and phenicols are authorized for oral administration to fish in Europe, although oxytetracycline gets particular attention due to its wide application in the aquaculture industry (Leal et al., 2016). Among these, sulfamethoxazole, trimethoprim and amoxicillin are included in the new watch list of CECs intended to improve knowledge of the occurrence and spread of antimicrobials in the environment (Decision, 2020). Estrone, 17-beta-oestradiol and oestriol hormones were also studied as target compounds, with the first two being included in the prior list of CECs of Decision 2018/840/EU (Decision, 2018). Although the use of hormones in aquaculture farms is prohibited in the EU territory, due to their possible impacts on farmed fish and human health, other countries such as Canada, Australia, New Zealand, Argentina and the United States still permit their use for artificial fish reproduction and sex reversal (Hoga et al., 2018). Studies on AOTs also include the removal of other substances such as geosmin and 2-methylisoborneol, known as taste and odour compounds (T&O). The excessive eutrophication of fish ponds promotes the development of cyanobacteria, and as a consequence, the generation of these and other T&O compounds is frequently detected in aquaculture facilities (Zhong et al., 2011).

The concentrations of the above mentioned substances were in a large range, varying between 0.0042 and 200000 $\mu g \cdot L^{-1}$. In fact, AOTs have generally been investigated using matrices with overestimated concentration doses of the target compounds. Moreover, the effects of any matrix constituent on the performance of AOTs (e. g. removal efficiencies) are not considered when synthetic effluents are used. Consequently, the treatment efficiency is often overestimated when synthetic solutions are used. In general, studies that do not spike the effluents correspond to those that implement AOTs in aquaculture facilities (Good et al., 2017; Pettit et al., 2014; Rodriguez-Gonzalez et al., 2019; Zhang et al., 2020a). The selection of high concentrations in most studies is related to the unavailability of the highly sensitive analytical apparatus required to assess the treatment performance at ng–$\mu g \cdot L^{-1}$ levels of MPs. Therefore, future research should involve testing concentrations of lower magnitudes, instead of studying conditions not adjusted to real scenarios. Investment in modern and powerful analytical technologies must be considered to enable the study of suitable conditions (e. g. catalyst dose, treatment time, and intensity of radiation) favouring treatment scale-up.

Analysing the efficiencies of AOTs (Table 1), it is clear that each of the listed studies had a large spectrum of removal efficiencies for the respective target MP. This is mainly due to the optimisation steps under different tested conditions (such as amount, composition and particle size of the catalyst, type and time of irradiation, initial concentrations of MPs, presence of inorganic ions, and treatment time). For instance, photolysis of oxytetracycline (in a real matrix) using simulated sunlight had significant removal variations (20 %–75 %), with its degradation being influenced by total suspended solids, pH, and salinity of effluents (Leal et al., 2016). On the other hand, higher removal (78 %–100 %) of oxytetracycline (in deionised water) was achieved by both solar photolysis and solar TiO_2 photocatalysis in pilot-scale compound parabolic collectors, requiring an accumulated UV energy of 7 and 1 $kJ \cdot L^{-1}$, respectively. However, additional experiments at the bench scale emphasised that the presence of PO_4^{3-} can affect the efficiency of the photocatalytic process (Pereira et al., 2013). Another study with deionised water indicated that the efficiency of solar photolysis of oxytetracycline and oxolinic acid was lower than 53 %. Solar TiO_2 and porphyrin-TiO_2 photocatalysis showed considerably higher removal of oxolinic acid, but the enhancement in the removal of oxytetracycline was negligible (Gaeta et al., 2020). These results are in accordance with those reported elsewhere (Pereira et al., 2013). The removal of tetracycline was evaluated in a hybrid solution with Cu (II) using TiO_2 photocatalysis, showing that the initial concentration of the antibiotic can considerably affect the performance of the process. Increasing the concentration from 20 to 200 $mg \cdot L^{-1}$ resulted in a reduction of photocatalytic performance by approximately 45 % (Wang et al., 2020). Moreover, the effect of pH on the photocatalytic degradation of the T&O compounds geosmin and 2-methylisoborneol was highlighted in another study, which also showed a negative effect of the real matrix on geosmin removal (Pettit et al., 2014). Likewise, the matrix has a significant influence on the degradation rate and cost effectiveness of UV/H_2O_2 and UV/O_3 removal of geosmin and 2-methylisoborneol (Klausen and Grønborg, 2010). In general, these compounds were highly removed after the total treatment time, except in a specific case of a UV-TiO_2 continuous reactor at bench-scale RAS (Rodriguez-Gonzalez et al., 2019). Several operating parameters were identified as causing the limited performance, because a similar batch reactor implemented at full scale showed better removal of the same compounds (Rodriguez-Gonzalez et al., 2019). When water containing formalin was treated using TiO_2-rGO UV photocatalysis, both inorganic ions and organic matter interfered with the treatment performance. This interference mainly occurs in salt water when compared with freshwater, due to the higher concentrations of inorganic ions that compete with the target contaminant for photogenerated $HO^{\bullet}$ radicals and adsorption sites at the catalyst surface (Leal et al., 2020). GO/CN-TiO-CNT photocatalytic membranes were more efficient in removing both sulfamethoxazole and bisphenol A (~80 %) than CN-TiO-CNT (Zhang et al., 2018). For metronidazole removal, TiO_2 UV photocatalysis was better than UV photolysis, with the activity of the catalysts in terms of degradation in the following order: Evonik-P90 ≈ Evonik-P25 > ECT-1023t > Hombikat. In this case, the results of the synthetic *vs.* non-synthetic matrix did not show any influence of the real matrix on the removal process (Santana et al., 2017). AOTs mediated by $SO_4^{\bullet-}$ (UV/persulfate, UV/peroxydisulfate) also exhibited good removal of target MPs, with studies highlighting that inorganic ions such as Cl^-, HCO_3^-, and Br^- might play an important role in the treatment performance. The treatment of hormones by UV photolysis showed good removal percentages of these compounds mainly for higher UV doses (500–1000 $mJ \cdot cm^{-2}$), and the addition of H_2O_2 did not consistently improve the treatment efficiency (Bennett et al., 2018). In turn, ozone could significantly eliminate amoxicillin, oxytetracycline, and oxolinic acid (O_3 doses: 20–41 μM), but higher removal of florfenicol was obtained under UV_{254} radiation (Choi et al., 2020). For the degradation of rifampicin antibiotic by the Fenton process, parameters such as the initial concentration of MP, temperature, pH, H_2O_2 dosage, and catalyst loading did not affect the performance; real matrix, or reuse of the

catalyst being the main factors that decrease process efficiency (Liu et al., 2021). In summary, the application of AOTs to remove MPs from aquaculture farms is at an early stage of investigation, generally still using very high spiked concentrations of MPs and/or synthetic media at lab-scale equipment. Nevertheless, the reported results demonstrate the potential of these innovative technologies, and more studies are necessary at full-scale to assess their performance under real conditions, as well as a comprehensive cost analysis. In fact, two studies involving AOTs (O_3, UV/O_3 and UV/H_2O_2) at full-scale RAS (Good et al., 2017; Klausen and Grønborg, 2010), highlighted the capacity of these technologies to remove MPs, indicating that further research is needed for the possible integration of AOTs into the existing water treatment systems, creating a new treatment approach that can remove MPs.

2.2.2. Removal of other pollutants

Intensive aquaculture involves problems such as infrequent water renewal, overfeeding, fast growth and inefficient removal of dead fish, promoting the proliferation of bacterial diseases (Poblete-Chavez et al., 2016). Therefore, AOTs are mainly used in aquaculture for water disinfection, as shown by a considerable number of studies focused on bacterial removal (Fig. 2). Considering the high costs of AOTs and the less stringent quality standards of released effluents in comparison to those that are recirculated in aquaculture activities, the application of AOTs is not very common in non-RAS. Given that aquaculture farms with recirculating systems have a higher risk of fish infection, AOTs are mainly applied in RAS to prevent bacterial dissemination and problems during fish production (Gullian et al., 2012; Powell et al., 2015; Sharrer and Summerfelt, 2007). The use of antibiotics to control fish diseases caused by harmful bacteria is one of the major drawbacks of the aquaculture sector in terms of water pollution. However, the administration to fish can be greatly reduced (as encouraged by EU strategies for sustainable aquaculture) if AOTs are implemented as a measure to ensure the disinfection of water, which would correspondingly decrease the amount of antibiotics needed.

In addition to the removal of bacteria, AOTs have also been applied to eliminate other conventional pollutants from aquaculture waters (Fig. 2). Ozone-based processes have often been used in full-scale RAS to enhance global water quality, and in general, high removal rates of organic matter, total suspended solids, colour and inorganic compounds are achieved (Schroeder et al., 2011; Tango and Gagnon, 2003). Two publications reviewing the application of ozonation in aquaculture facilities with RAS to remove these and other conventional pollutants are already available in the literature (Gonçalves and Gagnon, 2011; Summerfelt and Hochheimer, 1997). Both review articles highlight the high capacity of ozone to inactivate a range of bacterial, viral, fungal, and protozoan fish pathogens. However, the ozone concentration and its exposure length (contact time), pathogen loads, and levels of organic matter are parameters that require attention to avoid gross tissue damage and stock mortalities of cultured organisms. Summerfelt and Hochheimer (1997) showed that even very low ozone concentrations (9–80 $\mu g \cdot L^{-1}$) can be toxic to some fish species. In this sense, selecting the optimum ozone dose is still a matter of concern in these facilities because of the high costs of generation of this oxidant and the potential effects of residual ozone. Several studies have been performed to establish the proper criteria for the selection of the most cost-effective amount of ozone (Park et al., 2015; Spiliotopoulou et al., 2017). Electrochemical AOTs have mainly been applied to remove ammonia from aquaculture waters; two studies have assessed pilot implementation in RAS (Gendel and Lahav, 2013; Lahav et al., 2015). Studies on UV-based processes have also evaluated the removal of ammonia at the laboratory scale (Lin et al., 2019; Yu et al., 2012; Zhu et al., 2011). Regarding Fenton-based processes, a Fenton-coagulation batch reactor showed good performance in removing organic matter, turbidity and inorganic ions from aquaculture wastewater (Gomes et al., 2020). Thus, considering that AOTs have been used to eliminate bacteria and other conventional pollutants from aquaculture farms, and that other studies demonstrate their great potential to remove organic MPs when investigating different matrices, AOTs can be seen as an interesting alternative for the removal of both conventional contaminants and organic MPs from aquaculture waters.

3. Constructed wetlands

3.1. Background and operating principles

CWs are engineered systems that recreate the conditions and processes occurring in natural wetlands, which are well recognised by their capacity for improving water quality (Vymazal and Kröpfelová, 2008). Since CWs are constituted by water, substrate, plants (in most of the cases), and microorganisms, several biotic and abiotic processes may occur simultaneously (Fig. 3), contributing to the elimination of different types of compounds (Gorito et al., 2017; Lin et al., 2002).

Microbial degradation and uptake by plants are the main biotic mechanisms for the removal of contaminants from water passing through CWs, whereas abiotic processes include sedimentation, substrate adsorption, volatilization and photodegradation (Lin et al., 2002). Nonetheless, the occurrence of such processes is highly dependent on several factors, namely the specific characteristics of the compounds and the conditions of the surrounding environment (Gorito et al., 2017). Regarding the properties of pollutants, substances with higher vapour pressure values, for instance, are more likely to volatilise than others (Suthersan, 2001). Furthermore, hydrophobic compounds are easily adsorbed onto soil, mineral surfaces, and biofilms, whereas hydrophilic compounds are mostly removed by other processes (Zhang et al., 2014). The surrounding environment also plays an important role in the removal of pollutants by CWs. For instance, parameters such as pH, temperature, ionic strength, and oxygen availability can affect adsorption, uptake, and microbial degradation mechanisms (Stottmeister et al., 2003). The growth of plants as well as microbial activity in CWs requires optimal pH values (near neutral), and high pH can affect the adsorption behaviour of pollutants due to their dissociation and subsequent attachment to substrate/sediment by ion exchange. Moreover, the dissolved oxygen and temperature have a great impact on macrophytes and microorganisms, with the high availability of oxygen promoting aerobic degradation by microbial consortium, and the warm temperature (15–25 °C) enhancing the action of some organisms such as nitrifying and proteolytic bacteria (Ilyas and Hullebusch, 2020). The performance of CWs is also influenced by climatic conditions, which can affect evapotranspiration, photosynthesis and microbial activity (Varma et al., 2021). Given all these factors, CWs can be adapted according to the characteristics of the target contaminants to be removed and the environmental conditions available in their locations or implementation sites.

In general, CWs are differentiated according to their configurations, namely: (i) their hydrology (water surface flow and sub-surface flow), (ii) plant growth (free-floating, floating-leaved, submerged, or emergent) and (iii) the flow path (horizontal or vertical). Moreover, other operational parameters such as operating mode, hydraulic residence time, plant species, and substrate matrix are also important for their characterization (Gorito et al., 2017). In fact, both the configuration and operational conditions of CWs can significantly influence their performance. For instance, surface flow CWs can be a good solution for compounds susceptible to photodegradation because the water is directly exposed to sunlight, whereas subsurface flow CWs are a better alternative for biodegradable pollutants because the water passage in the substrate matrix triggers several biological and chemical interactions that promote their removal. Moreover, in subsurface flow CWs, the vertical path can improve microbial degradation due to the higher oxygenation of the different substrate layers when compared with horizontal ones (Zhang et al., 2014). In addition, the operational conditions of CWs, such as the operating mode and hydraulic residence time, can also be adjusted according to the type of treatment. CWs operating in

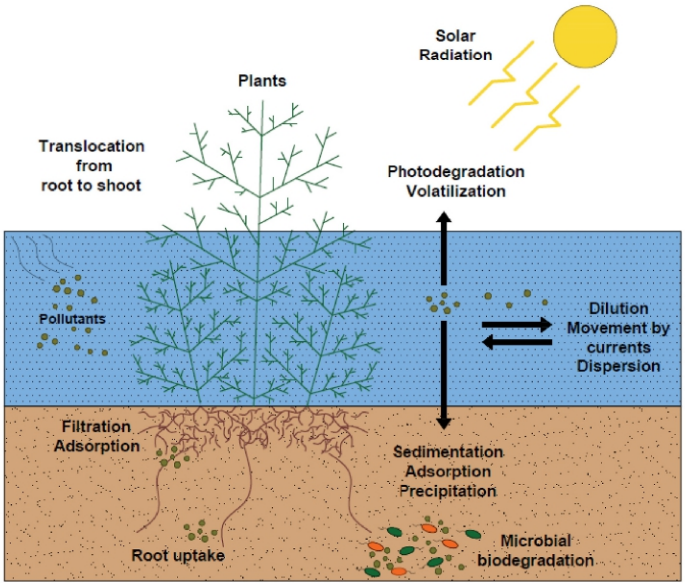

Fig. 3. Biotic and abiotic processes removing MPs from CWs systems – adapted from Gorito et al. (2017) with permission of Elsevier.

batch mode generally provide better performance than those under continuous feed because a more aerobic environment in the first case is promoted through draining and filling operations, enhancing the microbial activity and consequently the degradation of pollutants (Gorito et al., 2017). Pollutant biodegradation often involves a complex series of biochemical reactions that depend on the microbial communities present in the substrate. Taking into consideration the surrounding conditions of the media (for instance, aerobic and anaerobic zones), organic MPs (used as a source of energy by microorganisms) and nutrients can be removed by several mechanisms (Meng et al., 2014; Rajan et al., 2019). For instance, aerobic bacteria oxidize pollutants utilizing oxygen as the final electron acceptor, while the anaerobic bacteria perform the function of degradation through other final electron acceptors (such as CO_2, SO_4^{2-} and NO_3^-) or fermentation and methanogenesis (Rajan et al., 2019). Regarding the time of treatment, a period range of 4–15 d has been reported to be suitable for achieving good performance of CWs (Zhang et al., 2014). The selection of plant species and the substrate matrix is important to remove specific types of pollutants and to support the environment for microbial activity (Gorito et al., 2017). *Typha* ssp. and *Phragmites* ssp. are the most popular species used in CWs (Li et al., 2014), while the type of substrate matrix and their respective depths are very adaptable according to the implemented CWs (Dordio et al., 2009; Kumar and Dutta, 2019; Song et al., 2009).

In general, the applications of CW systems have important advantages, such as moderate capital costs, low energy consumption, small maintenance requirements and benefits for wildlife habitats (Huang et al., 2019). Considering their impressive performances in removing more common pollutants from wastewater, such as nitrogen, phosphorus, organic matter and bacteria, their applicability for the removal of organic MPs has only recently begun to be studied (Gorito et al., 2017; Li et al., 2014; Zhang et al., 2014). Traditionally, CWs have been used to treat wastewater in rural communities, small industries and farms; however, their application in aquaculture facilities is also increasing (Lin et al., 2002; Piñeyro et al., 2019).

3.2. Treatment of aquaculture waters by CWs

A total of 59 studies on the application of CWs in aquaculture farms have been published so far (without any temporal restriction on the *Scopus* survey, Fig. 4). The elimination of conventional pollutants represents most of the published studies (91 %); however, removal of organic MPs has gained increasing attention, representing nearly 9 % of the total studies dealing with the treatment of aquaculture waters by CWs.

3.2.1. Removal of organic MPs

Among the studies dealing with the treatment of aquaculture effluents by CWs (Table 2), two have been conducted at the microcosm-scale (Bóto et al., 2016; Gorito et al., 2018), one at the pilot-scale (Huang et al., 2019) and the other three at full-scale (Hsieh et al. 2013, 2015; Zhong et al., 2011). Moreover, from these last three, those conducted by Hsieh et al. (Hsieh et al. 2013, 2015) investigated the applicability of CWs for the treatment of aquaculture effluents in combination with domestic and livestock wastewaters. Thus, the CWs studied in these reports were not designed for their specific application in aquaculture farms, but for removing MPs from mixed effluents resulting from different activities.

Antibiotics are the most widely studied organic MPs, mainly the classes of fluoroquinolones, sulphonamides, and tetracyclines. These classes are all widely used in aquaculture facilities. Some studies have included other substances, such as endocrine disruptors (Hsieh et al. 2013, 2015) and other compounds that can be found in aquaculture inlet waters (Gorito et al., 2018). Many of the target MPs that have been studied so far are included in Directive 2013/39/EU (alachlor, atrazine, chlorfenvinphos, isoproturon, nonylphenol, octylphenol, perfluorooctanesulfonic acid, and simazine) (Directive, 2013) and in the Directive 2018/840/EU (azithromycin, ciprofloxacin, clarithromycin, clothianidin, erythromycin, methiocarb, thiacloprid, thiamethoxam, 17 beta-oestradiol and 17-alpha-ethinyloestradiol) (Decision, 2018). Moreover, trimethoprim, venlafaxine, sulfamethoxazole and

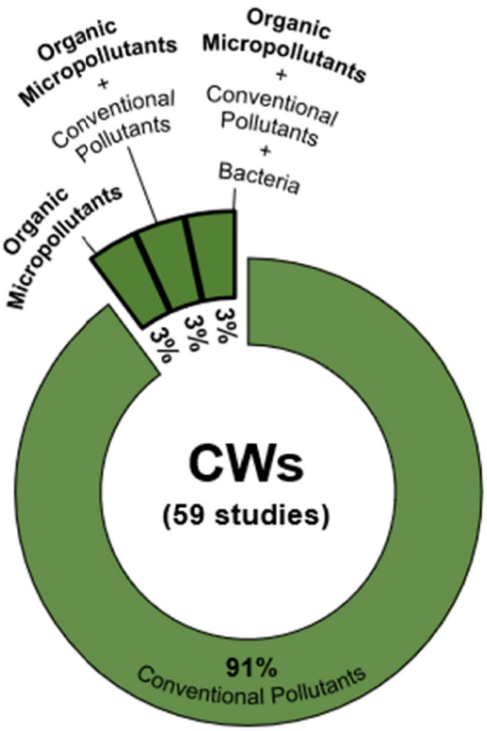

Fig. 4. Total number of studies dealing with application of CWs in aquaculture farms (59) and the distribution of studies regarding the type of contaminant studied (i.e., organic MPs, bacteria, or more conventional pollutants - e.g. organic matter, total suspended solids, and nutrients). The results were retrieved from the *Scopus* database using the keywords *"constructed wetlands"* and *"aquaculture"* (accessed until October 2020).

ciprofloxacin were included in the recent Directive 2020/1161/EU (Decision, 2020). Only one study reported the removal of geosmin and 2-methylisoborneol, which are T&O compounds naturally occurring in aquaculture facilities. These substances represent a serious problem for the sector because the quality of the fish can be compromised by its unpleasant flavour, and the use of undesirable compounds (such as algicides) is required in some cases to avoid this situation (Zhong et al., 2011).

The removal of target MPs by CWs has mainly been studied in freshwater aquaculture farms. Only a single study (Bôto et al., 2016) has been performed with marine aquaculture effluents (non-RAS); however, the high levels of salinity it tested did not affect CW performance. All the studies were conducted with non-synthetic water, independently of the scale of the study, demonstrating the concern for testing CWs under near-real conditions. The inlet concentrations of target pollutants studied in CWs ranged from a few to several $\mu g \cdot L^{-1}$ (0.01–100 $\mu g \cdot L^{-1}$). Only two studies were performed under spiked conditions (non-spiked experiments also included) (Bôto et al., 2016; Gorito et al., 2018). Pollutant concentration can affect the removal efficiency of processes, that is, biodegradation and adsorption. For instance, high concentrations may lead to destabilisation of the microbial community, disfavouring biodegradation, whereas saturation of the binding sites can limit adsorption (Gorito et al., 2018). In any case, the registered removal

Table 2
Studies on the applicability of CWs for the removal of organic MPs from aquaculture effluents.

Target Micropollutant	Type of aquaculture (category of water)	Concentration ($\mu g \cdot L^{-1}$)	Removal (%)	Ref.
Enrofloxacin	Non-RAS	100	>99	Bôto et al. (2016)
Oxytetracycline	(Marine; non-synthetic)	100	>99	
Acetamiprid	Non-RAS	0.06–0.09	100	Gorito et al. (2018)
Alachlor	(Freshwater;	0.01–0.12	100	
Atorvastatin	non-synthetic)	0.08–0.43	100	
Atrazine		0.0003–0.08	94–100	
Azithromycin		0.03–0.05	90–98	
Bezafibrate		0.08–0.09	98–100	
Carbamazepine		0.11–0.13	100	
Cephalexin		0.02–0.07	100	
Ceftiofur		0.03–0.06	100	
Chlorfenvinphos		0.05–0.14	98–100	
Citalopram		0.06–0.13	100	
Clarithromycin		0.002–0.11	90–100	
Clindamycin		0.02–0.07	100	
Clofibric acid		0.06–0.09	97–100	
Clothianidin		0.06–0.09	100	
Diclofenac		0.07–0.09	83–100	
Diphenhydramine		0.12–0.18	90–100	
EHMC		0.3–1.8	26–87	
Enrofloxacin		0.5–0.7	100	
Erythromycin		0.09–0.12	100	
Fluoxetine		0.00–0.08	100	
Isoproturon		0.0001–0.09	93–98	
Ketoprofen		0.04–0.12	100	
Methiocarb		0.6–0.9	100	
Metoprolol		0.07–0.11	100	
Norfluoxetine		0.007–0.12	100	
Ofloxacin		0.05–0.08	100	
PFOS		0.009–0.2	0–100	
Propranolol		0.1–0.13	88–100	
Simazine		0.057–0.064	91–100	
Thiacloprid		0.05–0.07	100	
Thiamethoxam		0.05–0.07	100	
Tramadol		0.03–0.06	94–100	
Trimethoprim		0.001–0.02	100	
Venlafaxine		0.05–0.08	100	
Warfarin		0.10–0.16	92–100	
Enrofloxacin	Non-RAS	0.03–0.06	75–82	Huang et al. (2019)
Sulfamethoxazole	(Freshwater; non-synthetic)	0.07–0.22	55–77	
Nonylphenol diethoxylates	Non-RAS (n.d; non-synthetic)	0.003–0.041	~0	Hsieh et al. (2013)
Nonylphenol monoethoxylates		0.003–0.035	~0	
Nonylphenol		0.001–0.152	16	
Octylphenol		0.001–0.901	38	
Ciprofloxacin	Non-RAS (n.d; non-synthetic)	0.02	100	Hsieh et al. (2015)
Chloramphenicol		0.06	98	
Chlortetracycline		not detected	n.d.	
Enrofloxacin		not detected	n.d.	
Leucomalachite green		n.d	n.d	
Malachite green		n.d	n.d.	
Oxolinic acid		0.01	100	
Oxytetracycline		0.22	97	
Sulfamonomethoxine		0.09	87	
Sulfadimethoxine		0.08	91	
Sulfamerazine		not detected	n.d.	
Sulfamethazinte		not detected	n.d.	
Tetracycline		not detected	n.d.	
Bisphenol A		0.9	89	
Nonylphenol diethoxylates		0.2	85	
Nonylphenol monoethoxylates		0.3	77	
Nonylphenol		1.7	90	
Octylphenol		1.1	85	
E2		0.2	95	

(*continued on next page*)

Table 2 (*continued*)

Target Micropollutant	Type of aquaculture (category of water)	Concentration ($\mu g \cdot L^{-1}$)	Removal (%)	Ref.
E3		0.2	77	
EE2		0.02	32	
Geosmin	RAS	not detected	n.d	Zhong et al. (2011)
2-methylisoborneol	(Freshwater; non-synthetic)	not detected–0.08	67–100	

n.d., not defined; EHMC, 2-ethylhexyl 4-methoxycinnamate; PFOS, perfluorooctanesulfonic acid; E2, 17-beta-oestradiol; E3, estriol; EE2,17-alpha-ethinyloestradiol.

efficiencies did not show evidence of lower CW performance in the presence of more concentrated effluents (Bòto et al., 2016; Gorito et al., 2018; Huang et al., 2019; Zhong et al., 2011).

A wide range of removals (0 %–100 %) of the target MPs has been verified, but it is important to consider that the number of compounds under analysis is also large and includes substances with very different characteristics. In general, CWs showed excellent performance in the removal of organic MPs. However, some of these compounds (17-alpha-ethinyloestradiol, 2-ethylhexyl 4-methoxycinnamate and sulfamethoxazole) were more resistant to the treatment, with low removal rates (between 32 and 54 %) (Gorito et al., 2018; Hsieh et al., 2015; Huang et al., 2019). As mentioned above, the distinct conditions of CWs and the composition of effluents can lead to different removal efficiencies of organic MPs. For instance, by analysing nonylphenol and octylphenol, it was possible to verify that two studies of the same research group (Hsieh et al. 2013, 2015) found very dissimilar removal of these compounds. When comparing the performance of CWs in removing nonylphenol diethoxylates, nonylphenol monoethoxylates, nonylphenol, and octylphenol in both reports, a series of three successive surface flow CWs (Hsieh et al., 2015) proved to be much more efficient (76 %–90 %) than an individual subsurface flow CW (0 %–38 %) operating with low concentrations of MPs (Hsieh et al., 2013). In a study by Hsieh et al. (2013), the ecological risks were evaluated by the risk quotients of nonylphenol and octylphenol derivatives before and after treatment; its decrease from influents to effluents indicates that CWs can effectively remove these phenolic endocrine disruptors. Furthermore, another study showed that CWs planted with single plant species are more efficient at removing enrofloxacin and sulfamethoxazole than CWs with mixed planting patterns (Huang et al., 2019). In the same report, sorption was the main removal mechanism of enrofloxacin, favouring the use of *Phragmites australis* instead of *Iris pseudacorus* because of its deeper and denser root system that enlarges the area for biofilm growth. In contrast, sulfamethoxazole has a low sorption capacity, with degradation (and transformation) playing a significant role in its removal in comparison to substrate adsorption and plant uptake. In this case, *Iris pseudacorus* provided a better environment for the microbial community to degrade or transform sulfamethoxazole (Huang et al., 2019). Indeed, two other studies showed excellent performance of CWs removing enrofloxacin with a single plant species, specifically *Phragmites australis*, treating both freshwater (Gorito et al., 2018) and marine (Bòto et al., 2016) aquaculture effluents.

All the reports detailed in Table 2 (including surface and subsurface flow configurations) correspond to operating periods equal to or higher than 1 month, and the successive loads of pollutants did not affect the efficiency of the systems. Moreover, most of the studies dealing with the removal of organic MPs (four out of six) also considered the elimination of other types of pollutants (such as total suspended solids, organic matter, nitrogen, phosphorus, ARB, and ARGs), which were also efficiently removed by CWs (Bòto et al., 2016; Gorito et al., 2017; Huang et al., 2019; Zhong et al., 2011). The reported information shows encouraging results for the implementation of CWs to remove organic MPs from aquaculture waters; however, further investigation is needed to fully understand the mechanisms responsible for the elimination of these compounds. In this way, the design and operational parameters can be optimised through appropriate criteria to obtain better treatment efficiencies for a wider list of MPs.

3.2.2. Removal of other pollutants

The potential of CWs to eliminate more conventional environmental contaminants such as nitrogen, phosphorus, organic matter and total suspended solids has been extensively described in the literature (Dotro et al., 2017; Polprasert, 2004; Vymazal et al., 2006). It is interesting to note that concerns about pollution caused by aquaculture, namely organic waste and nutrients released by their effluents, have increased over the last few years. As highlighted in the guiding document launched by the EC in 2016 (European Commission, 2016), organic matter and nutrients (as excretory products and uneaten fish food) can cause eutrophication of receiving waters, jeopardising ecosystems and natural dynamics of aquatic environments. Furthermore, even low concentrations of ammonia and nitrite ions (0.5 $mg \cdot L^{-1}$) can also be toxic to aquatic organisms (Buhmann and Papenbrock, 2013). Thus, it is important to implement measures to mitigate organic enrichment and nutrient input impacts, including treatment technologies such as CWs (European Commission, 2016).

The removal of conventional pollutants represents 91 % of CW studies in aquaculture farms (Fig. 4). These works include the treatment of both marine and freshwater aquaculture effluents in the laboratory, pilot (predominant) and full-scale CWs. Moreover, different configurations (surface and subsurface flow CWs) and operational conditions (such as nutrient loading rates, hydraulic regimes and plant species) of the CWs were also tested. It has been found that these parameters can affect the performance of wetland systems, but the efficiencies were in general sufficient to guarantee appropriate levels of discharge of conventional pollutants owing to the high removal efficiencies achieved. As verified for organic MPs, the high salinity of marine aquaculture effluents did not affect the effectiveness of the biological treatment, with the *Salicornia* plant species being frequently used for the treatment of this type of effluent (Li et al., 2018). The removal of nutrients and organic matter (assessed through chemical oxygen demand, biochemical oxygen demand and total organic carbon) is strongly dependent on the microbial activity. In fact, a considerable number of studies have focused on the composition and activity of the microbial consortium (Li et al., 2018; Liu et al., 2016; Lopardo et al., 2019; Zhimiao et al., 2019). Most of the studies analysed the removal of nitrogen compounds from external carbon sources (Lopardo et al., 2019; von Ahnen et al., 2020) or iron addition (Zhimiao et al., 2019) representing successful alternatives to enhance nitrification-denitrification processes. Despite the capacity of CWs to remove bacteria, only two studies have focused on the elimination of ARB (Bòto et al., 2016) and ARGs (Huang et al., 2019) from aquaculture waters, registering removals of approximately 95 % and 57 %, respectively.

Overall, the reviewed data indicate that CWs are highly efficient in removing conventional pollutants (such as organic matter, nitrogen, phosphorus, and total suspended solids) from aquaculture effluents. The functioning of CWs must be routinely controlled; if necessary, adaptations can be easily made to optimise the efficiencies obtained. Compared with other treatment technologies frequently applied in aquaculture farms (such as mechanical filters, trickling filters, submerged biofilters and rotating biological contactors), CWs may be an appealing alternative because of their attractive costs, environmental friendliness and efficiency in removing either conventional pollutants or organic MPs.

4. Conclusions and perspectives

The removal of organic MPs by AOTs and CWs from aquaculture waters is a recent area of study, and more research is needed to determine the feasibility of their full-scale application. The potential of both categories of technologies for the removal of MPs from contaminated

water/wastewater is now recognised, but information specific to aquaculture is still limited, with only 28 and 6 published studies having focused on AOTs and CWs, respectively. Although these reports show attractive results in terms of MPs elimination, the investigated conditions are still far from reality, for example, testing very high concentrations of MPs, synthetic water samples, installations at the micro-scale and batch treatments. In the specific case of AOTs, future research should be performed with lower concentrations of organic MPs, being directed to pilot and full-scale applications because the reviewed information is very focused on the pollutant itself, neglecting the influence of other aspects such as the real matrix, installation design and costs. Since light-driven and ozone-based AOTs have been applied at full scale aquacultures to remove other types of pollutants, investigating their performance regarding MP removal can be a valuable option. With this approach, it would be possible to reach valid conclusions about the treatment feasibility and the main parameters where improvement is possible. In relation to CWs, additional research is needed to increase the knowledge on the removal mechanism of MPs. Groups of MPs with similar characteristics can be an interesting alternative; at the same time, several CW designs and operational factors should be extensively studied.

It is unquestionable that the contamination of aquaculture waters by MPs and their respective treatments are topics that need attention. The AOTs and CWs, reviewed herein, are very distinct technologies that can remove these substances. However, knowledge about the best option for different type of aquaculture is lacking. Fig. 5 represents the main strengths of both systems, as well as their suitable application according to the type of aquaculture.

As a low-cost, ecological and biological solution, CWs are an interesting option for the removal of organic MPs in non-RAS, especially when these farms have spacious outdoor areas that favour their implementation, and as a consequence, the creation of additional ecosystems and habitats is possible. As mentioned above, CWs are affected by external factors such as temperature, humidity, and sunlight exposure; thus, long acclimatisation periods may occur. Furthermore, slower treatment is also expected because CW performance is dependent on the natural processes occurring in the system. For this reason, CWs seem to be a more compatible treatment for non-RAS, since these types of facilities are easily implemented in rural areas (such as inland flow-through systems) and do not require advanced or rapid treatment technologies. In contrast, AOTs are more compatible with RAS because, in general, these farms are more industrialized and modernised (often installed distant from natural water sources), with optimised profits and strict requirements, which demand efficient equipment to treat water before reuse.

It is interesting to mention that combining CWs and AOTs could also be an attractive alternative to remove MPs from aquacultures, since the individual advantages of both technologies are maximised, that is, the low cost of CWs and the high treatment efficiency of AOTs. Thus, CWs might function as: (i) pre-treatment of AOTs, that is, CWs removing most MPs and the more recalcitrant ones being eliminated by AOTs; or (ii) as post-treatment of AOTs, that is, CWs treating a more biodegradable effluent and removing possible by-products generated by AOTs. The combination of CWs and AOTs has been discussed in some studies to remove MPs (Cedillo-Herrera et al., 2020; Herrera-Melián et al., 2012; Lancheros et al., 2019); however, their application to aquaculture aqueous matrices has not yet been investigated. CWs and AOTs (singly applied or combined) are promising options for removing organic MPs from aquaculture farms, being simultaneously efficient in eliminating other relevant pollutants in full-scale systems. Therefore, research on this type of treatment option should be reinforced to maximise the performance and reduce the costs for the treatment of aquaculture waters. In this way, sustainable food production can be favoured, and environmental pollution can be prevented.

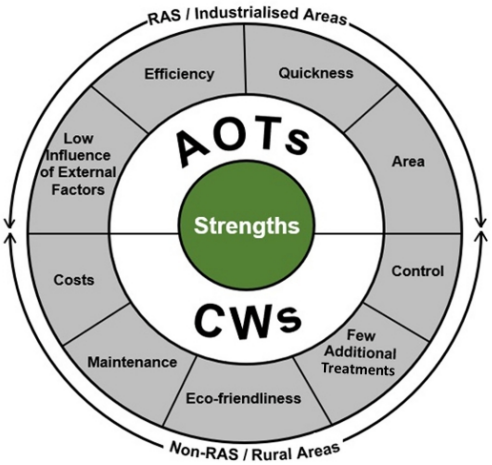

Fig. 5. Main strengths of AOTs and CWs, and suitable application according to the type of aquaculture (i.e., RAS in industrialized areas and non-RAS in rural areas).

Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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"Constructed wetland microcosms for the removal of organic micropollutants from freshwater aquaculture effluents?"

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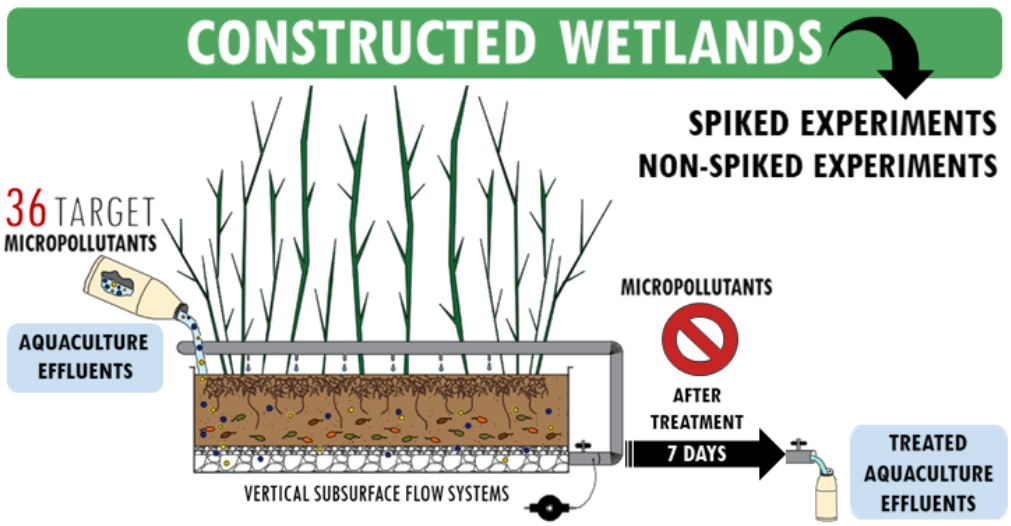

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Constructed wetland microcosms for the removal of organic micropollutants from freshwater aquaculture effluents

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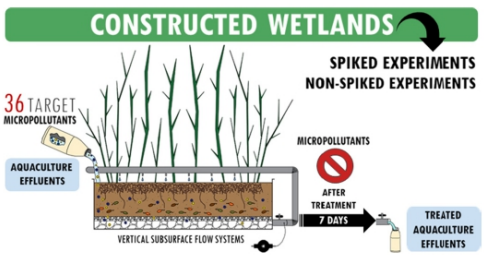
HIGHLIGHTS

- Constructed wetland microcosms studied for the removal of organic micropollutants.
- Spiked (36 micropollutants) and non-spiked aquaculture effluents were investigated.
- Most micropollutants found in effluents were removed to below the detection limit.
- Weekly removal efficiencies were higher than 87% for most spiked micropollutants.
- The UV filter EHMC was the only recalcitrant micropollutant.

GRAPHICAL ABSTRACT



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ABSTRACT

The presence of organic micropollutants (MPs) in the aquatic environment is strongly related to their difficult elimination by conventional water and wastewater treatment processes. Therefore, alternative treatment technologies are required to overcome this problem. In this domain, constructed wetlands (CWs) have gained increasing attention in the last years, mainly due to the low-cost, simple operation/maintenance and environmental friendliness of these systems. However, studies on the application of CWs to remove MPs from freshwater aquaculture effluents are still scarce. In this work, planted (*Phragmites australis*) vertical subsurface flow CWs, at microcosm scale, were investigated for the removal of MPs found in non-spiked freshwater aquaculture effluents, namely atrazine, isoproturon, perfluorooctanesulfonic acid (PFOS), clarithromycin, erythromycin, fluoxetine, norfluoxetine, and 2-ethylhexyl-4-methoxycinnamate (EHMC). A wider multi-component set of 36 MPs was also studied by adding these MPs at 100 ng L^{-1} to the same matrix (alachlor, atrazine, chlorfenvinphos, isoproturon, PFOS, azithromycin, clarithromycin, erythromycin, diclofenac, methiocarb, acetamiprid, clothianidin, thiacloprid, thiamethoxam, EHMC, simazine, atorvastatin, bezafibrate, carbamazepine, cephalexin, ceftiofur, citalopram, clindamycin, clofibric acid, diphenhydramine, enrofloxacin, fluoxetine, ketoprofen, metoprolol, norfluoxetine, ofloxacin, propranolol, tramadol, trimethoprim, venlafaxine, and warfarin). High weekly removal efficiencies (>87%) were observed for all MPs in both non-spiked and spiked experiments, with the exception of EHMC (removal rates between 0 and 86%). These results emphasize the potential of

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CWs to remove MPs from freshwater aquaculture effluents, but also the need to enhance the performance of these systems for the elimination of some recalcitrant MPs, such as EHMC, which was found at high concentrations in the studied effluents.

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1. Introduction

Water is a vital resource for life on Earth, having an immeasurable economic, social and environmental value. However, the current human use of this essential resource is no longer sustainable. For instance, human activities are generating large volumes of wastewater, which must be properly treated to avoid contamination of natural water resources. In the last two decades, organic micropollutants (MPs) have gained increasing attention due to the ineffective elimination of many of them in conventional water and wastewater treatment plants, and the consequent detection in the aquatic environment (Luo et al., 2014). MPs in the aquatic systems, usually between ng L^{-1} and μg L^{-1}, encompass several classes of substances, such as pharmaceuticals and personal care products (PPCPs), steroid hormones, industrial chemicals, pesticides, among others. These compounds are continuously released into the watercourses, posing a serious risk to both wildlife and human health, as for example, proliferation of antibiotic resistant bacteria and/or genes and endocrine disruption (Raghav et al., 2013). The European Union (EU) has demonstrated an evident concern in this domain by launching two documents, the Directive 2013/39/EU (Directive, 2013) and more recently, the Decision 2015/495/EU (Decision, 2015). The Directive 2013/39/EU includes a list of 41 organic priority substances/classes of substances (PSs) with their respective environmental quality standards (EQS), and 8 so-called "certain others pollutants" with EQS to be considered (Ribeiro et al., 2015). Furthermore, this Directive also highlights the importance of monitoring the occurrence of other MPs, still not included in this list and known as contaminants of emerging concern (CECs). In this context, a Watch List of 10 substances/group of substances (total of 17 CECs), which should be monitored in surface waters within the EU, was established by the European Commission in the Decision 2015/495/EU (Barbosa et al., 2016a).

To prevent the continuous discharge and presence of MPs in water bodies, as well as their possible undesirable effects, unconventional water treatment technologies are imperative. Chemical oxidation technologies and advanced oxidation processes, adsorption by activated carbon, membrane bioreactors and other membrane processes (e.g., microfiltration, ultrafiltration, nanofiltration, and reverse osmosis) are some examples of technologies already applied at full-scale (Gorito et al., 2017). However, in most cases, a high investment is required, emphasizing the need of exploring more affordable solutions (Zhang et al., 2014), such as constructed wetlands - CWs (Liu et al., 2015). Most reports dealing with CWs show the potential of these systems to eliminate specific types of contaminants, such as total suspended solids (TSS), organic matter, nutrients and metals (Gorito et al., 2017). However, more recently, several studies also reported promising results for the removal of MPs, namely pesticides (Anderson et al., 2013; Matamoros et al., 2007b; Warsaw et al., 2012), veterinary medicines (Carvalho et al., 2013; Hsieh et al., 2015; Matamoros et al., 2008), PPCPs (Anderson et al., 2013; Park et al., 2009; Yi et al., 2017) and industrial compounds (Šíma et al., 2013; Toro-Vélez et al., 2016). Despite this, only less than half of the EU relevant substances (PSs and CECs) were investigated so far and in many different matrices: 24 out of a total of 41 organic PSs, plus 2 of the 8 pollutants with EQS to be considered, and 8 CECs out of the 17 referred in the Watch List (Gorito et al., 2017). Moreover, only a few of these studies reported the removal of some of these MPs in a multi-component mixture (Carlson et al., 2013; Hsieh et al., 2015; Matamoros and Salvadó, 2012; Qiang et al., 2013).

Attending to the dispersion of MPs in the aquatic environment, many food industries end up using process water polluted with these compounds, as is the case of aquaculture farms. Nowadays aquaculture provides near half of the fish consumed worldwide, and an increase >60% is expected over the next 15 years (Webb et al., 2012). Typically, intensive aquaculture involves breeding control and the artificial supply of food and medicines, which may contain MPs that might posteriorly be released in the effluents of this farming activity (Muñoz et al., 2010). Thus, MPs can be found in both aquaculture influents and effluents and also in the resulting food-products, affecting the environment and posing a risk for aquatic organisms and human health (Gavrilescu et al., 2015). In this context, appropriate treatment processes to remove MPs are needed in aquaculture farms, being the CWs an attractive option. However, it is interesting to note that, to the best of our knowledge, only two studies pointed out the CWs as a possible treatment to eliminate MPs from freshwater aquaculture effluents, namely nonylphenol (Hsieh et al., 2013; Hsieh et al., 2015), octylphenol, 17-beta-estradiol and 17-alpha-ethinylestradiol (Hsieh et al., 2015).

In this work, the MPs present in the waters collected at the entrance and exit of an aquaculture freshwater facility were determined to assess which of the selected MPs were present in its influents and effluents. Then, the efficiency of CWs to treat the raw (non-spiked) effluent, as well as the effluent spiked with 36 organic MPs, was investigated to evaluate the potential of these biological systems to remove a mixture of a larger number of MPs. Effluents, instead of aquaculture influents, were studied because in most cases they have a more complex matrix and most MPs appearing in aquaculture influents are expected to be present in the respective effluents. Target MPs in this study included PSs and CECs listed in Directive 2013/39/EU and Watch List of Decision 2015/495/EU, respectively, as well as other relevant MPs selected due to their frequency of detection in water bodies, persistence and ecotoxicity. Two sets of experiments were carried out in microcosms simulating a vertical subsurface flow CW (VSSF-CW), one using spiked (100 ng L^{-1} of each selected MP) and another using non-spiked aquaculture effluents, during four one-week cycles of CWs treatment.

2. Materials and methods

2.1. Chemicals and materials

The 36 MPs targeted in this study included 6 PSs identified in the Directive 2013/39/EU (alachlor, atrazine, chlorfenvinphos, isoproturon, perfluorooctanesulfonic acid (PFOS), simazine), 10 CECs defined in the Watch List of Commission Decision 2015/495/EU (azithromycin, clarithromycin, erythromycin, diclofenac, methiocarb, acetamiprid, clothianidin, thiacloprid, thiamethoxam, 2-ethylhexyl-4-methoxycinnamate (EHMC)) and a set of 20 CECs (atorvastatin, bezafibrate, carbamazepine, cephalexin, ceftiofur, citalopram, clindamycin, clofibric acid, diphenhydramine, enrofloxacin, fluoxetine, ketoprofen, metoprolol, norfluoxetine, ofloxacin, propranolol, tramadol, trimethoprim, venlafaxine, warfarin). The physicochemical properties of the analytes are present in Table S1 (Supplementary Information). All abovementioned reference standards, as well as the internal standards (atrazine-d_5, acetamiprid-d_3, methiocarb-d_3, clothianidin-d_3, diclofenac-d_4, azithromycin-d_3, ofloxacin-d_3, fluoxetine-d_5, ketoprofen-d_3), were of high purity grade (>98%) and were purchased from Sigma-Aldrich (Steinhein, Germany). Stock standard solutions with a concentration of ca. 1000 mg L^{-1} were prepared by dissolving known amounts of

the standards in ethanol, methanol or acetonitrile, depending on their solubility. Similarly, solutions of 1000 mg L^{-1} of each isotopically labelled internal standard were prepared, by dissolving the respective standard in methanol (except fluoxetine-d_5, which was already in solution) or in 1% of NaOH solution in the case of ofloxacin-d_3. Two working solutions were prepared using ethanol as solvent, one containing each target analyte at 2.5 mg L^{-1} and another containing the individual internal standards at 10 mg L^{-1}. Ethanol HPLC grade was acquired from Fisher Scientific (Leicestershire, UK), whereas methanol and acetonitrile (MS grade) were purchased from VWR International (Fontenay-sous-Bois, France). Formic acid and sulphuric acid were supplied by Merck (Darmstadt, Germany). Oasis® Hydrophilic-Lipophilic-Balanced (HLB) cartridges (150 mg, 6 mL) were acquired from Waters (Milford, Massachusetts, USA).

2.2. Sampling

Influent and effluent samples from a Portuguese freshwater aquaculture facility (through-flow system) were collected every week (ca. 15 L, grab samples) during February 2017 (1st, 8th, 16th and 22nd) to evaluate the presence of MPs, being the aquaculture effluents used to perform the CW microcosms experiments. To replicate the typical conditions of natural wetlands and their surrounding environment, *Phragmites australis* plants already grown, sediments in direct contact with plant roots and sand were collected in January 2017 at Lima river margins (Portugal), and immediately transported to the laboratory for subsequent assembly of microcosms.

2.3. CW systems

2.3.1. Setup

CW microcosms were assembled at bench-scale in VSSF-CW configuration, as described elsewhere (Carvalho et al., 2013), and adapted to automatically operate with a recirculating water system (flow rate ca. 10 mL min^{-1}), employing a pump and six outlets dripping on substrate top (Fig. 1a). Six microcosms were set up using plastic containers (40 cm × 30 cm × 30 cm) filled with a first layer of gravel (4 cm), a second layer of lava rock (2 cm) and, finally, a third previously prepared layer of the roots bed substrate (16 cm), into which *P. australis* were transplanted (ca. 40 sprouts per microcosm). Gravel was used at the bottom to prevent clogging of the draining tubes, while lava rock was applied in the middle of layers to increase the porosity of the substrate, promote the retention of contaminants and potentiate the development of biofilms, which have an important role in the degradation of MPs. Sediments in contact with plant roots and sand collected at Lima river were mixed (proportion 1:2, to increase substrate porosity) and homogenized to prepare the roots bed substrate for CW microcosms, the top layer. This layer of soil supports the plants (implementation of plants belowground tissues), also maintaining the native microbial consortium associated with this plant species. The plants (*P. australis*) were arranged in accordance with their stems length, aiming at an equal distribution in their transplantation into the CW systems. As plants were fully developed, significant plant growth was not expected in the relatively short time of the experimental trial, and for that reason, a considerable plant density was used to maximize their effect on CW functioning. The plastic containers were wrapped with aluminium foil, to prevent natural light penetration through the container walls and the possible photodegradation of the compounds, mimicking CWs natural conditions. Microcosms were kept under greenhouse-like conditions, namely natural day:night regime, with a temperature variation between 10 °C and 20 °C. Before the experiments, CW microcosms were acclimated for 1 week with a Hogland nutrient solution (level just above the substrate surface), in order to maintain suitable nutritional conditions for both plants and substrate. Afterwards, the systems were filled with freshwater aquaculture effluents.

2.3.2. Operation and sampling

Six microcosms were assembled for spiked (three microcosms) and non-spiked experiments (three microcosms), each one treating 2 L of aquaculture effluents. This volume was established because it enabled the complete saturation of the substrate, maintaining the water level just above the substrate surface (saturation rate of ca. 100%). One set of triplicate CW systems was supplemented with 2 L of aquaculture effluents (as collected) and the other set of triplicate CW systems was supplemented with the same volume of aquaculture effluents spiked with 80 μL of a working standard solution containing each target MP at 2.5 mg L^{-1}. The resulting concentration (100 ng L^{-1} of each MP) was chosen taking into consideration our recent review addressing the occurrence of PSs and CECs in surface waters (Sousa et al., 2018) and our previous studies dealing with MPs in aquatic systems

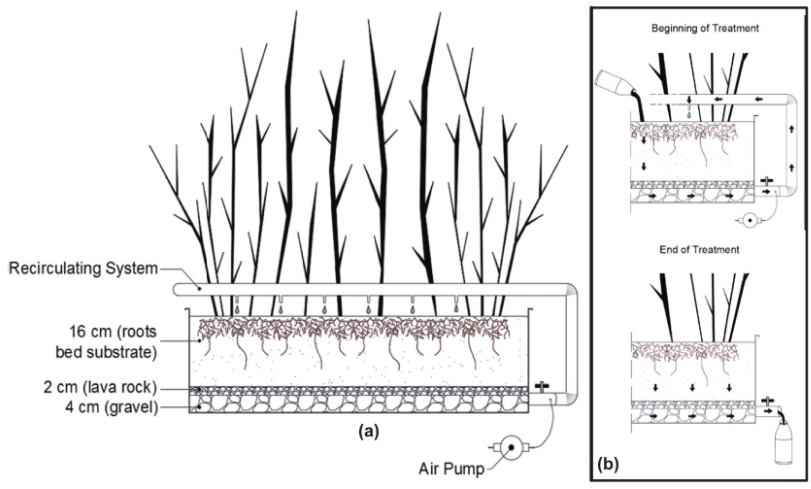

Fig. 1. CW microcosm setup (a), operation and sampling at the beginning and at the end of each weekly treatment (b).

(Barbosa et al., 2016b; Ribeiro et al., 2016), where many of the selected MPs have been reported from ng L^{-1} to μg L^{-1} levels.

After addition of the effluents on the substrate top of the CW systems (Fig. 1b), aquaculture effluents were treated for 1 week. This commonly used hydraulic retention time (HRT) is adequate for an efficient removal of pollutants, according to Zhang et al. (2014). During this time, effluents were automatically recirculated in the system as mentioned in Section 2.3.1 (7 a.m.–7 p.m.), to prevent the development of anoxic areas and to promote the removal of MPs. The CWs performance was evaluated during 1 month, i.e., four 1-week cycles. The regular addition of new aquaculture effluents into the CW microcosms, at the beginning of each week, allowed simulating the cumulative effect of a new load of pollutants added cyclically to the system, which may affect the CWs performance over time, as occurs in full-scale systems. In this sense, microcosms were weekly drained and refilled with spiked or non-spiked fresh aquaculture effluents, collected as indicated in Section 2.2. At the end of each week of treatment, the liquid phase of each CW microcosm was drained into glass bottles (Fig. 1b), to which deionized water was added to achieve a final volume of 2 L, equalizing the initial volume for a proper analysis (an average of 600 and 500 mL of deionized water was needed each week, in spiked and non-spiked CWs, respectively, resulting from water losses of 30 and 25% for each one of the systems). Small amounts of deionized water were also added to CW microcosms along the week, whenever necessary, to maintain ca. 100% saturation of the substrate bed.

All samples collected during the CW experiments, as well as the aquaculture influent samples, were stored at 4 °C and filtration was performed in <24 h, using 1.2 μm nylon membrane filters (Membrane Solutions, Texas, USA) to eliminate suspended solids before analysis.

2.4. Chemical analysis (MPs, dissolved organic carbon (DOC) and ions)

The concentration of the target MPs in the inlet and outlet samples of CW microcosms and in the freshwater samples of the aquaculture facility was determined using an analytical methodology that couples solid phase extraction and ultra-high performance liquid chromatography-tandem mass spectrometry (SPE-UHPLC-MS/MS). Water samples were pre-cleaned and concentrated by using a SPE procedure described elsewhere (Barbosa et al., 2016b). Briefly, Oasis® HLB cartridges were used to extract 500 mL of sample adjusted to pH 3, with ethanol as eluting solvent. MPs analysis was carried out in a UHPLC Shimadzu Corporation equipment connected to a triple quadrupole mass spectrometer detector (Ultra-Fast Mass Spectrometry series LCMS-8040). Methanol and water acidified with 0.10% formic acid (50/50, v/v) were used as mobile phase for the chromatographic separation. Capillary voltage, drying gas flow, nebulizing gas flow, desolvation and source temperature of the mass spectrometer were respectively 4.5 kV, 10 dm^3 min^{-1}, 3 dm^3 min^{-1}, 250 °C and 400 °C. Method detection limits (MDLs) and method quantification limits (MQLs) of the target analytes are presented in Table S2 (Supplementary Information).

The removal percentages of each of the 36 target MPs in the individual CW microcosms were determined using Eq. (1).

$$\text{Removal efficiency } (\%) = \frac{(C_{\text{Inlet}} - C_{\text{Outlet}})}{C_{\text{Inlet}}} \times 100 \quad (1)$$

where C_{Inlet} and C_{Outlet} are the concentrations of the target MPs entering and leaving CW microcosms, respectively. The average weekly removal efficiency of each set of experiments (spiked and non-spiked aquaculture effluents) was determined by calculating the mean of each triplicate CW microcosms.

Dissolved organic carbon (DOC) was determined following the standard procedure 5310 B of the Standard Methods for Examination of Water and Wastewater (APHA et al., 1998), and using a Shimadzu TOC-L apparatus.

The concentrations of cations (sodium, potassium, ammonium, calcium, magnesium) and anions (chloride, bromide, bromate, nitrite, nitrate, phosphate, sulphate) in solution were determined by ionic chromatography using, respectively, Metrosep C4 Cationic Exchange and Metrosep A Supp 7 Anionic Exchange columns (250 mm × 4.0 mm) in a Metrohm 881 Crompaed IC Pro equipment. The pH values were measured using a pH meter pHenomenal® pH 1100 L (VWR, Germany).

3. Results and discussion

3.1. Occurrence of target MPs in aquaculture facility influents and effluents

The influent and effluent samples weekly collected in the aquaculture facility (i.e. non-spiked) were analysed to assess which of the selected MPs were present at the entrance and exit of the farm.

Ten MPs out of a target group of 36 were detected during the 4 weeks of sampling (Table 1), including 3 PSs listed in the Directive 2013/39/EU (atrazine, isoproturon and PFOS), 4 CECs included in the Watch List of Decision 2015/495/EU (azithromycin, clarithromycin, erythromycin and EHMC) and 3 other CECs (diphenhydramine, fluoxetine and norfluoxetine). The pesticides and pharmaceuticals were the MPs found at lower concentrations (most of them at nearly 1 ng L^{-1}). Moreover, the industrial compound PFOS was quantified at a concentration ten times higher, while concentrations up to 530 ng L^{-1} were found for the UV filter EHMC. With the exception of azithromycin and diphenhydramine, which were apparently removed from water in the aquaculture facility, no considerable differences between the inlet and outlet aquaculture water samples were observed in each sampling week, which means that this aquaculture farm is not a source of the MPs under study. Despite this, some differences were observed from week to week, which can be caused by the different anthropogenic activities and meteorological conditions occurring during the sampling period. The fact that a few MPs were only detected in the aquaculture influents may indicate their adsorption or degradation within the aquaculture facility or their bioaccumulation in aquatic biota. However, the distribution in the aquaculture facility is not a focus of this work. Notwithstanding, for most of the target MPs, the present results clearly indicate that MPs entering in the feed-freshwater, were passing through the aquaculture facility, which emphasizes that water treatments are needed.

As abovementioned, the presence of MPs in surface waters is mainly related to the inefficient removal of these compounds in wastewater treatment plants and also to the industrial and agricultural activities (common practices near the sampling site). In the particular case of EHMC, which is often used as an ingredient in PPCPs (sunscreens, beauty creams, hair sprays, shampoos, among others) or as furniture protector, the presence of some industries close to the aquaculture farm (namely timber, plastic, personal hygiene, fragrance and cosmetics industries) may explain the high concentration of this compound when compared with those values of other substances.

3.2. Removal of MPs in CW microcosms

Aquaculture effluents were used in CWs experiments, since in most cases the effluents have a more complex matrix than the freshwater entering into the aquaculture facilities (these effluents can contain higher levels of organic matter or nutrients, which can affect CWs performance), and also because most of the MPs determined in the influents were also present in the effluents.

A set of experiments was conducted using effluents as collected (Fig. 2a) and another set by spiking the same effluents with the selected 36 MPs at 100 ng L^{-1} each (Fig. 2b), with the aim of simulating a worst case scenario and because the MPs not found in the realistic effluent under study may be found in the effluents of other aquaculture farms. In Fig. 2, the closed circles refer to the initial MP concentration in the

Table 1
Concentrations of the target MPs (ng L^{-1}) in the influents (**I**) and effluents (**E**) of the selected freshwater aquaculture facility (mean and standard deviation, $n = 2$); * or ** represent one measurement of the target analyte, being the other below the Method Detection Limit (MDL) or Method Quantification Limit (MQL), respectively. The compounds listed in footnote[a] were not detected in any sampling week.

Organic MPs	Sampling period			
	Week 1 (February 1st, 2017)	Week 2 (February 8th, 2017)	Week 3 (February 16th, 2017)	Week 4 (February 22nd, 2017)
Pesticides				
Atrazine	**I** = 0.43 ± 0.01	**I** = 0.19 ± 0.04	**I** = 0.12 ± 0.01	**I** = 0.4 ± 0.2
	E = 0.42 ± 0.01	**E** = 0.5 ± 0.2	**E** = 0.26 ± 0.01	**E** = 0.2 ± 0.1
Isoproturon	**I** < 0.13 (MQL)	**I** < 0.13 (MQL)	**I** < 0.04 (MDL)	**I** < 0.04 (MDL)
	E < 0.13 (MQL)	**E** < 0.13 (MQL)	**E** < 0.04 (MDL)	**E** < 0.04 (MDL)
Industrial compound				
PFOS	**I** = 10.0 ± 0.7	**I** = 10 ± 1	**I** = 11 ± 2	**I** < 0.91 (MDL)
	E = 11 ± 2	**E** = 9.54 ± 0.07	**E** = 11 ± 2	**E** < 0.91 (MDL)
Pharmaceuticals				
Azithromycin	**I** = 1.12 ± 0.09	**I** = 2.8 ± 0.2	**I** = 1.30 ± 0.03	**I** = 1.03 ± 0.09
	E < 0.15 (MDL)	**E** < 0.15 (MDL)	**E** < 0.15 (MDL)	**E** < 0.15 (MDL)
Clarithromycin	**I** = 0.40*	**I** = 1.1 ± 0.4	**I** < 0.39 (MQL)	**I** = 3.28**
	E < 0.39 (MQL)	**E** < 1.92**	**E** < 0.39 (MQL)	**E** < 0.39 (MQL)
Diphenhydramine	**I** = 1.91 ± 0.01	**I** < 0.02 (MDL)	**I** = 1.4 ± 0.6	**I** < 0.02 (MDL)
	E < 0.02 (MDL)	**E** < 0.02 (MDL)	**E** < 0.02 (MDL)	**E** < 0.02 (MDL)
Erythromycin	**I** < 0.13 (MDL)	**I** < 0.13 (MDL)	**I** < 0.13 (MDL)	**I** < 0.13 (MDL)
	E < 0.13 (MDL)	**E** < 0.39 (MQL)	**E** < 0.39 (MQL)	**E** < 0.13 (MDL)
Fluoxetine	**I** < 0.10 (MDL)	**I** = 1.93 ± 0.08	**I** < 0.10 (MDL)	**I** < 0.10 (MDL)
	E < 0.10 (MDL)	**E** = 2.71**	**E** < 0.10 (MDL)	**E** < 0.10 (MDL)
Norfluoxetine	**I** < 1.61 (MDL)	**I** = 8.1 ± 0.2	**I** < 1.61 (MDL)	**I** < 1.61 (MDL)
	E < 1.61 (MDL)	**E** = 6.52*	**E** < 1.61 (MDL)	**E** < 1.61 (MDL)
Organic UV filter				
EHMC	**I** = 314 ± 84	**I** = 246 ± 1	**I** = 532 ± 25	**I** = 495 ± 39
	E = 307 ± 161	**E** = 252 ± 21	**E** = 452 ± 6	**E** = 506 ± 2

[a] The other 26 target MPs (alachlor, chlorfenvinphos, simazine, diclofenac, methiocarb, acetamiprid, clothianidin, thiacloprid, thiamethoxam, atorvastatin, bezafibrate, carbamazepine, cephalexin, ceftiofur, citalopram, clindamycin, clofibric acid, enrofloxacin, ketoprofen, metoprolol, ofloxacin, propranolol, tramadol, trimethoprim, venlafaxine and warfarin) were not detected in any sample.

effluent. Some deviations from 100 ng L^{-1} in spiked experiments were observed due to the presence of these compounds in the collected effluent samples or to matrix effects. The removal efficiencies (grey bars) of the investigated MPs throughout the 4 weeks of treatment demonstrated a high elimination performance for most of the target compounds, in both non-spiked and spiked experiments, EHMC representing the sole exception in both cases.

In the particular case of the non-spiked experiments (Fig. 2a), high removal efficiencies were obtained for the majority of the detected MPs. The pesticide atrazine was fully eliminated in all cycles of treatment (i.e., to concentrations below the MDL, 0.05 ng L^{-1}). In the case of PFOS, its removal was almost complete, except in the first week of treatment, which may be related to the acclimation of the biological systems in the initial stage. In fact, these biological systems may require a period of adaptation before attaining their full performance, as observed in other studies (Bôto et al., 2016). Removals nearly 100% were also observed for the pharmaceuticals clarithromycin, fluoxetine and norfluoxetine. However, these compounds were only quantified in the effluent samples collected in the second week, hampering a detailed analysis and the comparison of the removal efficiencies between different cycles. The UV filter EHMC was quantified at hundreds of ng L^{-1} in all effluent samples and was poorly removed (<55%) in the CW microcosms. These preliminary experiments using non-spiked effluents (as collected), demonstrated the potential of the CWs to eliminate most of the MPs present in the studied effluent samples.

For spiked experiments (Fig. 2b), it is noticeable the adaptation period of the CW systems at the beginning of the experiments, with an overall removal efficiency slightly lower for many MPs, as suggested by the results obtained for PFOS in the non-spiked experiments. However, the overall efficiencies in the four 1-week cycles were higher than 87% for all compounds, excluding EHMC, which was also the most recalcitrant MP in the non-spiked experiments. Specifically, 21 out of the 36 MPs were totally eliminated (i.e. to levels below the MDL, meaning removal rates ≈100%) in all weeks of spiked experiments, namely acetamiprid, alachlor, atorvastatin, bezafibrate, carbamazepine, cephalexin, ceftiofur, citalopram, clindamycin, clothianidin, enrofloxacin, erythromycin, ketoprofen, methiocarb, metoprolol, norfluoxetine, ofloxacin, thiacloprid, thiamethoxam, trimethoprim, and venlafaxine. In the case of acetamiprid, atorvastatin, cephalexin, citalopram, clothianidin, methiocarb, norfluoxetine, thiacloprid, and thiamethoxam, no studies were found in the literature regardless of the matrix, invalidating a possible comparison with the obtained results. For the other MPs (alachlor, bezafibrate, carbamazepine, ceftiofur, clindamycin, enrofloxacin, erythromycin, ketoprofen, metoprolol, ofloxacin, trimethoprim, and venlafaxine), studies in the literature showed lower removal rates than those described in this work (Berglund et al., 2014; Hijosa-Valsero et al., 2011; Hijosa-Valsero et al., 2016; Li et al., 2014; Matamoros et al., 2007b; Vymazal et al., 2017). However, a direct comparison is difficult because the tested operating conditions were considerably different, as well as the type of matrix employed (which are known to significantly affect the removal rates achieved in CWs). In the case of the MPs bezafibrate, metoprolol, venlafaxine, erythromycin, ceftiofur, and enrofloxacin, the following removals were respectively obtained for conditions similar to those tested in this work: 66%, 80%, 57%, >92% (Rühmland et al., 2015), >85% and >98% (Almeida et al., 2017), but using, respectively, domestic and livestock wastewater as matrix. The higher inlet concentrations of bezafibrate and venlafaxine studied by Rühmland et al. (2015) might justify the lower removal rate (66 and 57%, respectively), when compared to the removals obtained in the present study. Regarding carbamazepine, the published works generally reported poor performances of CWs to remove it, including studies employing VSSF-CWs, the same configuration herein reported (Li et al., 2014; Matamoros et al., 2007a). Therefore, a more detailed analysis would be needed to understand these marked differences.

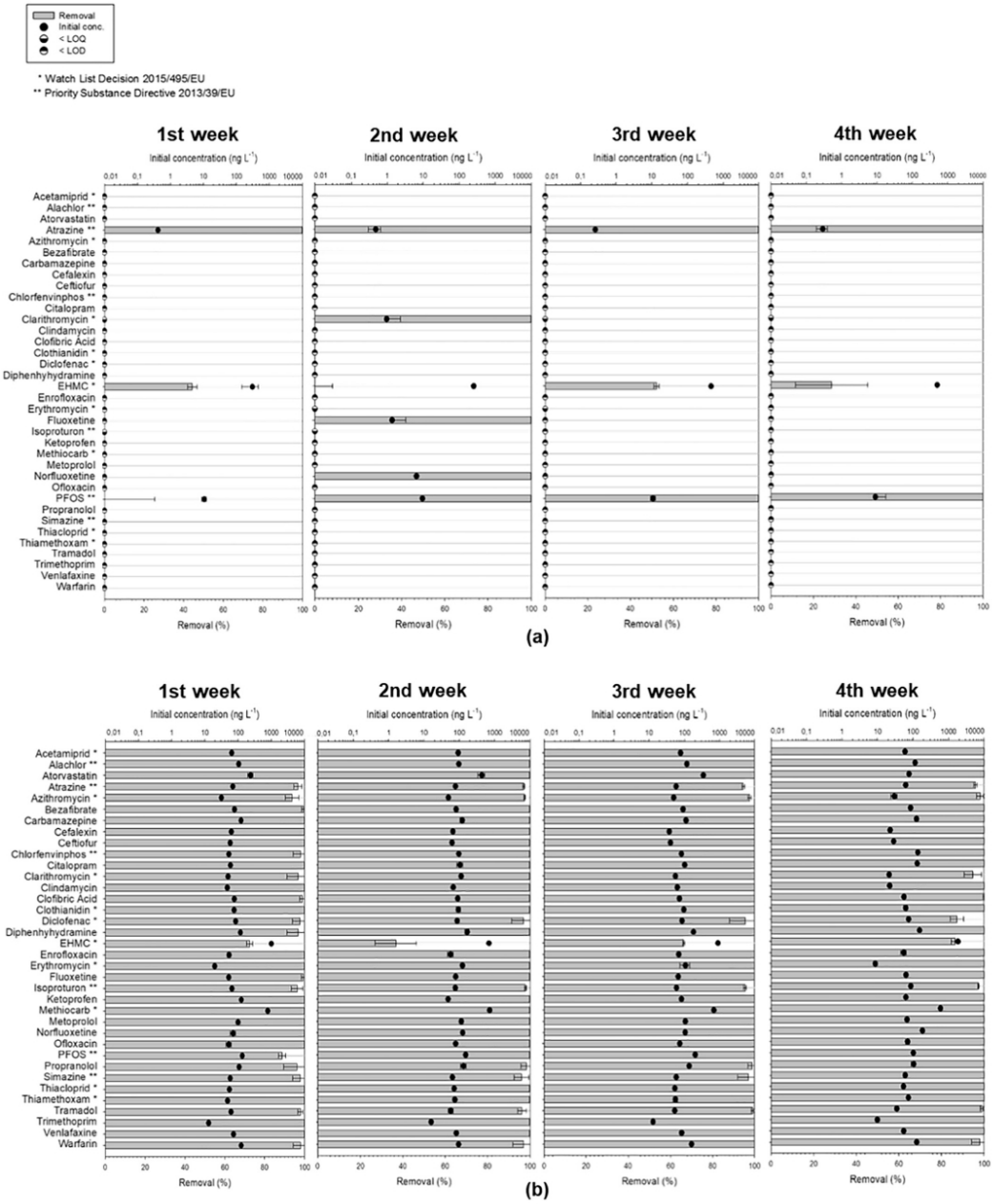

Fig. 2. Weekly removal efficiencies and concentrations of the 36 target MPs during the four 1-week cycles of CW microcosms treatment, using non-spiked (a) and spiked (b) aquaculture effluents.

For the MPs that were not eliminated from water to levels below the MDL (Fig. 2b), atrazine, azithromycin, diclofenac, isoproturon, propranolol, simazine, tramadol, and warfarin, the removal efficiencies were always higher than 87%. A recent review survey shows very dissimilar removals for the PSs atrazine (0–100%) and simazine (20–96%) and removals up to 50% for isoproturon (Gorito et al., 2017). Therefore, the

present study exhibited a better performance of CWs to remove isoproturon, i.e., almost total elimination. In turn, removal efficiencies for the Watch List CEC azithromycin (94–98%) were similar to those described by Berglund et al. (2014). Although that study applied different conditions, namely a surface-flow CW (SF-CW) instead of the configuration used in the present study (VSSF-CWs), the removal performances were similar. These results may indicate that photodegradation (that can occur in SF-CWs) and biodegradation (that can occur in VSSF-CWs) were not probably the main elimination processes for this specific MP. High values of octanol-water coefficient (K_{ow}) and pK_a of azithromycin suggest its ability to sorb into soil and/or to be uptaken by plants, common processes in both configurations. For diclofenac, removal rates between 88 and 98%, slightly lowering over time, were observed. Published works dealing with its removal by CWs reported very different results, ranging from 0 to 96% but typically below 60% (Gorito et al., 2017). For the same configuration used in the present study, Reyes-Contreras et al. (2012) achieved a maximum efficiency of 55% and observed that CWs performances decreased over time, as well as in cold seasons. Studies applying SF-CWs configurations have been demonstrating better results for the removal of diclofenac, probably due to its susceptibility to photolysis when exposed to sunlight irradiation, in comparison with other elimination processes (Matamoros et al., 2012; Rühmland et al., 2015). Although the configuration used in this study does not promote photodegradation, diclofenac was efficiently removed, suggesting its microbial degradation, as already evidenced in other studies (Matamoros et al., 2007a; Radke et al., 2010). Lastly, for the pharmaceuticals propranolol, tramadol, and warfarin, very similar removal rates were observed, always higher than 96%. According to Vystavna et al. (2017), propranolol tends to accumulate in the sediments and plants of CWs due to its hydrophobicity. In that study, removals up to 80% were obtained using a combined vertical and horizontal flow CW system (VSSF/HSSF-CWs), being the removal of this pollutant favoured by an increase of HRT and plants cover. Similar efficiencies were reported in other studies (Anderson et al., 2013; Reyes-Contreras et al., 2012). For tramadol, the literature describes removals up to 80% (VSSF-CWs; HRT = 11 days) (Rühmland et al., 2015) and for warfarin, removals up to 44% (HSSF-CWs; HRT = 6 days) (Vymazal et al., 2017).

Other MPs (chlorfenvinphos, clarithromycin, clofibric acid, diphenhydramine, fluoxetine, and PFOS) also had removal rates lower than 100% in some periods of treatment (between 89% and 99%), mainly in the first week corresponding to the adaptation phase (Fig. 2b). Thus, this group of compounds was more susceptible to the adaptation period, but totally eliminated in the course of treatment. Information about removal of chlorfenvinphos and diphenhydramine by CWs is limited and unclear (Blankenberg et al., 2007; Du et al., 2014). Removal efficiencies of clarithromycin, fluoxetine and PFOS are reported in several studies, presenting dissimilar values depending mainly on the operating conditions (Gorito et al., 2017; Li et al., 2014). Clofibric acid has been classified as recalcitrant in CW systems, phytoremediation and adsorption being the main processes contributing to its removal (Zhang et al., 2014). Most studies on the removal of clofibric acid were performed in HSSF-CWs, and for that reason, more research on the VSSF-CW configuration is needed for a reliable comparison (Zhang et al., 2014).

As verified in the non-spiked experiments, the organic UV filter was also recalcitrant in the spiked ones, being the MP with the lowest CW removal percentages (between 37 and 86%). Nonetheless, EHMC was the MP found at the highest concentration in the studied aquaculture effluents. In fact, EHMC is one of the CECs listed in the Watch List of Commission Decision 2015/495/EU and it has been reported at relatively high concentrations in surface waters (up to 4000 ng L^{-1}) (Sousa et al., 2018), as herein shown, but no studies were found in the literature dealing with the application of CWs for its removal (Gorito et al., 2017).

The obtained results highlight the potential of CWs to remove a significant number of MPs; however, research is still needed to fully understand the processes behind these removals. As discussed above, the CW configuration has a significant impact on the achieved performance. So, additional studies are being carried out in which different configurations, plants, substrate composition and HRT are tested to identify and intensify the processes responsible for the removal of recalcitrant MPs, such as EHMC, as well as other MPs often detected at hundreds or thousands of ng L^{-1} in surface waters (such as alachlor, azithromycin, clarithromycin, clothianidin, diclofenac, erythromycin, thiacloprid, thiamethoxam and simazine) (Sousa et al., 2018).

3.3. DOC and ions monitoring

The DOC contents and concentrations of nutrients are relevant parameters which may largely affect the natural interactions inherent to biological systems. Mean DOC contents (Fig. 3) of the influents were 4.9 ± 0.8 mg L^{-1} and 9.0 ± 0.9 mg L^{-1} for non-spiked and spiked experiments, respectively. The higher values in the spiked experiments, in comparison to the non-spiked ones, are due to the addition of the methanolic solution (containing all the target MPs) to the aquaculture effluents. Moreover, the marked differences between non-spiked influents and effluents treated during the first week, in terms of DOC contents, were probably due to plants and microorganisms activities. After the first week, the DOC content in CW microcosm effluents was similar (ca. 10 mg L^{-1}) for both non-spiked and spiked experiments. Although organic carbon can be used by microbial communities and by plants for cellular respiration and biomass growth, DOC also can be leached into the water flowing through CW systems due to plants and bacteria grow, die and decay (Scholz et al., 2016). The few studies published on DOC variation during CWs treatment indicate very dissimilar results, but the potential of these biological systems as a possible source of DOC has recently received considerable attention (Scholz et al., 2016).

Studies dealing with the removal of nutrients by CWs have been particularly focused on nitrogen and phosphorus. In the present work, twelve ions species, including sodium (Na^+), potassium (K^+), ammonium (NH_4^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), chloride (Cl^-), bromide (Br^-), bromate (BrO_3^-), nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{3-}), and sulphate (SO_4^{2-}), were selected to monitor during all the weekly cycles (Fig. 4).

The nutrients are typically eliminated by CWs through biotic and abiotic processes, specially occurring around the rhizosphere of vegetation. Therefore, they are adsorbed, absorbed, transformed, trapped and subsequently removed from water in CW systems (Stottmeister et al., 2003). Most of the analysed ions, namely Ca^{2+}, Mg^{2+}, PO_4^{3-} and NO_3^- (with inlet concentrations under 4 mg L^{-1}), were considerably removed by the CW microcosms during the 4 cycles of treatment in non-spiked (Fig. 4a) and spiked (Fig. 4b) experiments. In both cases, as expected, the removal trend over the weeks was very similar (≈100%). Although it was not possible to determine the NH_4^+ concentrations in CW microcosm effluents by using ionic chromatography due to matrix effects (high concentration of Na^+), a preliminary analysis by a spectrophotometric method (as in Bôto et al. (2016)) showed high removal rates (>70%) in both non-spiked and spiked experiments (data not shown). For K^+, BrO_3^-, Br^- and NO_2^-, removals were not evaluated because the concentrations of these ions were below the MDL (respectively 0.30, 0.08 and 0.08 mg L^{-1}) or below the MQL (0.25 mg L^{-1}), respectively.

The removal of nutrients is affected by several factors, such as their properties, availability of oxygen, temperature, vegetation type, HRT, substrate composition and nutrient loading rate (Stottmeister et al., 2003). Primary macronutrients nitrogen and phosphorus (which include NH_4^+, NO_2^-, NO_3^- and PO_4^{3-}) are predominantly removed by nitrification-denitrification processes and adsorption and/or precipitation into soil/CW substrates, respectively (Kyambadde et al., 2005; Stottmeister et al., 2003). In contrast, Ca^{2+} and Mg^{2+} (secondary macronutrients) are mainly absorbed by plants (Kozak et al., 2014). Although nutrients elimination rates vary considerably with the operating conditions used in CWs and influent loads, the removal yields

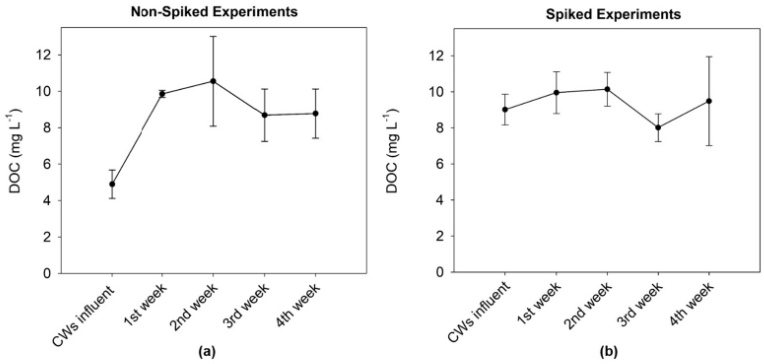

Fig. 3. DOC contents in CW microcosm influents and effluents during the four 1-week cycles of treatment, using non-spiked (**a**) and spiked (b) aquaculture effluents.

obtained in the present study were quite similar to those already reported for bench-scale (Almeida et al. 2017) and full-scale (Vymazal, 2007) CW systems. Therefore, the assembled CW microcosms were working properly, simulating a real CW system along the experimental period.

The concentrations of the anions SO_4^{2-} and Cl^- considerably increased from few mg L^{-1} in the influents to hundreds and thousands of mg L^{-1} in the respective effluents. Little attention has been paid to the removal of these ions in CWs, being the information about these two micronutrients scarce in the literature (Stottmeister et al., 2003). Anaerobic respiration is the main process responsible for SO_4^{2-} removal, and for that reason, this occurs always followed by NO_3^- depletion (García et al., 2010; Whitmire and Hamilton, 2005). Whitmire and Hamilton (2005) suggested that SO_4^{2-} production during the treatment may be due to the NO_3^- removal processes, which stimulate sulphur oxidation or, on other hand, inhibit SO_4^{2-} reduction by denitrification. Regarding Cl^-, the existing studies are inconclusive (Olukanni and Kokumo, 2013; Rozema et al., 2016) and thus, more research is needed on this topic. For Na^+, although quantification by ionic chromatography was not possible due to matrix effects (partial co-elution with NH_4^+), a clear increase in the peak area was observed, indicating a significantly higher concentration of Na^+ in CW microcosm effluents in comparison to the influents. In fact, the root bed substrate could release some of these ions (by a washing effect), as the sediment and sand were collected in a location of the river that can still suffer from salty water intrusion, resulting from the influence of the sea, into which this river drains.

Overall, these results showed that the performance of CW microcosms to remove nutrients was not affected by the presence of a significant amount of MPs in the feeding water. Therefore, besides the removal of MPs, these biological systems can remove conventional pollutants from aquaculture effluents, like nutrients, and contribute for a more environmentally responsible and sustainable aquaculture sector.

4. Conclusions

The investigated VSSF-CW system demonstrated a great potential to remove organic MPs from freshwater aquaculture effluents. In non-spiked experiments, most MPs quantified in aquaculture effluents (atrazine, PFOS, clarithromycin, fluoxetine, and norfluoxetine) were highly removed (≈100%), the UV filter EHMC being the only exception (removals < 55%). Furthermore, the spiked experiments with all the target MPs added simultaneously to the freshwater aquaculture effluents confirmed these good performances (weekly removal rates of MPs

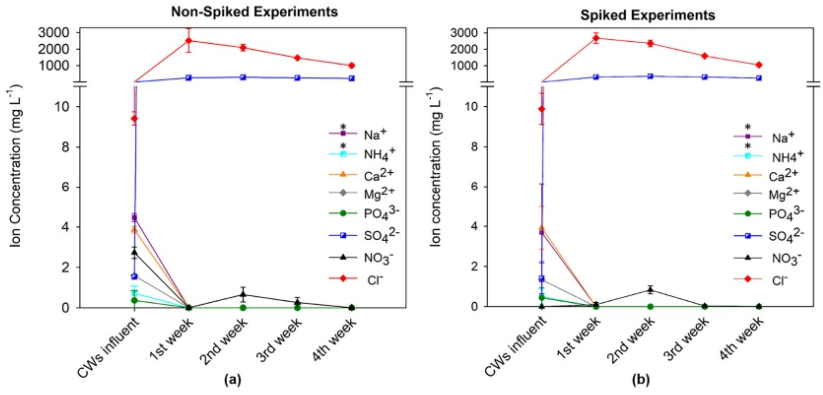

Fig. 4. Concentrations of ions in CW microcosm influents and effluents during the four 1-week cycles of experiments, using non-spiked (a) and spiked (b) aquaculture effluents. In both cases K^+, BrO_3^-, Br^- were always below MDL (respectively 0.30, 0.08 and 0.08 mg L^{-1}) and NO_2^- was always below MQL (0.25 mg L^{-1}). *Cations Na^+ and NH_4^+ were only determined in CW influents, due to matrix effects verified after CW treatment.

higher than 87%, once again with exception of EHMC). Although the DOC content was not decreased by the CW systems, high removals of nutrients were generally obtained, revealing that the large number of MPs in spiked effluents did not affect the performance of these systems. The comparison of the results with those obtained in other reports is difficult, because the tested conditions are variable according to the aim of each specific study. Further research is needed to better understand the processes that are responsible for the removal of MPs in CWs, and to optimize these systems.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.06.371.

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A4.

"Quenchers in advanced oxidation technologies for analysis of micropollutants by liquid chromatography coupled to mass spectrometry: Sodium sulphite or catalase?"

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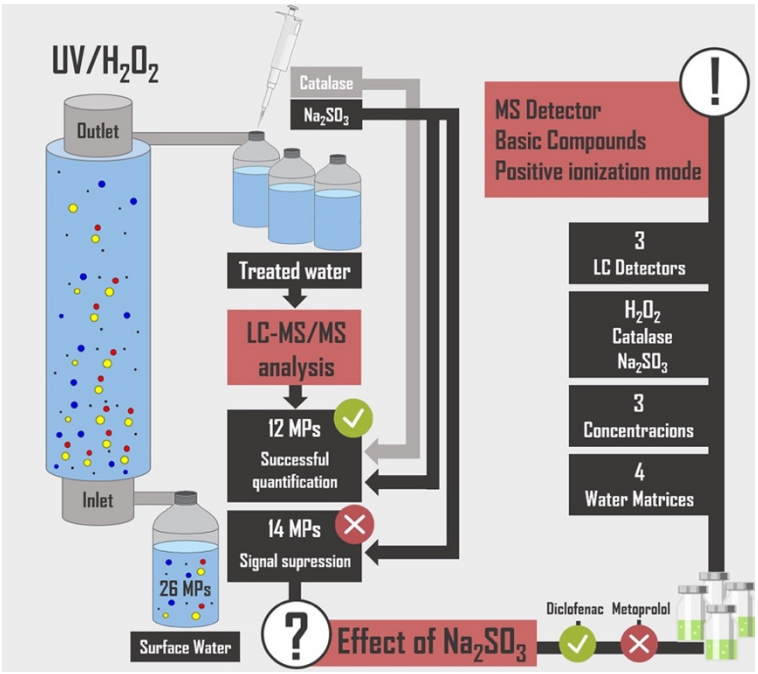

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Quenchers in advanced oxidation technologies for analysis of micropollutants by liquid chromatography coupled to mass spectrometry: Sodium sulphite or catalase?

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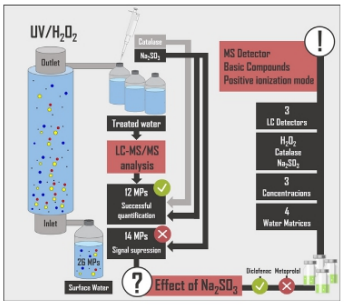
HIGHLIGHTS

- Sodium sulphite is not recommended as quencher in AOTs when using LC-MS/MS.
- Catalase should be used as quencher of H_2O_2, when analysing by LC-MS/MS.
- Matrix matched analytical validated methods are always recommended.
- Analytical method validation must consider all the experimental steps of AOTs.

GRAPHICAL ABSTRACT



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ABSTRACT

This work aimed to investigate the possible effect of 2 quenchers commonly used in H_2O_2-based advanced oxidation technologies (AOTs), i.e. catalase and sodium sulphite (Na_2SO_3), on the analytical signal of 3 detectors coupled to liquid chromatography (LC): tandem mass spectrometry (LC-MS/MS), fluorescence detection (LC-FD) and LC-diode array detection (LC-DAD). The observation of analytical interferences for a group of compounds when studying the removal by continuous mode UV/H_2O_2 of 26 micropollutants (MPs) from a spiked surface water (SW), for which the residual H_2O_2 in the samples was quenched by Na_2SO_3, triggered the need of understanding these effects and thus catalase was used as comparative quencher. From the 26 MPs having a wide range of polarity and pK_a, those monitored after electrospray ionization (ESI) under positive ionization (PI) mode and presenting a pK_a higher than 5.9 revealed a great signal suppression, but only when using Na_2SO_3 as H_2O_2 quencher. In this sense, we further explored this effect by selecting 2 MPs, metoprolol and diclofenac, which had respectively signal suppression and no interference in the LC-MS/MS response. These MPs were analysed before and after addition of H_2O_2 and catalase or Na_2SO_3 in reaction vials, using: (i) different detectors coupled to

Ozonation
Advanced oxidation processes

LC, namely LC-MS/MS with ESI under PI and negative ionization (NI) modes, LC-FD and LC-DAD; (ii) different environmental matrices (SW, drinking water, wastewater) and ultrapure water; and (iii) different magnitude levels (0.1–10 mg L^{-1}). The results demonstrated a remarkable signal suppression in LC-MS/MS analyses under PI mode for those compounds with pK_a higher than 5.9, confirming the interfering effect of H_2O_2/Na_2SO_3. To the best of our knowledge, the analytical interference in the LC-MS/MS analysis, after adding Na_2SO_3 to quench H_2O_2 in AOTs experiments was never reported before and the results presented herein support the recommendation to use catalase instead of Na_2SO_3 as quencher in AOTs studies.

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1. Introduction

The environmental occurrence and fate of organic micropollutants (MPs) in aquatic compartments have been extensively reported in the last few decades (Luo et al., 2014). MPs include a wide range of substances, such as pesticides, pharmaceuticals, synthetic and natural hormones, among others. These substances are continuously introduced into the waterbodies (typically at residual levels between ng L^{-1} and μg L^{-1}) and many of them are considered pseudopersistent since they are not eliminated in conventional water/wastewater treatment plants, contributing to the deterioration of water quality, threatening ecosystems, wildlife and human health. Advanced oxidation technologies (AOTs), such as UV/H_2O_2, the Fenton process, ozonation and heterogeneous photocatalysis, are some alternatives under study to eliminate this type of MPs from contaminated water/wastewaters (Ribeiro et al., 2015; Barbosa et al., 2016a).

Attending to the past analytical limitations and costs to quantify organic pollutants at very low concentrations in environmental matrices, most studies dealing with AOTs have been performed: (i) with ultrapure water (UPW), distilled water or synthetic solutions mimicking environmental waters; and (ii) spiking one or a few model pollutants at mg L^{-1} levels (concentrations well above those occurring in the environment). In this type of studies, liquid chromatography (LC) coupled to the simple and widely available UV or diode array detectors (DAD) has been employed to quantify these pollutants. However, the great advances on analytical methodologies allowed applying more realistic approaches, by extending the research to multiple MPs in environmental matrices at the concentration levels that they are actually occurring (Outsiou et al., 2017).

Nowadays, LC coupled to tandem mass spectrometry (LC-MS/MS) by atmospheric pressure ionization is a key analytical tool for quantification of MPs (Ribeiro et al., 2015; Sosa-Ferrera et al., 2012; Barbosa et al., 2018; García-Galán et al., 2016). This technique combines the advantages of LC as a versatile separation methodology for polar, nonvolatile or thermally unstable compounds, and MS as an extremely sensitive detection and identification technique (Rosen, 2007; Comerton et al., 2009). MS is based on the formation of ions from the analytes, which are then fragmented and analysed according to their mass to charge ratio (m/z). Electrospray ionization (ESI) of target analytes by a strong electric field applied in the liquid sample has been often used in environmental samples analysis (Pitt, 2009). With this, charged droplets are originated, from which the ions at the surface are ejected to the gaseous phase and fragmented for quantification (Ho et al., 2003). During this complex process, factors such as surface activity (affinity for electrospray droplet surfaces) and solvation energy can affect the response of analytes to ionization (Henriksen et al., 2005), often resulting in the suppression or less frequently in the enhancement of their signals (Comerton et al., 2009; Uclés et al., 2017). In this regard, the main drawback of the LC-ESI-MS technique is the matrix effect in the ionization, which must be considered to avoid erroneous interpretation of results (Comerton et al., 2009; Ćirić et al., 2012). This effect results from the competition between the analytes and the co-eluting interfering species (Gosetti et al., 2010), and thus the following aspects are able to cause them: (i) the matrix itself (e.g., shown by comparison of UPW and wastewater (WW)); (ii) the sample pre-treatment procedure; (iii) the reactants needed for the AOT under study (e.g., oxidants, catalysts); and/or (iv) the reactants used to quench the radicals in order to stop the oxidative reaction (e.g., H_2O_2 or O_3 quenchers, such as Na_2SO_3 and catalase). The traditional methods for quenching residual H_2O_2 after oxidative reactions are based on the addition of reactants as free chlorine, catalase, Na_2SO_3 or $Na_2S_2O_3$, depending on the post-AOT water analysis (Keen et al., 2013; Liu et al., 2003). Given the complexity of reaction between H_2O_2 and $S_2O_3^{2-}$ (slow reaction, proceeding through multiple intermediate steps and exhibiting oscillatory properties under certain conditions) (Keen et al., 2013), several authors usually select Na_2SO_3 instead of $Na_2S_2O_3$ (Lima et al., 2016; Noreen et al., 2012; Wang et al., 2013; Ribeiro et al., 2013). Nonetheless, the application of these two inorganic scavengers has been recently discouraged for quenching residual H_2O_2 after AOTs due to several possible undesirable effects, namely the detrimental high concentrations of salts for mass spectrometry analysis (Keen et al., 2013).

In this work, the analytical interference of the oxidant H_2O_2 and respective quenchers catalase and Na_2SO_3 was studied, using different detectors coupled to LC. Initially, a surface water (SW) spiked with a group of 26 MPs, each at a concentration of 100 ng L^{-1}, was treated by continuous mode UV/H_2O_2 process, but the detection signals were completely suppressed for more than half compounds and respective internal standards in both control (H_2O_2 without UV, where removal is not expected) and UV/H_2O_2 experiments, being their removal rates inconclusive. We hypothesized that a possible interference of Na_2SO_3 used as quencher could take place in the LC-MS/MS, an idea that was reinforced by the absence of such effect when using catalase instead of Na_2SO_3. In this sense, a second approach consisting in batch experiments using reaction vials was performed to assess the effect of adding oxidant (H_2O_2) and combined reactants (H_2O_2/Na_2SO_3, H_2O_2/catalase) on the LC-MS/MS, LC-DAD and LC-FD (Fluorescence Detection) signals of 2 compounds with distinct characteristics and responses when using $NaSO_3$ in both control and UV/H_2O_2 experiments. These 2 compounds were selected out of the target 26 MPs: metoprolol (positive interference) and diclofenac (without interference). The effect of each component (H_2O_2, H_2O_2/Na_2SO_3, H_2O_2/catalase) was also investigated considering different water matrices (SW, drinking water (DW) and WW, vs UPW) and different concentration levels: 0.1 mg L^{-1} (LC-MS/MS and LC-FD); 1.0 mg L^{-1} (LC-MS/MS, LC-DAD and LC-FD); and 10 mg L^{-1} (LC-MS/MS, LC-DAD and LC-FD).

2. Materials and methods

2.1. Chemical and materials

All reference standards of the MPs under study (acetamiprid, atrazine, azithromycin, bezafibrate, carbamazepine, citalopram, clarithromycin, clindamycin, clofibric acid, diclofenac, diphenhydramine, fluoxetine, isoproturon, ketoprofen, metoprolol, norfluoxetine, ofloxacin, perfluorooctanesulfonic acid (PFOS), propranolol, simazine, thiacloprid, thiamethoxam, tramadol, trimethoprim, venlafaxine, and warfarin; >98% purity), were purchased from Sigma-Aldrich (Steinhein, Germany). The physicochemical properties of the analytes can be found in Table S1 (Supplementary Material). The respective deuterated compounds used as internal standards (atrazine-d5, acetamiprid-d3,

diclofenac-d4, azithromycin-d3, ofloxacin-d3, fluoxetine-d5, ketoprofen-d3; >98% purity), catalase from bovine liver powder (2000–5000 units/mg protein) and hydrogen peroxide (30%, w/w) were obtained from Sigma-Aldrich (Steinhein, Germany). Sodium sulphite (98.5% purity) was acquired from Acros Organics (New Jersey, USA). Stock solutions of ca. 1000 mg L^{-1} were prepared for each reference and internal standard and 2 working solutions were prepared by their dilution in ethanol, one containing all target MPs at 2.5 mg L^{-1} and another containing all internal standards at 10 mg L^{-1}. Stock solutions of ca. 10 mg L^{-1} were also prepared by dissolution of known amounts of diclofenac and metoprolol in each matrix under study (i.e., UPW, SW, DW and WW).

Formic acid was purchased from Merck (Darmstadt, Germany). Methanol and acetonitrile (MS grade) were acquired from VWR International (Fontenay-sous-Bois, France), whereas ethanol HPLC grade was obtained from Fisher Scientific UK Limited (Leicestershire, UK). UPW (resistivity > 18 mΩ cm) was supplied by a Milli-Q water system. Oasis® HLB (Hydrophilic-Lipophilic-Balanced) cartridges (150 mg, 6 mL) were acquired from Waters (Milford, Massachusetts, USA).

2.2. Surface, drinking and wastewater samples

All water samples were collected in April 2017 using pre-rinsed amber glass bottles, which were then transported at 4 °C to the laboratory and kept refrigerated until use. WW was collected from a secondary clarifier of a municipal wastewater treatment plant located in Northern Portugal, SW samples were collected from Crestuma-Lever reservoir, and DW was collected from the water supply network of Porto. WW and SW samples were previously filtered through 1.2 μm glass microfiber filters GF/C, 47 mm (Whatman™, United Kingdom) and DW (chlorine content: 47.5 mg L^{-1}) was used without any pretreatment.

2.3. UV/H_2O_2 experiments (A): set-up and procedure

Continuous UV/H_2O_2 experiments to treat SW (Fig. 1A) and the respective controls (H_2O_2 without UV) were conducted in a column (70 cm height × 3 cm i.d.) packed with glass rings, continuously fed with SW sample (15 mL min^{-1}) and pure oxygen, and connected to a loop column to simulate a perfectly mixed reactor, as described

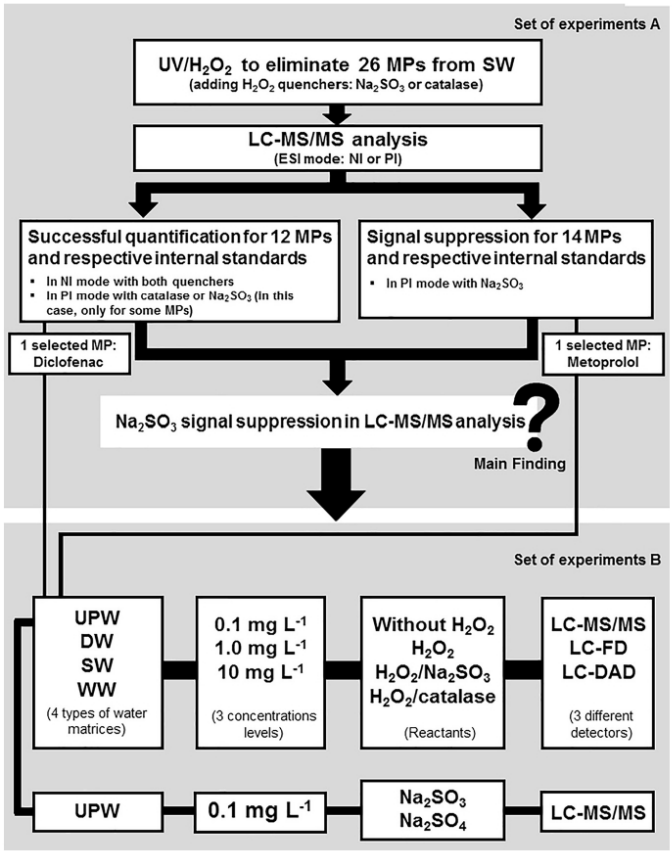

Fig. 1. Scheme of the experimental approach, including continuous UV/H_2O_2 experiments (A) and batch experiments (B) to evaluate the matrix effects and interference of reactants used in (A). Electrospray ionization (ESI); negative ionization (NI); positive ionization (PI).

elsewhere (Moreira et al., 2016). The main column was equipped with eight 10 W UV high intensity light emitting diodes (LEDs, λ_{max} = 382 nm).

The experiments were conducted in duplicate, with 6 L of SW spiked with 100 ng L^{-1} of each target analyte. The selection of this concentration was based on our recent review reporting that many of the target MPs have been found worldwide in SW at ng L^{-1}–μg L^{-1} levels (Sousa et al., 2018). Each experiment was conducted during 180 min after reaching the steady state (t_0), which corresponds to approximately 2 × retention time (90 min) of the spiked SW passing through the reactor in order to represent the sample accurately, i.e. when no more dilution effect occurs and the outlet solution has reached a steady concentration. Sample triplicates were collected. At t_0, H_2O_2 was added according to the stoichiometric amount with respect to the content of dissolved organic carbon (DOC) in SW and the LEDs were switched on in the case of UV/H_2O_2 experiments. A stoichiometric excess of Na_2SO_3 with respect to H_2O_2 (5% excess in relation to the stoichiometric amount) or 5 mL of fresh catalase solution (0.1 g L^{-1}) based on a previous work (Moreira et al., 2018), were added to each triplicate sample, to quench the residual H_2O_2.

2.4. Study of the analytical interference of reactants (B)

With the aim of understanding the interference related to the addition of Na_2SO_3 in the analysis of more than half of the MPs by LC-MS/MS in the set of experiments (A) (Fig. 1A.), which could result from either its residual presence or the product of its oxidation (Na_2SO_4, Reaction 1), experiments were performed using UPW spiked with the 2 MPs (diclofenac and metoprolol) at 0.1 mg L^{-1}, by adding only Na_2SO_3 or Na_2SO_4 solutions to the samples, without any sample preparation (pre-filtration or pre-concentration).

$$Na_2SO_3 + H_2O_2 \rightarrow Na_2SO_4 + H_2O \quad \text{(Reaction 1)}$$

The possible interference of H_2O_2, H_2O_2/Na_2SO_3 and H_2O_2/catalase was further investigated, evaluating the signal obtained in 4 matrices (SW, UPW, DW, and WW) spiked with 2 MPs at 3 concentration levels (0.1, 1.0, 10 mg L^{-1}) by LC-MS/MS, LC-FD and LC-DAD (Fig. 1B.), without any sample pre-concentration. Metoprolol and diclofenac were the MPs selected for this set of batch experiments due to their different behaviour in the LC-MS/MS analysis, after quenching the samples of (A) with Na_2SO_3. All experiments were performed in 1.5 mL reaction vials, under dark conditions in triplicate.

Solutions containing the 2 MPs (diclofenac and metoprolol) at 10 mg L^{-1} in UPW, DW, SW and WW (Section 2.1) were diluted in the respective matrix to obtain concentrations of 0.1 and 1.0 mg L^{-1} in the 4 matrices. H_2O_2, H_2O_2/Na_2SO_3 or H_2O_2/catalase were added to 1.5 mL vials (batch mode) containing 1 mL aliquots of each matrix spiked at the 3 concentration levels. A stoichiometric excess of H_2O_2 (5% excess in relation to the stoichiometric amount) was added to each sample, which was determined based on the DOC content of UPW, DW, SW and WW (0.5 ± 0.1, 1.6 ± 0.1, 2.6 ± 0.5 and 16.8 ± 0.9 mg L^{-1}, respectively). Posteriorly, a volume of 100 μL of a Na_2SO_3 solution of 3.3 mg L^{-1} or of a 0.1 g L^{-1} catalase solution (Moreira et al., 2018), was added to evaluate the effect of the reactants H_2O_2/Na_2SO_3 and H_2O_2/catalase, respectively. The effect of the oxidant itself (H_2O_2) was also assessed, by adding to aliquots containing H_2O_2, 100 μL of the respective matrix to obtain the same dilution factor. Blanks were prepared by adding the same volume of each matrix, instead of H_2O_2, H_2O_2/Na_2SO_3 or H_2O_2/catalase. All samples were analysed by LC-MS/MS, LC-FD and LC-DAD, as triplicate measurements. The effects on the signal, namely the matrix effects (DW, SW and WW) and the interference caused by the addition of the oxidant (H_2O_2) and the reactants (H_2O_2/Na_2SO_3 and H_2O_2/catalase) were determined using the Eqs. (1)–(3).

$$\text{Matrix effect [DW, SW and WW]}\ (\%) = \frac{A_{(\text{Spiked Matrix}-\text{Blank Matrix})}}{A_{(\text{Spiked UPW}-\text{Blank UPW})}} \times 100 \quad (1)$$

$$\text{Oxidant effect } [H_2O_2]\ (\%) = \frac{\left(A_{(\text{Spiked Matrix}-\text{Blank Matrix})} - A_{(\text{Spiked Matrix with Oxidant}-\text{Blank Matrix})}\right)}{A_{(\text{Spiked Matrix}-\text{Blank Matrix})}} \times 100 \quad (2)$$

$$\text{Reactant effect } [H_2O_2/Na_2SO_3, H_2O_2/\text{catalase}]\ (\%) = \frac{\left(A_{(\text{Spiked Matrix}-\text{Blank Matrix})} - A_{(\text{Spiked Matrix with Reactant}-\text{Blank Matrix})}\right)}{A_{(\text{Spiked Matrix}-\text{Blank Matrix})}} \times 100 \quad (3)$$

where $A_{\text{Spiked UPW}}$ and $A_{\text{Blank UPW}}$ are the peak areas of the spiked and non-spiked UPW samples, respectively; $A_{\text{Spiked Matrix}}$ and $A_{\text{Blank Matrix}}$ are the peak areas of the spiked and non-spiked matrices, respectively, without addition of H_2O_2; $A_{\text{Spiked Matrix with Oxidant}}$ is the peak area of spiked matrices after addition of H_2O_2; and $A_{\text{Spiked Matrix with Reactant}}$ is the peak area of spiked matrices, after adding H_2O_2/Na_2SO_3 or H_2O_2/catalase. These measurements were made for each matrix and MP concentration. Values of 100% indicate absence of interference, while values higher or lower than 100% indicate a signal enhancement or suppression, respectively.

2.5. Analytical methodologies

The concentration of the target MPs in the first set of experiments (A) was determined using a validated analytical method described elsewhere (Barbosa et al., 2018), based on offline solid phase extraction and ultra-high-performance liquid chromatography-tandem mass spectrometry (SPE-UHPLC-MS/MS). Briefly, SW samples (500 mL) previously filtered (Section 2.2) were acidified to pH 3 with sulphuric acid, followed by pre-cleaning and extraction with SPE using Oasis® HLB cartridges and ethanol as eluting solvent. Chromatographic analysis was carried out in a UHPLC Shimadzu Corporation equipment connected to a triple quadrupole mass spectrometer detector (Ultra-Fast Mass Spectrometry series LCMS-8040). A Kinetex™ XB-C18 100 Å column (100 × 2.1 mm i.d.; 1.7 μm particle diameter) supplied by Phenomenex, Inc. (California, USA) was used for chromatographic analysis. A gradient of methanol and water acidified with 0.1% formic acid was used as mobile phase at 0.20 mL min^{-1}. Capillary voltage, drying gas flow, nebulizing gas flow, desolvation and source temperature of the mass spectrometer were respectively 4.5 kV, 10 dm^3 min^{-1}, 3 dm^3 min^{-1}, 250 °C and 400 °C. Retention time, method detection limits (MDLs), method quantification limits (MQLs) and recoveries of the target analytes are presented in Table S2 (Supplementary Material).

Regarding the second set of experiments (B), metoprolol and diclofenac were determined in the samples without any pre-concentration step (only filtration of WW and SW, as described in Section 2.2), by UHPLC-MS/MS and also with a LC System coupled to DAD (Shimadzu SPD-M20A) and FD (Shimadzu RF-20Axs). For these analyses, the mobile phase consisted on a mixture of 0.1% aqueous formic/acetonitrile (50:50, v/v) at isocratic mode and at a flow rate of 0.20 mL min^{-1}. Excitation and emission wavelengths (λ_{Exc}; λ_{Em}) of the FD were set as follows: λ_{Exc} 230 nm/λ_{Em} 290 nm from 0 to 4.5 min; λ_{Exc} 285 nm/λ_{Em} 360 nm from 4.5 to 6.5 min; and λ_{Exc} 230 nm/λ_{Em} 290 nm from 4.5 to 6.5 min.

A pH meter pHenomenal® pH 1100 L (VWR, Germany) was required for the pH adjustments. DOC was measured using a Shimadzu TOC-5000A analyser (Shimadzu Scientific Instruments, Japan), following the standard procedure 5310 B of the Standard Methods for Examination of Water and Wastewater (APHA et al., 1998).

3. Results and discussion

3.1. UV/H_2O_2 experiments (A): removal of organic MPs and interference of reactants

SW samples spiked with a set of 26 MPs at 100 ng L^{-1} were treated by UV/H_2O_2, being MPs concentrations determined afterwards. The final concentrations and removals rates (not shown) were inconclusive for 14 compounds (azithromycin, carbamazepine, citalopram, clarithromycin, clindamycin, diphenhydramine, fluoxetine, metoprolol, norfluoxetine, ofloxacin, propranolol, tramadol, trimethoprim and venlafaxine), since the analytes and their surrogates used as internal standards had a complete or large signal suppression in the LC-MS/MS analysis, in both control (H_2O_2 without UV) and treated samples (UV/H_2O_2). Control samples allowed confirming that the absence of signal for the target MPs was not related to their elimination during UV/H_2O_2, which could have resulted in concentrations below the MDLs. Indeed, in the control samples, the decrease on the initially spiked concentrations (Fig. 2) was not expected due to the inability of H_2O_2 to degrade MPs in the absence of an UV irradiation source or other oxidants as O_3. Moreover, this suppression effect was not observed in the initial spiked samples (i.e., without any treatment) in which all target MPs could be analysed, giving an insight that some interference could have resulted from the treatment process, namely the addition of H_2O_2 and Na_2SO_3 in both control and treated samples. Interestingly, this analytical interference was only observed for part of the MPs, corresponding to those eluted first in the chromatographic column and ionized in ESI under positive ionization (PI) mode. Although the exogenous suppressor resulting from addition of H_2O_2/Na_2SO_3 has been verified for a considerable number (14) of MPs (Fig. 2), other 12 compounds including diclofenac did not show any analytical interference. In this sense, and taking into account the behaviour of the 26 target MPs included in this set of experiments (A), the specific properties of these compounds may be related to the described interference.

Many studies have investigated the phenomenon of matrix effect. However, its cause is still not fully understood (Zhou and Kang, 2013). Several factors contributing to analyte signal suppression or enhancement have been pointed out, including: (i) the competition between analytes and interfering compounds for the available charges and access to the droplet surface; (ii) the formation of solid particles due to the use of non-volatile additives or, in the case of less-volatile target compounds, the difficulty to convert the charged droplets in gas-phase ions; and (iii) the formation of analyte ions or neutral complexes when additives or matrix components act like ion pairing reagents; (iv) among others (Cappiello et al., 2010; Shaw et al., 2014).

According to (Shaw et al. (2014)), compounds with high mass, polarity and basicity are more likely to be impacted by matrix effect. The distribution of the 26 target MPs according to their pK_a and log K_{ow} (Fig. 3) shows that pK_a is the dominating factor, describing the respective response of these compounds in the presence of Na_2SO_3 as H_2O_2

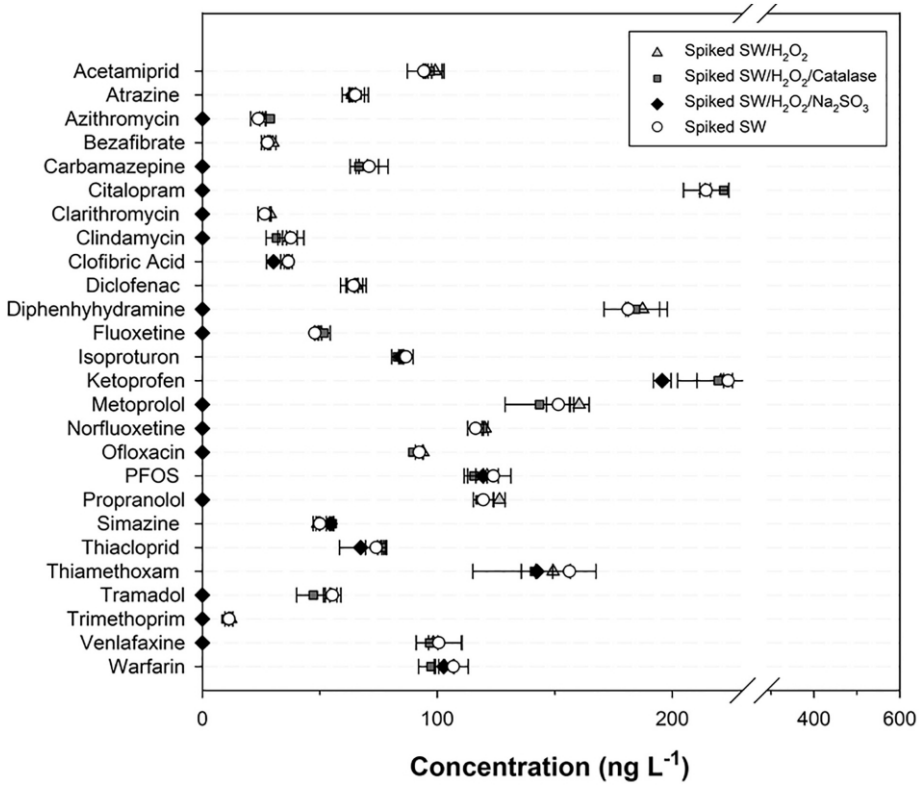

Fig. 2. Concentrations of MPs (ng L^{-1}) in the initial sample (Spiked SW) and in the control samples (Spiked SW/H_2O_2), before and after addition of H_2O_2-quenchers: Na_2SO_3 (Spiked SW/H_2O_2/Na_2SO_3) and catalase (Spiked SW/H_2O_2/Catalase).

quencher. In fact, pK_a was already described as one of the most influential parameters in predicting the ionization in ESI source via monoprotonation, under PI mode (Oss et al., 2010). MPs with low pK_a (<5.9) showed no interference of this reactant (#1–12 in Fig. 3; Table S1), whereas MPs with higher pK_a (>5.9) were affected by it (#13–26 in Fig. 3; Table S1). It is interesting to mention that acid analytes (i.e., with low pK_a) have a natural tendency to originate deprotonated forms, being expected to be ionized in the negative ionization (NI) mode. In turn, basic analytes (i.e., with high pK_a) tend to create cations and their ionization is likely to occur in the PI mode (Henriksen et al., 2005). It was noticeable that the MPs analysed under PI mode and eluted first, which are basic, had a detection interference in the samples containing H_2O_2/Na_2SO_3 (Fig. 3), suggesting that the alkaline pH of the samples containing Na_2SO_3 might reduce their ionization or a matrix effect of co-eluting substances could be the origin of such interferences. Some exceptions were observed for several neutral and acidic MPs analysed under PI mode, namely for pesticides acetamiprid, atrazine, isoproturon, thiacloprid, thiamethoxam, simazine and the anticoagulant warfarin, which did not have any analytical interference. In contrast, all acidic MPs monitored under NI mode (clofibric acid, PFOS, bezafibrate, diclofenac and ketoprofen) had a good response on the MS detector in the presence of H_2O_2/Na_2SO_3.

Therefore, we hypothesized that: (i) the remaining Na_2SO_3 in solution used as reaction quencher (in excess) or the product of the reaction between H_2O_2 and Na_2SO_3, (Na_2SO_4) could interfere with the PI taking place in the ESI interface of the LC-MS/MS; (ii) raising the sample pH after addition of H_2O_2 and Na_2SO_3 might reduce the ionization of basic molecules; (iii) some adduct could be produced, resulting from the presence of the reactants in the sample solution; or (iv) less likely the reaction between H_2O_2 and Na_2SO_3 in solution might originate some reactive species, which slightly altered the structures of MPs.

In this sense, control experiments (H_2O_2 without UV, where degradation of MPs was not expected) were repeated to understand the role of Na_2SO_3, by testing the addition of Na_2SO_3 or catalase quenchers and the absence of any quencher (Fig. 2). The obtained data showed a clear interference in the quantification of some MPs when using Na_2SO_3, specifically in the case of the compounds abovementioned, all eluted at the very beginning of the chromatographic run and ionized under PI mode. In turn, the catalase also used as quencher did not cause any interference in the analysis of MPs by LC-MS/MS, with similar initial and final concentrations of MPs being registered (Fig. 2). Although the treatment efficiency was not the focus of this work, it should be mentioned that UV/H_2O_2 treatment using catalase as quencher had removal rates below 21% for all compounds (not shown). Considering that MPs were spiked at 100 ng L^{-1} and that the validation of the method was performed with the same type of water collected at different times, the inaccuracy found for the initial concentration of some MPs (Fig. 2) was ascribed to a slight different matrix effect between samples collected in different days.

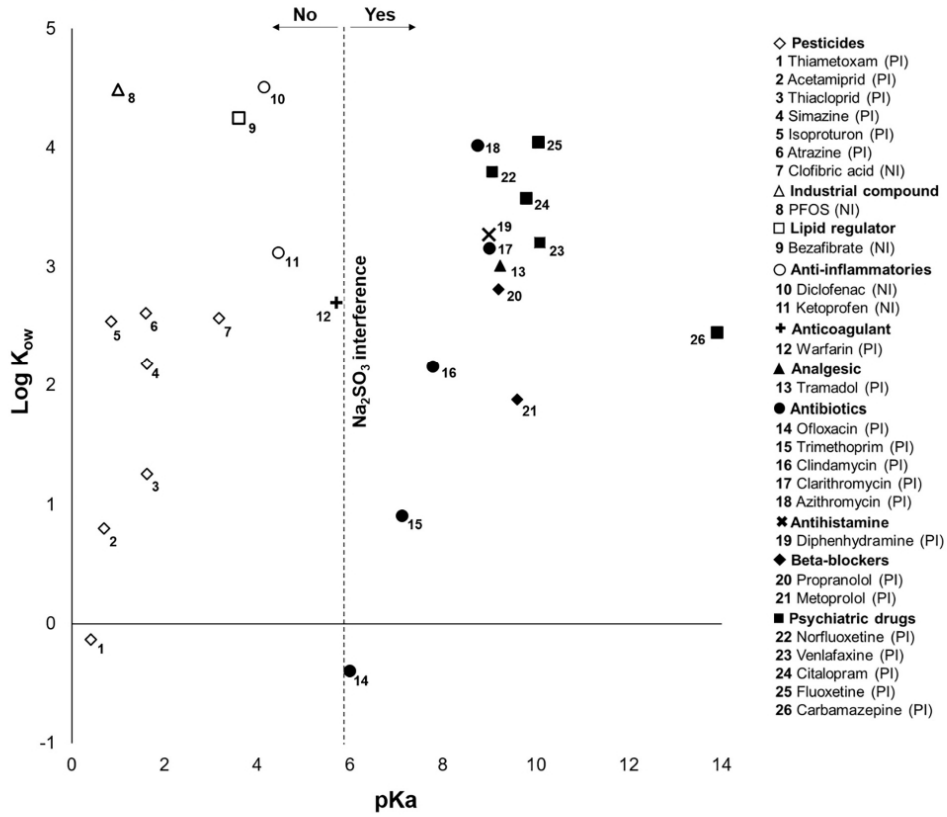

Fig. 3. Representation of target MPs according to their pK_a and log K_{ow}, grouped by absence (No) or presence (Yes) of interference by adding H_2O_2/Na_2SO_3 to the SW sample spiked at 100 ng L^{-1}. PI and NI are positive and negative ionization modes, respectively.

These results reinforce that the matrix-matched validated analytical methods using internal calibration methods to calculate removal efficiencies is crucial to avoid misinterpretations of the results, particularly bias conclusions about treatment efficiency of AOTs. Moreover, these results suggest that Na_2SO_3 is not a good option as quencher in AOT experiments when LC-MS/MS is employed, maybe due to a suppression effect taking place in the ESI interface of LC-MS/MS (matrix effect or pH effect) or due to the formation of adducts or by-products. To test these hypotheses, reinforced by the absence of such effect when using catalase instead of Na_2SO_3, the effects of each single component (H_2O_2; H_2O_2/Na_2SO_3 and H_2O_2/catalase) on the LC-MS/MS signals of 2 MPs selected from the set of the 26 target MPs, one analysed after ESI under PI mode and with verified analytical interference (metoprolol) and another analysed after ESI under NI mode and without interference (diclofenac), were studied (Section 3.2), as shown in Fig. 1B. The analytical interference on other detection modes (LC-DAD and LC-FD) was also evaluated.

3.2. Matrix effects and interference of reactants (B) in chromatographic analysis

As referred above, metoprolol (ESI+) and diclofenac (ESI−) were selected as model compounds due to their different behaviour, when using Na_2SO_3 as quencher of H_2O_2, leading to signal suppression only in the case of metoprolol (Fig. S1, Supplementary Material). In this set of experiments (B), the sample pre-treatment was restricted to the pre-filtration of WW and SW and SPE was not used as sample pre-treatment in order to confirm that the analytical interference resulted from some effect related to the addition of H_2O_2/Na_2SO_3. To understand whether this effect was due to Na_2SO_3 or to the product (Na_2SO_4) that could be formed by reacting with H_2O_2 (Reaction 1), UPW spiked with the MPs at 0.1 mg L^{-1}, and either Na_2SO_3 or Na_2SO_4, were analysed by LC-MS/MS (Fig. 1B). The results showed that neither the compounds alone (remaining Na_2SO_3 in solution nor the product Na_2SO_4), nor the sample pH (≈9) due to the addition of Na_2SO_3 were responsible for this effect, suggesting that other product of the reaction between H_2O_2 and Na_2SO_3 might be causing an analytical interference, but more research is needed to elucidate which species can be formed and lead to this effect. The matrix effect (SW, DW and WW, vs UPW), the impact of adding the oxidant (H_2O_2) and the quenchers (H_2O_2/Na_2SO_3, H_2O_2/catalase) were deeply investigated using 3 detection modes (MS, DAD and FD) and 3 initial concentrations of MPs (0.1 mg L^{-1}, 1.0 mg L^{-1} and 10 mg L^{-1} for LC-MS/MS, LC-DAD and LC-FD). This strategy aimed at representing 3 levels of concentrations in the different water matrices and understanding the effects on the detection modes, including MS/MS, and on the 2 conventional detectors DAD and FD.

In general, a similar trend was found for matrix effects occurring in the 3 detectors for diclofenac (Fig. 4), which was a slight signal enhancement in both spiked DW at the 2 highest concentrations tested, as well as at all concentrations in SW. This fact can be attributed to matrix

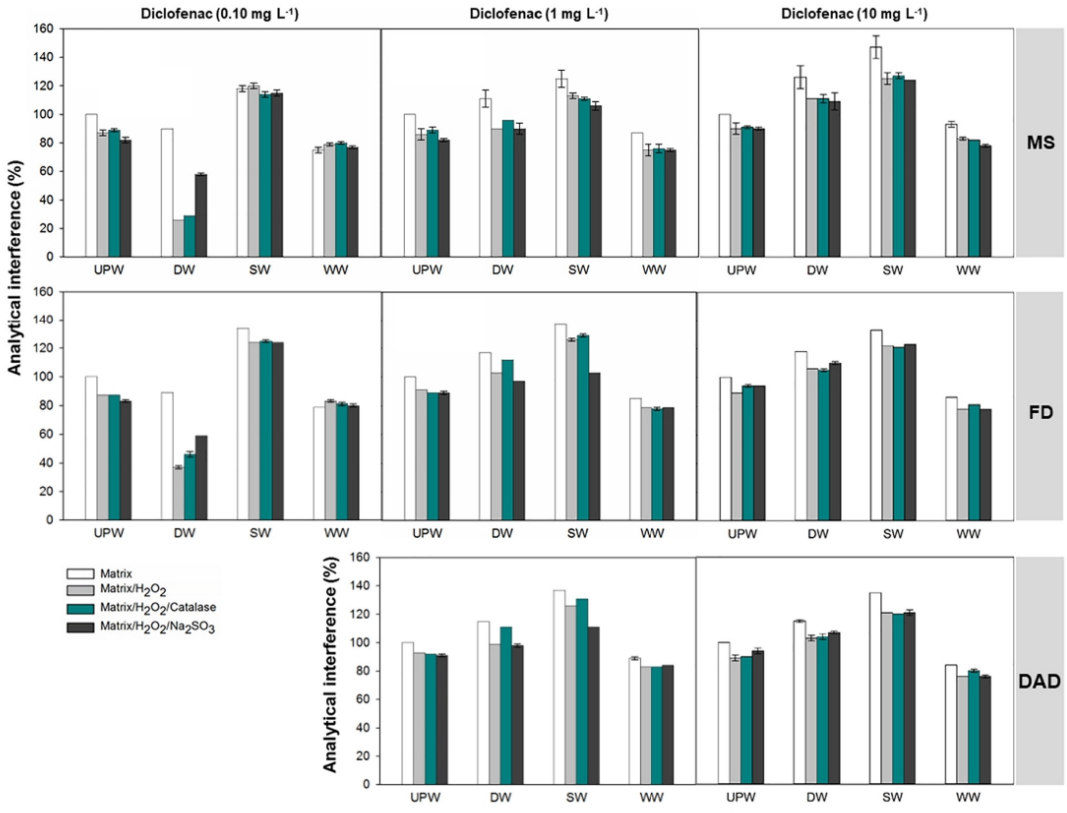

Fig. 4. Analytical interference (%) resulting from the matrix, the oxidant (H_2O_2) and the quenchers (H_2O_2/Na_2SO_3, H_2O_2/catalase) in MS, FD and DAD detectors, for diclofenac spiked at 0.1 mg L^{-1} (left), 1.0 mg L^{-1} (centre) and 10 mg L^{-1} (right) in UPW, DW, SW and WW samples.

effects in the ESI source of co-eluting species present in the matrix in the case of LC-MS/MS and to species absorbing in the same wavelengths selected to analyse diclofenac, in the case of LC-DAD and LC-FD. In turn, WW led to a small suppression of the signal for all the concentrations and detectors. Regarding the effects of the oxidant (H_2O_2) and the quenchers (H_2O_2/Na_2SO_3, H_2O_2/catalase), a very slight loss of signal was observed for all detectors that can be ascribed to the addition of H_2O_2 and not to these quenchers, since the effect was similar in the presence and absence of quenchers. Additionally, the DW samples had a large signal suppression when H_2O_2 was added, more notorious at the lowest concentration of diclofenac, which can be justified by the chlorine present in the matrix that might react with H_2O_2. This effect was mitigated when Na_2SO_3 was added, since this reactant is known as a dechlorinating agent able to reduce the residual chlorine used as a disinfectant in the DW supply (Barbosa et al., 2016b; Wang et al., 2011) and consequently prevent the suppression. In fact, the average chlorine content of DW samples (spiked and non-spiked) was 47.5 mg L^{-1}, whereas it decreased to values below the quantification limit (<0.18 mg L^{-1}) in those samples to which Na_2SO_3 was added. Thus, these results confirm that diclofenac is not impacted by the addition of Na_2SO_3 to the treated samples containing H_2O_2.

In the case of metoprolol (Fig. 5), the matrix effect in the LC-MS/MS occurred mostly for DW, with 32% at 0.1 mg L^{-1}, 63% at 1.0 mg L^{-1} and 93% at 10 mg L^{-1}, suggesting that this effect in DW was concentration-dependent. The results would be different if any quencher of chlorine was used as pre-treatment of DW, before the experiments. Moreover, this suppression of the signal was also verified in spiked DW analysed by using DAD (60% at 1 mg $L^{-1}$and 91% at 10 mg L^{-1}) and FD (62% at 0.1 mg L^{-1}, 84% at 1 mg $L^{-1}$and 93% at 10 mg L^{-1}), maybe due to the chlorine occurring in tap water, showing the importance of matrix-matched validation of analytical methods. In the case of WW, the analytical interferences in the LC-MS/MS were negligible. In turn, it was observed a slight enhancement of the signal for metoprolol in the case of SW (106–114% for DAD and 100–111% for FD) and WW (118–124% for DAD and 121–131% for FD), in the same detectors (DAD and FD), maybe due to some species absorbing in the same wavelengths selected to analyse metoprolol, namely natural organic matter.

For all matrices and concentrations, H_2O_2/Na_2SO_3 led to a decrease on the signal of metoprolol in the MS detector, which was more evident when the spiked metoprolol concentration was lower. This signal decrease may be ascribed to some species resulting from the addition of H_2O_2/Na_2SO_3, leading to a suppression of ionization in the ESI source or adducts formation, which was confirmed by the absence of signal reduction in both DAD and FD detectors. This fact showed that the formation of by-products was not related to the analytical interference, otherwise it would be expected in all detectors. The interference of Na_2SO_3 and H_2O_2/Na_2SO_3 on the signal of paraquat in LC-DAD was reported before (Santos et al., 2015) as dependent on the concentration

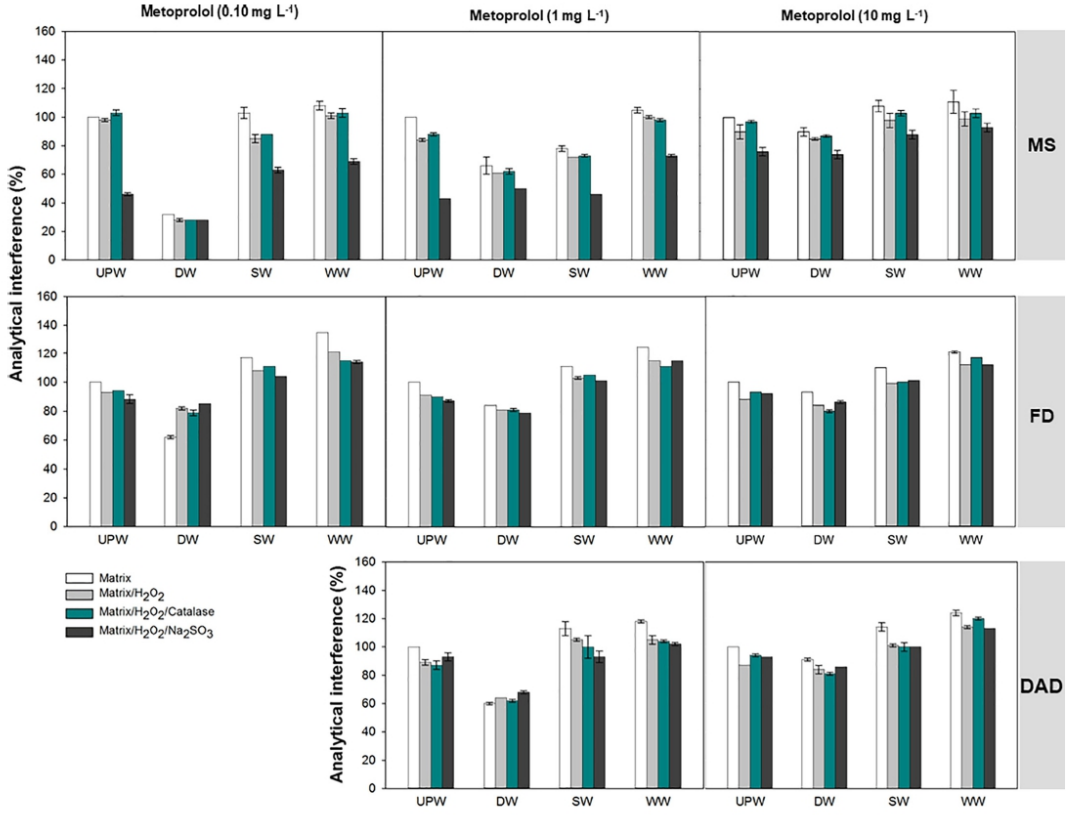

Fig. 5. Analytical interference (%) resulting from the matrix, the oxidant (H_2O_2) and the quenchers (H_2O_2/Na_2SO_3, H_2O_2/catalase) in MS, FD and DAD detectors, for metoprolol spiked at 0.1 mg L^{-1} (left), 1.0 mg L^{-1} (centre) and 10 mg L^{-1} (right) in UPW, DW, SW and WW samples.

of Na_2SO_3 and attributed to the shift effect in the maximum wavelength or in the absorbance signal. However, this effect of the quencher both in the presence and absence of H_2O_2 was only verified at concentrations of Na_2SO_3 one order of magnitude higher than those here tested.

The signal suppression was attenuated in the case of DW, maybe due to the consumption of Na_2SO_3 by the chlorine present in tap water, which was thus less or not available to interact and cause suppression in the ionization source. Interestingly, the addition of oxidant in the presence of chlorine, did not originate the signal suppression verified for diclofenac in DW when H_2O_2 was added.

The obtained results confirmed the interference of H_2O_2/Na_2SO_3 in the LC-MS/MS analysis of metoprolol, as had previously observed in the set of experiments (A) (Section 3.1). The formation of adducts cannot be disregarded, but the fact that all the affected compounds are ionized under PI mode have a $pK_a > 5.9$ and are eluted in the same range of time corresponding to the beginning of the chromatographic run, points out that matrix effects can justify the analytical interferences observed. Nonetheless, the suppression effect arising from the use of H_2O_2/Na_2SO_3 in MS detector is still not described in the literature, being addressed for the first time in this study. Overall, the present report clearly indicates that the application of Na_2SO_3 as H_2O_2 quencher should be replaced by catalase, when it comes to samples to be analysed by LC-MS/MS. These findings highlight the crucial role of the matrix effect interpretation in order to avoid wrong conclusions.

4. Conclusions

This study shows for the first time the suppression effect in the MS detection of a wide set of compounds having different chemical moieties, which is caused by the Na_2SO_3 commonly added as a H_2O_2 quencher to samples resulting from experiments with AOTs. In the case of LC-MS/MS, the signal of MPs monitored under NI mode and those with lower pK_a was not affected, whereas MPs with higher pK_a (monitored under PI mode) eluted in the beginning of the chromatographic run, had a strong signal suppression. This suggests that analytes pK_a may be the governing parameter on this effect, in accordance with previous studies reporting pK_a as one of the most important factors to predict the ionization in ESI source via monoprotonation, under PI mode. For instance, diclofenac as model MP with low pK_a did not have noteworthy suppression effects, whereas metoprolol had suppression in the ESI source of LC-MS/MS, which seemed to be concentration-dependent. For all matrices and concentrations of metoprolol, H_2O_2/Na_2SO_3 led to a suppression of ionization in the ESI source and a consequent decrease on the signal in the MS detector, which was marked for the lowest concentration. The results suggest that some product of the reaction between the H_2O_2 and Na_2SO_3 might cause this suppression in the ionization source of compounds with higher values of pK_a or some adducts can be formed, but more investigation is required to understand this effect. Moreover, the suppression was mitigated in the case of DW, due to the consumption of Na_2SO_3 by the chlorine present in tap water, which avoided the suppression in the ionization source.

The conclusions of this report are relevant for studies focused on treatment processes implementing H_2O_2 or O_3, which have to consider that: (i) Na_2SO_3 is not a good option as quencher, due to an analytical interference taking place in the LC-MS/MS; (ii) catalase should be used instead to avoid interferences in the MS detection; and (iii) matrix-match validation and internal calibration are crucial to calculate removal efficiencies and avoid bias conclusions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.07.278.

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"Ozone-based water treatment (O_3, O_3/UV, O_3/H_2O_2) for removal of organic micropollutants, bacteria inactivation and regrowth prevention?"

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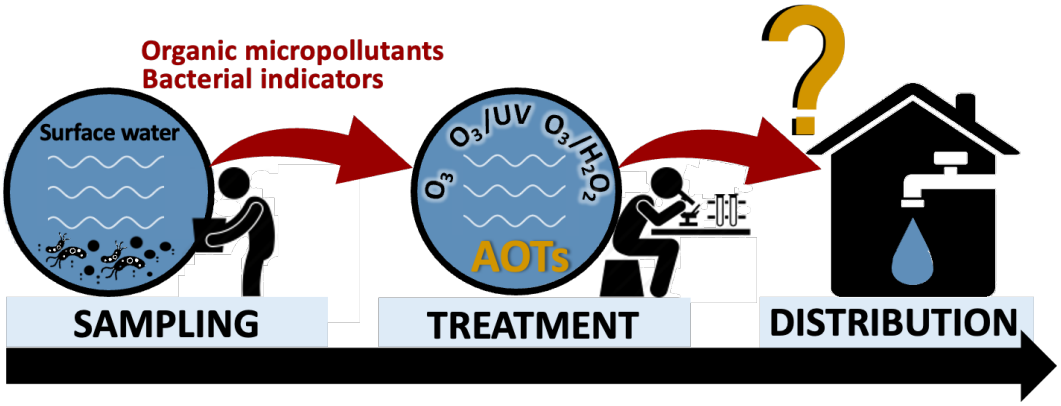

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Ozone-based water treatment (O_3, O_3/UV, O_3/H_2O_2) for removal of organic micropollutants, bacteria inactivation and regrowth prevention

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ABSTRACT

Ozone-based water treatments were investigated to simultaneously remove organic micropollutants (OMPs) and bacteria from surface water samples collected in a river reservoir supplying a drinking water treatment plant. Among the 21 environmentally relevant OMPs selected, 15 were detected at low (< 20) ng L^{-1} level. Surface water samples were then spiked with these 21 OMPs (100 ng L^{-1}) and the ozone-based treatments were tested with excess of ozone and high hydraulic retention time (HRT: 41 min). Regardless of the treatment applied (O_3, O_3/UVA-LEDs, and O_3/H_2O_2), removal efficiencies higher than 85% were always obtained for 20 out of 21 OMPs (and lower than 50% for PFOS). Thus, single ozonation was considered the best treatment solution, being afterwards investigated in terms of water disinfection at milder operating conditions (3 mg O_3 L^{-1} and HRT: 10 min). Under these conditions, the removals of spiked OMPs were also higher than 85% (except for PFOS), whereas bacteria (*i.e.*, total heterotrophs at 37 and 22 °C, *E. coli* and enterococci) decreased to values lower than the permissible levels for drinking water, even upon storage for 3-days. However, lower ozone doses (1.5 mg O_3 L^{-1}) could not prevent the regrowth of total heterotrophs incubated at 37 °C, which reached levels five times higher than those recommended. Because drinking water treatments should not only consider OMPs abatement but also microbial inactivation for long periods of time, the results herein obtained point out the importance of using ozone doses capable of accomplish both targets when using single ozonation.

1. Introduction

The poor effectiveness of conventional water treatment systems to remove organic micropollutants (OMPs) is a matter of concern due to the possible adverse long-term effects that these substances may develop on ecosystems and human health. It is known that many OMPs are not completely removed in conventional wastewater treatment plants, resulting in a permanent entry and consequent persistence in watercourses [1,2]. Agriculture and livestock runoff, sewer leakage and landfill leachates also contribute to the proliferation of these substances into receiving waters [1], OMPs being found in surface, drinking, and ground waters (between ng L^{-1} and μg L^{-1} levels) [3–5]. In this context, EU Directives 2008/105 and 2013/39 [6] recommend the monitoring of OMPs in surface waters (SW), identifying substances of priority action (or priority substances – PSs) with environmental quality standards (EQS), and contaminants of emerging concern (CECs) for which information on their occurrence is yet required and the list is regularly updated in EU Decisions, such as Decision (EU) 2020/1161 of 4 August 2020 [7–9].

OMPs comprise several classes of substances widely used in human activities (*e.g.*, pharmaceuticals and personal care products (PPCPs), X-ray contrast media, steroid hormones, industrial compounds, flame retardants, artificial sweeteners, and pesticides), and scientific studies have confirmed their potential threat to ecosystems and human health, namely proliferation of antibiotic resistant bacteria and/or their related genes, endocrine disruption, and acute or chronic toxicity [3,10–14].

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Since many OMPs persist in aquatic systems, several water related activities are using waters containing residual amounts of OMPs (*e.g.*, aquaculture farms, dairy industries, and drinking water treatment plants – DWTPs), rising environmental and health concerns [15–17]. In the particular case of DWTPs, some OMPs in SW might end up in tap water, leading to possible human exposure, bioaccumulation and consequent adverse effects [1,18]. In this context, it is important to apply effective water treatment technologies for the removal of OMPs from SW [19].

In addition, microbial contamination of drinking water contributes to diseases dissemination and, therefore, its control through effective treatments in DWTPs is indispensable [20]. For this reason, in the EU, the Directive 98/83/EC (amended by Directives 2015/1787 and 2020/2184) sets microbiological quality standards for potable water, including indicators of faecal contamination, namely *Escherichia coli* and enterococci, and total heterotrophs as target bacteria [21–23] [21,22,23]. Chlorination is the most common disinfection treatment for drinking water, but the by-products that chlorine originates, by reacting with natural organic matter, are a reason of concern due to their possible carcinogenicity, teratogenicity, and genotoxicity [24]. Adsorption using activated carbon [25–28] and membrane technologies (such as microfiltration, ultrafiltration, nanofiltration and reverse osmosis) [27,29] have shown good removal efficiencies of OMPs in DWTPs, and some of those can also be employed for disinfection [30]. However, the adsorbent must be regenerated in the first case [31,32], and a proper treatment or disposal of the concentrate generated is needed in the second case [29]. Advanced oxidation technologies (AOTs), such as ozonation, have been shown as an interesting option in DWTPs for disinfection [33–35]. Moreover, the OMPs can be oxidized by the highly reactive species that are generated by these processes, rather than transferring these pollutants from one to another phase [36,37]. Combining ozone with hydrogen peroxide (H_2O_2) or UV radiation may be beneficial options, since both may accelerate the decomposition of ozone into highly reactive species [36,38]. Although ozone based-processes might form reaction by-products, the overall quality of the water is often improved [36,38].

In fact, AOTs are promising technologies that can be used for many applications like purification and disinfection of water, gases, and soils, sterilization of medical rooms and instruments, disinfection of plant and animal products, among others [39–44]. However, regarding DWTPs, studies on the removal of OMPs are still very limited when more realistic conditions are considered (*e.g.,* real matrices, wide range of OMPs, and treatment operating in continuous mode). Furthermore, when the simultaneous removal of OMPs and disinfection is considered to treat SW in particular, only three studies were found in the literature for this type of water matrix [19,45,46]. Two of these studies were focused on UV/H_2O_2 without ozone [45,46], and another was our previous investigation on photocatalytic ozonation [19], none of these reports targeting the disinfection to comply with national and EU regulations on water for human consumption.Thus, the novelty of the present work relies on the comparison of three ozone-based treatments to remove multi-class OMPs from SW and conclude about the potential of the best cost-effective treatment to simultaneously inactivate bacteria according to the EU limits for water consumption.

The main research objectives are: (i) to evaluate the occurrence of OMPs in a SW reservoir supplying a DWTP in Northern Portugal (*i.e.*, 5 PSs of EU Directive 2013/39 [6], 8 CECs included in the watch lists of EU Decisions [7–9], and 8 other CECs of particular interest due to their occurrence and persistence in the environment – Table S1); (ii) to compare ozone-based treatments (O_3, O_3/UVA-LEDs and O_3/H_2O_2) operating in continuous mode for the removal of all target OMPs spiked in SW; and (iii) to investigate milder operating conditions to achieve a simultaneous removal of OMPs and water disinfection.

2. Materials and methods

2.1. Chemicals and materials

All reference standards of OMPs under study (alachlor, atrazine, azithromycin, carbamazepine, clarithromycin, clindamycin, clothianidin, diclofenac, diphenhydramine, fluoxetine, isoproturon, methiocarb, metoprolol, norfluoxetine, perfluorooctanesulfonic acid (PFOS), propranolol, simazine, thiacloprid, thiamethoxam, tramadol and venlafaxine; Table S1, Supplementary Material) were purchased from Sigma-Aldrich (Steinhein, Germany). The respective deuterated compounds used as internal standards (atrazine-d5, acetamiprid-d3, clothianidin-d3, diclofenac-d4, fluoxetine-d5, methiocarb-d3, propranolol-d7; >98% purity) were also obtained from Sigma-Aldrich (Steinhein, Germany). Stock solutions of each reference and internal standard (*ca.* 1000 mg L^{-1}) were prepared in methanol, ethanol or acetonitrile, depending on the solubility of each substance. Two working solutions, one containing all reference OMPs (2.5 mg L^{-1}) and another including all internal standards (5 mg L^{-1}), were prepared by dilution of the individual stock solutions in ethanol.

Ethanol HPLC grade was acquired from Fisher Scientific (Leicestershire, UK), whereas methanol and acetonitrile MS grade were purchased from VWR International (Fontenay-sous-Bois, France). Formic acid and sulphuric acid were supplied by Merck (Darmstadt, Germany). Oasis® Hydrophilic-Lipophilic-Balanced (HLB) cartridges (150 mg, 6 mL) were acquired from Waters (Milford, MA, USA). Hydrogen peroxide (30%, w/w) and catalase from bovine liver powder (2000–5000 units/mg protein) were obtained from Sigma-Aldrich (Steinhein, Germany). Ultrapure water (resistivity > 18.2 MΩ cm at 25 °C) was supplied by a Milli-Q water system from Millipore (Massachusetts, USA).

The culture media Slanetz and Bartley (SB) and Plate Count Agar (PCA) were purchased from VWR International (Fontenay-sous-Bois, France). Chromogenic Coliform Agar (CCA) was acquired from Scharlab (Barcelona, Spain) and 2,3,5-triphenyltetrazolium chloride solution (TTC) from Sigma-Aldrich (Steinhein, Germany). For confirmatory tests, Bile Esculin Azide Agar (BEA, ISO 7899–2) and N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) were provided by Sigma-Aldrich (Steinhein, Germany).

2.2. Surface water (SW) samples

All experiments were performed using SW samples collected from Douro River (41°04'39.4"N 8°28'21.0"W) between 2017 and 2019, over five sampling periods (January, April and October 2017, June 2018, and September 2019; $n = 5$). The physico-chemical parameters, namely pH, dissolved organic/inorganic carbon (DOC/DIC), ions and iron dissolved concentrations, are described in the Supplementary Material (Table S2). This waterbody supplies a DWTP that serves 1.39 million of people and treats an average flow of 270,000 m^3 day^{-1}. After collection, the samples were stored in pre-rinsed amber glass bottles, transported at 4 °C to the laboratory and kept refrigerated until use. In the case of microbiological assays, samples were collected in sterile flasks and immediately processed in the same day of collection. For determination of the target OMPs in SW, the samples were analysed as collected. Ozone-based (O_3, O_3/UVA-LEDs and O_3/H_2O_2) and control (UVA-LEDs) experiments were performed with SW spiked with the target OMPs at 100 ng L^{-1} each to start the runs with similar concentrations of OMPs, because SW was collected in different sampling periods, and also to simulate a worst-case scenario, *i.e.* the occurrence of all target 21 OMPs at hundreds ng L^{-1}. The SW spiking was conducted as described elsewhere [47]. Briefly, the ethanolic solution containing all target OMPs was added to an empty volumetric flask and the residual solvent was evaporated using a nitrogen flow. Afterwards, the SW was added into the flask, which was stirred and sonicated for 30 s. With this approach, we aimed to minimize the variations in DOC content resulting from the addition of the stock ethanolic solution of OMPs to SW.

2.3. Experimental set-up and procedure

Screening (O_3, O_3/UVA-LEDs or O_3/H_2O_2) and control (UVA-LEDs) experiments were performed in a flow-through glass bubble column reactor operating in continuous mode. Spiked SW was fed at the bottom of the reactor (Fig. 1a) by using a peristaltic pump (Watson-Marlow, UK; Fig. 1b), and constantly collected after treatment at the outlet stream located at the top of the reactor (Fig. 1c). This bubble column reactor (3.0 cm internal diameter × 70 cm height; Fig. 1d) was packed with glass Raschig rings (diameter: 3 mm and length: 3 mm; filling volume of 385 mL after packing) to promote the ozone-water mass transfer, as demonstrated in our previous publication [48]. A BMT 802X ozone generator (BMT Messtechnik, Germany) (Fig. 1g) was used to produce ozone from pure oxygen at a constant inlet concentration (50 g Nm^{-3}) and flow rate (15 Ncm^3 min^{-1} in the gas phase), and introduced into the reactor with a ceramic diffuser [19]. Ozone in the gas phase was monitored by using a BMT 964 ozone analyser (BMT Messtechnik, Germany) (Fig. 1h). To remove the remaining ozone from the gas phase leaving the reactor, gas washing bottles filled with potassium iodide solution were employed (Fig. 1i). A Q45H/64 dissolved ozone probe (Analytical Technology, USA; Fig. 1k) was calibrated by using the Indigo colorimetric method [49] and a T60 UV-Visible Spectrophotometer for absorbance measurements (PG Instruments; Lutterworth, UK). In the case of light-driven experiments, eight 10 W UVA high intensity light emitting diodes (LEDs, 15.5 mm × 23.0 mm) with a dominant emission line at 382 nm and long service life (intensity remains above 70% after 10,000 h work) were set along the reactor with a distance of 5 cm (Fig. 1f) [19,50]. The LEDs medium nominal irradiation was 390 W m^{-2} determined with a UV-Vis spectroradiometer (USB 2000 +, Ocean-Options, USA). In H_2O_2-based experiments, H_2O_2 was directly added to the spiked SW as a single pulse, and the dosage of H_2O_2 was selected according to the stoichiometric amount with respect to the DOC content (Fig. 1j).

In a typical run, the reactor was initially filled with distilled water. When spiked SW starts to be pumped into the reactor (t_0), a dilution effect is observed until steady-state conditions are achieved. Considering our previous studies conducted in the same installation - one focused on photocatalytic ozonation of SW collected in the same location [19] and another dealing with ozonation/peroxone treatment of urban wastewater and where different operating conditions were studied [51] - the liquid phase (SW) was pumped at 15 mL min^{-1}, corresponding to a hydraulic retention time (HRT) of 41 min and a steady state time (t_s) of 180 min. It is worthy to note that, for each type of treatment, the spiked SW was pumped through the reactor and once t_s was achieved, the treatment started (t_1) by bubbling ozone (O_3), switching on the LEDs (O_3/UVA-LEDs) or adding H_2O_2, (O_3/H_2O_2), according to the treatment under study. After t_1, another t_s of 180 min was considered until collection of the final samples (t_2, triplicates of 500 mL, meaning t_{2a}, t_{2b}, t_{2c}). For H_2O_2-based experiments, a stoichiometric excess (5 mL) of a fresh catalase aqueous solution (0.1 g L^{-1}) was added to each sample to quench any residual oxidant, as described elsewhere [52]. Each experiment was performed in triplicate and the efficiency was estimated considering the average of OMPs concentration abatement between t_1 and $t_{2(a, b, c)}$. Based on previous works [19,52], all these screening experiments were performed using a loop column (Fig. 1e) simulating a perfectly mixed reactor (details in Section 3.2). After selecting the best performing treatment, and in order to mimic a real single passage bubble column reactor, this loop column / recirculation (bold line, Fig. 1) was not employed in a second set of experiments performed under milder conditions (details in Section 3.3).

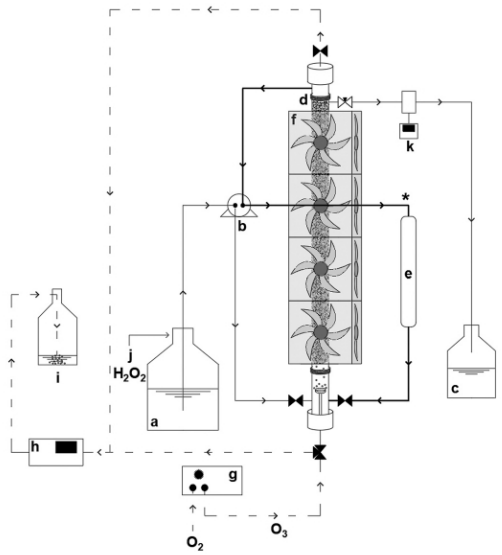

Fig. 1. Schematic representation of the experimental set-up of the bubble column reactor operating in continuous mode: (a) inlet of SW; (b) peristaltic pump; (c) outlet of SW; (d) glass column, packed with glass Raschig rings; (e) loop column*; (f) UVA-LEDs; (g) ozone generator; (h) ozone analyser; (i) gas washing bottles; (j) H_2O_2 addition; and (k) Q45H/64O_3 probe. *Bold line*: loop used in screening experiments of different treatment processes (Section 3.2) and not employed in those experiments conducted at milder operating conditions (Section 3.3).

2.4. Chemical analysis

An offline solid phase extraction followed by ultra-high-performance liquid chromatography-tandem mass spectrometry (SPE-UHPLC-MS/MS) analytical method was used to determine the concentration of the target OMPs in SW samples (including spiked and non-spiked SW) before and after treatment. The SPE protocol was performed as described elsewhere [53]. Briefly, the SW samples (500 mL) were filtered through 1.2 μm glass-fiber filters (47 mm GF/C, Whatman™, UK) and acidified to pH 3 with sulphuric acid. Oasis® HLB cartridges were used to pre-clean and extract the OMPs from the samples in a manifold provided by Waters (Milford, USA) and a Labconco® CentriVap (Kansas City, USA) was applied to dry the extracts. Ethanol was used as conditioning and eluting solvent of the cartridges (4 mL) and as restorative solvent of the dried sample extracts (250 μL). For chromatographic analysis, a Shimadzu Corporation apparatus (Tokyo, Japan) with LC (UHPLC, Nexera) and MS/MS detection (triple quadrupole mass spectrometer detector, LCMS-8040) was used. A Kinetex™ 1.7 μm XB-C18 100 Å column (100 × 2.1 mm i.d.) supplied by Phenomenex, Inc. (California, USA) was employed and the mobile phase (at a flow rate of 0.2 mL min^{-1}) consisted of ultrapure water and acetonitrile acidified with 0.1% formic acid, operating at gradient mode (15% of organic phase during 3 min, a linear gradient during 18 min up to 85%, which was kept during 2 min, after which the initial conditions were set again in 0.1 min to condition the column during 9.9 min). Column oven and autosampler temperatures were set at 40 °C and 4 °C, respectively. The volume of injection was 5 μL. Capillary voltage, drying gas flow, nebulizing gas flow, desolvation and source temperature of the mass spectrometer were 4.5 kV, 12.5 dm^3 min^{-1}, 2.5 dm^3 min^{-1}, 250 °C and 400 °C, respectively. The analysis was based on a triplicate matrix-matched calibrations curve, by adding selected amounts of each reference standard and a known and constant amount of internal standards. Retention time, range, method detection limits (MDLs) and method quantification limits (MQLs) of the target analytes are available in Table S3 (Supplementary Material).

A TOC-L analyser (Shimadzu Scientific Instruments, Japan) was used to determine the DOC content and a UNICAM-939 AA Spectrometer was employed to analyse the dissolved concentration of iron, respectively following procedures 5310B and 3030E/3111B of the Standard Methods for Examination of Water and Wastewater [54]. The concentration of cations (sodium, potassium, ammonium, calcium, magnesium) and anions (chloride, bromide, bromate, nitrite, nitrate, phosphate, sulphate) in solution were respectively determined by ionic chromatography using Metrosep C4 Cationic Exchange and Metrosep A Supp 7 Anionic Exchange columns (250 mm × 4.0 mm) in a Metrohm 881 Compact IC Pro equipment. The pH values were measured using a pH meter pHenomenal® pH 1100 L (VWR, Germany).

2.5. Microbiological assays

Microbiological analyses were performed considering the guidelines of the Portuguese legislation regarding water quality for human consumption [55] (transposing the Directive 2015/1787/EU [22]). Two independently collected SW samples were analysed (i) before, (ii) immediately after treatment, and (iii) after three days of storage in the dark at room temperature (for regrowth assays). Total heterotrophs, *E. coli* and enterococci were isolated and enumerated by the membrane filtration method, following EN ISO 6222, EN ISO 9308-1, and EN ISO 7899–2, respectively [22]. Adequate serial dilutions of each sample were prepared in sterile saline solution (0.85% NaCl). Volumes ranging from 100 mL to 1 mL of samples or dilutions thereof were filtered in duplicate, through cellulose nitrate membranes (0.22 μm pore size; Millipore, UK), which were further placed onto the culture medium and incubated according to the bacterial group: PCA for total heterotrophs (37 °C, 2 days, and 22 °C, 3 days); CCA for *E. coli* (37 °C, 1 day); and SB for enterococci (37 °C, 1 day). Oxidase test with 1% TMPD and incubation in BEA at 44.0 ± 0.5 °C for 4 h was used to confirm *E. coli* (oxidase negative) and enterococci (blackening of BEA), respectively.

3. Results and discussion

3.1. Occurrence of target OMPs in SW samples supplying a DWTP

Considering all collected samples ($n = 5$), 15 out of 21 target OMPs were detected at ng L^{-1} levels at different frequencies (Fig. 2), namely (i) 5 PSs of EU Directive 2013/39 (alachlor, atrazine, isoproturon, PFOS, simazine) [6]; (ii) 5 CECs included in the watch lists of EU Decisions 2015/495 (diclofenac) [7], 2018/840 (azithromycin, clarithromycin, thiacloprid) [8], and 2020/1161 (venlafaxine) [9]; and (iii) 5 other CECs (carbamazepine, clindamycin, diphenhydramine, fluoxetine, tramadol). Clothianidin, methiocarb, metoprolol, norfluoxetine, propranolol, and thiamethoxam, were never detected in these samples (Fig. 2).

Most of the OMPs found in SW are pharmaceuticals (azithromycin, clarithromycin, carbamazepine, clindamycin, diclofenac, diphenhydramine, fluoxetine, tramadol, venlafaxine), and their occurrence is probably related to their wide usage in medicine and ineffective elimination by conventional treatment of urban wastewater. In particular, the highest concentrations were observed for carbamazepine and tramadol (12.6 and 20.8 ng L^{-1}, respectively), whereas the lowest concentration was registered for fluoxetine (< 0.11 ng L^{-1}). A similar concentration was reported for tramadol (17 ng L^{-1}) in another study performed in the same river [56]. Carbamazepine was detected at a lower concentration (1 ng L^{-1}) in areas nearby the sampling site of the present study [57].

Other detected OMPs were pesticides (alachlor, atrazine, isoproturon, simazine, and thiacloprid), and their occurrence can be related to agriculture runoff. Atrazine (already banned) and isoproturon were always detected, the highest concentrations of this group of substances being found for isoproturon (between 3.2 and 9.2 ng L^{-1}), but still below the limit of 0.1 μg L^{-1} set for pesticides in EU legislation on the quality of water intended for human consumption [23]. Likewise, the sum of all pesticides concentrations was also below the maximum admissible (0.5 μg L^{-1}) by the same regulation [23].

The industrial compound PFOS was quantified up to 12.0 ng L^{-1}, and it has been detected in several watercourses due to its wide commercial application during the past sixty years, high solubility and

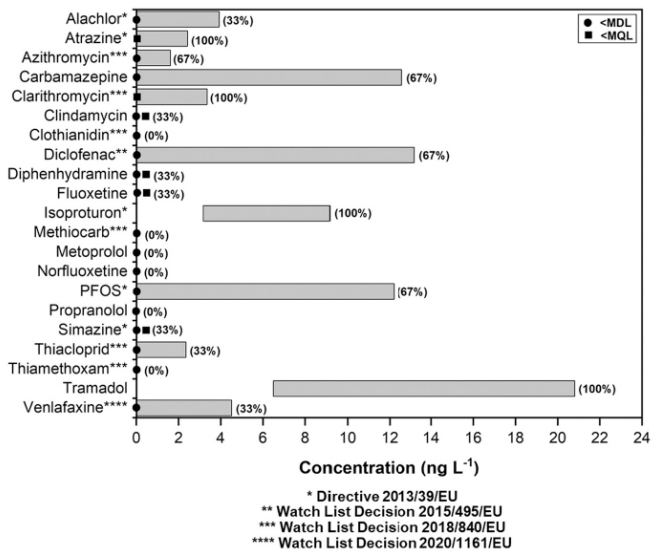

Fig. 2. Concentrations range (ng L^{-1}) and detection frequency (%) of each target OMP in SW collected during the sampling campaign.

recalcitrance to water treatment [58,59].

Overall, all the PSs included in the present work were detected during the sampling campaign, highlighting the concern about the possible effects of these substances in the aquatic environment. However, the concentrations of these OMPs were always below the Maximum Allowable Concentration - EQS (MAC-EQS) set in Directive 2013/39/EU [6]. Moreover, the detection of 10 out of 16 CECs, 5 of which already flagged in EU Decisions, urges the importance of monitoring studies as well as risk assessment for these substances that are still not included as PSs with EQS in any regulation. Considering the high variability of OMPs occurrence in SW (*i.e.* susceptible to the quality and volume of the discharged urban wastewaters, weather conditions, population habits, among others), spiked SW was used in the following studies simulating a worst-case scenario where all target OMPs would be present. The spiking was conducted with a known concentration (100 ng L^{-1}) of each of the 21 target OMPs, this concentration being selected taking into account the range of concentrations typically reported for OMPs in monitoring studies of SW [60].

3.2. Degradation of OMPs: selection of the best performing treatment

The removal rates of OMPs for each studied treatment (after reaching steady state, t_2), as well as the initial concentrations of analytes at t_1, are shown in Fig. 3. Slight deviations from the original spiked concentration (100 ng L^{-1}) were registered for some compounds. These deviations are mainly attributed to matrix effects and to the inherent occurrence of these OMPs in natural SW. As expected, low removals (< 10%) were obtained in the control experiment with UVA-LEDs (Fig. 3a). In contrast, very high removal efficiencies (> 85%) for most of the studied OMPs were obtained with the ozone-based treatments (Fig. 3b–d). PFOS was the exception and the most recalcitrant OMP regardless of the ozone-based treatment applied.

It is well known that UVC lamps are more effective to promote the direct photolysis of organic compounds than UVA and UVB lamps [61–63]. However, traditional UV mercury lamps are characterized by a short lifetime, low energy conversion efficiency and high pollution potential. For this reason, UV-LEDs have gained increasing interest presenting short warm-up time, no mercury disposal issues, stability and easy handling, operation at low voltage, and low power requirement [63]. LEDs have been investigated to remove organic pollutants from water matrices, but high intensity UVC-LEDs are still very expensive and only considered in few studies [64,65]. Looking for a less costly alternative, UVA-LEDs have been more often employed [66,67]. Photosensitizers contained in waters (e.g., humic substances dissolved in natural organic matter and inorganic species like nitrates) can generate highly reactive species, leading to indirect photochemical oxidation of OMPs, especially those for which indirect photolysis is the main reactive pathway [61,68]. Nonetheless, this phenomenon had no considerable impact for the SW under treatment, as can be observed by the low degradation of the OMPs in the control experiment with UVA-LEDs (Fig. 3a) and by comparison of the results obtained with O_3/UVA-LEDs and O_3 (Fig. 3b and c). Indirect photolysis of OMPs is very dependent on the water matrix, high removal efficiencies of some OMPs being reported by photolysis in other studies when using UVA-LEDs and urban wastewater as matrix [68].

Regarding the ozone-based experiments (O_3, O_3/UVA-LEDs and O_3/H_2O_2), 20 out of 21 OMPs were removed to levels below the MQL, regardless of the applied treatment, PFOS being the exception. Nonetheless, it is important to bear in mind that the high or partial degradation of OMPs may originate by-products that can be more or equally toxic. Depending on the pH, ozone will oxidize the organic molecules directly (more acidic pHs) or indirectly (more alkaline pHs). The latter occurs when molecular ozone is decomposed into hydroxyl radicals ($HO^{\bullet}$) or other reactive species such as oxide anion ($O_2^{\bullet-}$), hydroperoxyl ($HO_2^{\bullet}$) and ozonide anion ($O_3^{\bullet-}$) radicals [69]. Considering the treatments and the pH of the SW under study (8.5), the indirect mechanism is the main expected degradation pathway. O_3/H_2O_2 (or peroxone) may accelerate the formation of reactive species, for instance by the reaction

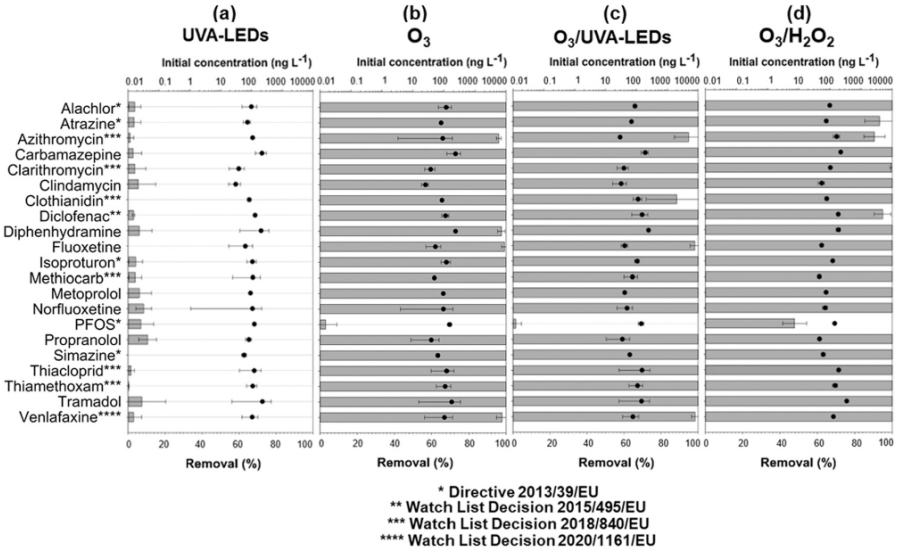

Fig. 3. Removal of OMPs in continuous mode with loop (simulating a perfectly mixed reactor): (a) UVA-LEDs (control), (b) O_3, (c) O_3/UVA-LEDs and (d) O_3/H_2O_2. Experimental conditions: spiked SW (100 ng L^{-1} of each OMP), HRT: 41 min, t_t: 180 min, O_3 in excess: 50 g Nm^{-3} in gas phase, and pH: 8.5.

of the H_2O_2 conjugate base (H_2O^-) with ozone generating $HO^•$ [51,69]. However, ozone alone was enough to remove (> 85%) 20 OMPs under study, and the removal of PFOS was below 50% even when H_2O_2 was added. Therefore, SW polluted with ng L^{-1} levels of OMPs can be treated with single ozonation. In fact, even ozone-resistant compounds (e.g., atrazine > simazine > metoprolol > isoproturon > clarithromycin), with kinetic constants rates (k_{O3}) lower than 10^5 L mol^{-1} s^{-1}, can be removed by ozonation because the other highly reactive species referred above are generated with this process, in particular at more alkaline pH values such as 8.5 [36,70]. The recalcitrance of PFOS towards ozonation is due to its strong carbon–fluorine bonds (Table S1) that make this compound a very stable and resistant pollutant. It was demonstrated that ozonation at pH 11 can increase the removal of PFOS (43–85%: 4 h treatment), as well as the application of O_3/H_2O_2 at the same high pH (>99%: 4 h treatment) [71]; however, an undesirable pH increase from 8.5 to 11 is needed in both cases. In fact, a recent review on advanced oxidation/reduction processes for the treatment of PFOS [72] identifies this substance as recalcitrant to ozone (removals < 2% in SW) and describes reductive photochemical/photocatalytic processes (mainly mediated by hydrated electrons in absence of oxygen) as more efficient to eliminate PFOS.

Beyond the chemical properties of the compounds, the water matrix can also affect the degradation of the OMPs due to the presence of scavengers. However, the high efficiencies obtained suggest that the matrix does not have a noticeable impact in this case, most likely due to low DOC content of SW (3.5 mg L^{-1}, Table S2). The efficiency of ozone-based processes has been extensively reported in the literature for wastewater treatment; however, there are much less studies for SW, in particular when considering multi-class OMPs and treatment under more realistic conditions [37,38,73]. One of the major drawbacks of the ozonation treatment of real matrices is the common incomplete mineralization of the organic compounds. In this context, the formation of bromate by the reaction of bromide present in natural water with O_3 and $OH^•$ represents a concern, a limit of 10 μg BrO_3^- L^{-1} being defined for water intended for human consumption [38]. Fortunately, this anion was never detected in the present study after O_3 treatment and, therefore, single ozonation was selected to be applied in the next set of experiments, for water disinfection and removal of OMPs under mild operating conditions. Nonetheless, depending on each water supply and characteristics (mainly in terms of bromide concentration), as well as treatment train in the plant, there are different approaches that can minimise the formation of bromate during ozonation, namely lowering the pH, adding ammonia, lowering the ozone exposure, and using chlorine-ammonia or peroxone processes (depending largely on the dose (and point) of H_2O_2 addition in comparison with that of ozone) [74–76].

3.3. Water disinfection and degradation of OMPs: testing mild operating conditions

This set of experiments was performed with a single passage of the liquid into the column, *i.e.* mimicking a more realistic ozonation bubble column, instead of recirculating a certain fraction of the liquid (Fig. 1). Adjustments on the HRT and ozone dose were also performed in relation to the first set of experiments (Section 3.2), to implement mild operating conditions and improve the environmental (and economic) sustainability of the treatment. Thus, a higher liquid flow rate of 33 mL min^{-1} was selected, resulting in a HRT of 10 min (corresponding to a t_s of 25 min), which is an ozone contact time usually applied at full-scale DWTPs [25]. The aim was to find a low ozone concentration that guarantees the removal of drinking water EU targeted bacteria, while attaining high removals of OMPs (> 85%). Since the OMPs represent only a small part of the total DOC content of the SW matrix, and numerous water matrix constituents might act as scavengers of highly reactive species [77], the ozone dose should be selected considering the DOC content of SW as expressed by the specific ozone dose parameter ($g_{O_3}g_{DOC}^{-1}$). In this way, the impact of the matrix is taken into account on the process efficiency, allowing more reliable comparisons between experiments using different SW collected at a different time and/or location [38,77–79].

3.3.1. Water disinfection

The ozonation efficiency regarding the inactivation of the microbial groups listed in national and European legislation [22,55] was investigated for a HRT of 10 min and ozone concentrations of 1.5 mgO_3 L^{-1} (~ 0.3 g_{O_3} g_{DOC}^{-1}) and 3 mg O_3 L^{-1} (~ 0.7 $g_{O_3}g_{DOC}^{-1}$). Information found in the literature describes ozone doses applied in DWTPs between 1.5 and 3 mg O_3 L^{-1} for disinfection purposes [80,81] and, for this reason, those were selected in the present work. The number of colony forming units (log CFU 100 mL^{-1}) of total heterotrophs (at 37 and 22 °C), *E. coli* and enterococci were determined (i) before, (ii) immediately after treatment, and (iii) after 3-days storage (regrowth) in the dark and at room temperature (Fig. 4). In contrast to the experiments dealing only with the removal of OMPs, biological assays were not undertaken with spiked bacteria in water since the preliminary analyses showed the presence of the target bacteria within quantifiable levels.

Inactivation of all targeted bacteria to values lower than the LOQ (0.5 CFU 100 mL^{-1}) was observed immediately after treatment for both dissolved ozone concentrations tested (Fig. 4). In the case of the higher

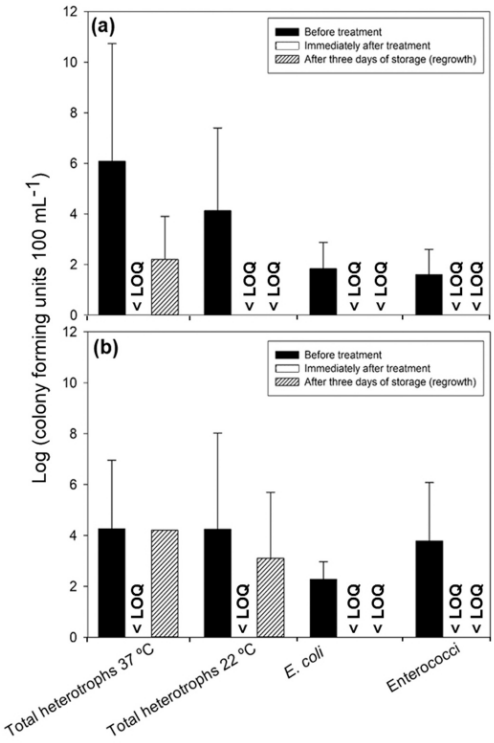

Fig. 4. Microbial analyses (total heterotrophs at 37 and 22 °C, *E. coli* and enterococci) in SW (i) before, (ii) immediately after ozonation treatment operating in continuous mode (HRT: 10 min), and (iii) after 3-days storage (regrowth) in the dark and at room temperature (LOQ: 0.5 CFU 100 mL^{-1}). Applied O_3 doses: (a) 3 mg O_3 L^{-1} and (b) 1.5 mg O_3 L^{-1}.

ozone dose (Fig. 4a), regrowth did not occur for total heterotrophs at 22 °C, *E. coli* and enterococci. In contrast, regrowth was observed after 3-days storage for total heterotrophs at 37 °C, quantified at approximately 10^2 CFU 100 mL^{-1} (*i.e.* 1 CFU mL^{-1}), but this value is below the maximum recommendable of 20 CFU mL^{-1} for the quality of water intended for human consumption [22,55]. These results proved the effectiveness of ozonation to inactivate these regulated microbial groups using 3 mg O_3 L^{-1} and 10 min of HRT.

When half of the dissolved ozone concentration (1.5 mg_{O_3} L^{-1}) was used (Fig. 4b), all microbial groups were again inactivated immediately after treatment. Total heterotrophs reactivated after 3-days storage, but in this case at both incubation temperatures (22 and 37 °C). Moreover, although the quantification of total heterotrophs incubated at 22 °C did not exceed the limit recommended (100 CFU mL^{-1}) [22,55], the same did not happen when incubated at 37 °C, resulting in values around 10^2 CFU mL^{-1}, *i.e.* five times higher than those advisable (20 CFU mL^{-1}) [22,55]. Although the addition of H_2O_2 together with low ozone doses (1.5 mg O_3 L^{-1}) could be an interesting option in comparison with the application of ozone alone at higher concentration (3 mg O_3 L^{-1}), studies have described ozone alone as a preferential bactericide to peroxone due to the higher ozone residuals produced [82–85]. Moreover, in one of our previous studies dealing with ozonation for the treatment of urban wastewater [51], significant differences in terms of *E. coli* inactivation were not observed when H_2O_2 was added to ozonation, these results being in agreement with those obtained for the degradation of OMPs in the same study, and also with those herein reported. Indeed, the microbial inactivation immediately after treatment does not represent a definitive removal since the available sources of carbon resulting from the partial oxidation of organic matter can still promote their reactivation, as already verified for total heterotrophs in a previous work [47]. Total heterotrophs cover a great diversity of microorganisms, which may justify their predominance after storage as already demonstrated in our previous study [19]. Thus, this group of bacteria proved to be a determinant indicator to select the best ozone dose for water disinfection. In the present study, an ozone concentration of 3 mg_{O_3} L^{-1} ensured the water disinfection and microbiological quality of water after treatment and storage for at least 3 days (2–6 log reduction). These conditions correspond to a cumulative disinfectant over time (*Ct*) value of 30 mg O_3 min L^{-1} (*i.e.* 3 mg O_3 L^{-1} × HRT of 10 min). This parameter has been widely used for water disinfection [86,87].

3.3.2. Degradation of OMPs

The degradation of the selected OMPs spiked in SW samples was performed under mild operating conditions (Fig. 5), *i.e.,* employing HRT of 10 min and a dissolved ozone concentration of 3 mg O_3 L^{-1}, corresponding to specific ozone doses between 0.7 and 1.3 g_{O_3} g_{DOC}^{-1}, depending on the DOC of collected SW. The idea was to remove these OMPs while ensuring water disinfection, *i.e.* bacteria inactivation as defined in Section 3.3.1. Once again, removals higher than 85% were registered for all target OMPs, except for the recalcitrant PFOS (Fig. 5). Thus, these milder operating conditions (HRT of 10 min and 3 mg O_3 L^{-1}) can be employed for both water disinfection and removal of OMPs.

It is important to refer that the specific ozone dose normalized with respect to DOC is a good parameter suggested in the literature to decide about the proper conditions to achieve the degradation of OMPs by ozonation. However, as demonstrated in the present work, the ozone dose must be also selected by taking into account the microbiological constitution of the SW that will be treated, *i.e.* applying the minimum dissolved ozone concentration able to inactivate bacteria to levels complying the regulation for water consumption [55]. Thus, the ozone dose must be selected considering both DOC and microbial inactivation (rather than OMPs only). Although ozonation has been applied in DWTPs for disinfection, and some studies have been published exclusively dealing with the removal of OMPs in these facilities, this is the first report dealing with single ozonation for the simultaneous removal

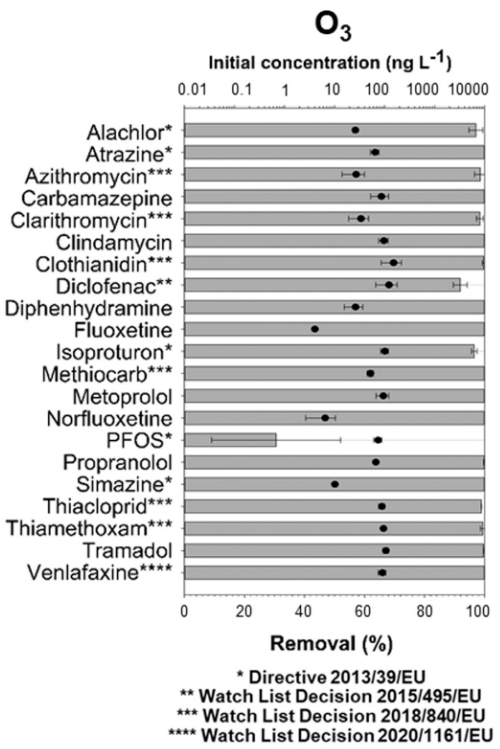

Fig. 5. Removal of OMPs by continuous ozonation (O_3) without loop. Experimental conditions: spiked OMPs in SW (100 ng L^{-1} of each OMP), HRT: 10 min, t_i: 25 min, O_3: 3 mg O_3 L^{-1} in liquid phase, and pH: 8.1.

of both chemical and biological contaminants from SW. Moreover, bacteria regrowth in the treated water was investigated to assess the efficiency of the treatment process if storage, before its use, is envisaged. This work was conducted having as target the compliance with national and EU regulations on water for human consumption. In this context, further studies are needed to evaluate the simultaneous removal of OMPs and biological contaminants by single ozonation at full-scale DWTPs, while performing toxicity bioassays (*e.g.*, mutagenicity, genotoxicity and oxidative stress) to assess if complementary treatments are needed.

4. Conclusions

Five priority substances of the EU Directive 2013/39 (alachlor, atrazine, isoproturon, PFOS, simazine), five contaminants of emerging concern listed in EU Decisions (azithromycin, clarithromycin, diclofenac, thiacloprid and venlafaxine) and other five OMPs of particular interest in the context of water policy (carbamazepine, clindamycin, diphenhydramine, fluoxetine, tramadol) were detected at low concentrations (< 20 ng L^{-1}) in surface water supplying a drinking water treatment plant in Northern Portugal. Among the studied treatment technologies (O_3, O_3/UVA-LEDs and O_3/H_2O_2), single ozonation was considered as the best solution, because it is the simplest option and it was effective for removing (> 85%) most of the OMPs (except PFOS) from spiked surface water without the generation of bromates.

Regardless of the ozone-based processes applied, PFOS was always the most recalcitrant compound, with lower removals (< 50%). Moreover, single ozonation using a low amount of O_3 (3 mg O_3 L^{-1}) with HRT of 10 min proved to be efficient for both removal of OMPs (> 85%, except PFOS) and disinfection. *E. coli*, enterococci, and total heterotrophic bacteria complied with the established limits for potable water immediately after treatment and after 3 days of storage, showing bacteria inactivation and regrowth prevention. Lower ozone doses are not recommended because reactivation of total heterotrophs at 37 °C after 3 days of storage of treated water showed bacterial values (10^2 CFU mL^{-1}) above those recommended for the quality of water intended for human consumption (20 CFU mL^{-1}).

CRediT authorship contribution statement

Ana M. Gorito: Investigation, Conceptualization, Formal analysis, Methodology, Writing - original draft. **Joana F.J.R. Pesqueira**: Conceptualization, Methodology, Writing - review & editing. **Nuno. F.F. Moreira**: Conceptualization, Methodology, Writing - review & editing. **Ana R.L. Ribeiro**: Conceptualization, Methodology, Writing - review & editing, Supervision. **M. Fernando R. Pereira**: Conceptualization, Writing - review & editing. **Olga C. Nunes**: Conceptualization, Writing - review & editing. **C. Marisa R. Almeida**: Conceptualization, Writing - review & editing, Supervision. **Adrián M.T. Silva**: Conceptualization, Resources, Project administration, Funding acquisition, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2021.105315.

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Appendix B

Co-authors authorization regarding the full agreement of the inclusion of the manuscripts in the present thesis

January 2022

To Whom it May Concern

Adrián M.T. Silva, Assistant Professor at the Faculty of Engineering of the University of Porto (FEUP), C. Marisa R. Almeida, Researcher at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), and Ana R. Lado Ribeiro, Researcher at the Associate Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials (LSRE-LCM), hereby authorize the PhD student Ana Margarida Gorito Gonçalves from the FEUP Doctoral Programme of Environmental Engineering, to include the following publication in her doctoral Thesis entitled "*Micropollutants in aquaculture: constructed wetlands and advanced oxidation technologies as prospective treatments*":

A review on the application of constructed wetlands for the removal of priority substances and contaminants of emerging concern listed in recently launched EU legislation, Environmental Pollution, 227 (2017) 428-443, doi: https://doi.org/10.1016/j.envpol.2017.04.060

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To Whom it May Concern

M. Fernando R. Pereira, Full Professor at the Faculty of Engineering of the University of Porto (FEUP), Adrián M.T. Silva, Assistant Professor at FEUP, C. Marisa R. Almeida, Researcher at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), and Ana R. Lado Ribeiro, Researcher at the Associate Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials (LSRE-LCM) hereby authorize the PhD student Ana Margarida Gorito Gonçalves from the FEUP Doctoral Programme of Environmental Engineering, to include the following publication in her doctoral Thesis entitled "*Micropollutants in aquaculture: constructed wetlands and advanced oxidation technologies as prospective treatments*":

Advanced oxidation technologies and constructed wetlands in aquaculture farms: What do we know so far about micropollutant removal? Environmental Research, 204 (2022) 111955, doi: https://doi.org/10.1016/j.envres.2021.111955

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January 2022

To Whom it May Concern

Adrián M.T. Silva, Assistant Professor at the Faculty of Engineering of the University of Porto (FEUP), Carlos Rocha Gomes, Assistant Professor at the Faculty of Sciences of the University of Porto (FCUP), C. Marisa R. Almeida, Researcher at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), and Ana R. Lado Ribeiro, Researcher at the Associate Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials (LSRE-LCM), hereby authorize the PhD student Ana Margarida Gorito Gonçalves from the FEUP Doctoral Programme of Environmental Engineering, to include the following publication in her doctoral Thesis entitled "*Micropollutants in aquaculture: constructed wetlands and advanced oxidation technologies as prospective treatments*":

Constructed wetland microcosms for the removal of organic micropollutants from freshwater aquaculture effluents, Science of the Total Environment, 644 (2018) 1171-1180, doi: https://doi.org/10.1016/j.scitotenv.2018.06.371

(Authors: Ana M. Gorito, Ana R. Ribeiro, Carlos Rocha Gomes, C.M.R. Almeida, and Adrián M.T. Silva)

Signatures

(Adrián M. T. Silva)

Assinado por: **CARLOS ALBERTO ROCHA GOMES**
Num. de Identificação: 07827130
Data: 2022.01.12 21:10:14+00'00'

(Carlos Rocha Gomes)

(Cristina M. R. Almeida)

(Ana R. Lado Ribeiro)

January 2022

To Whom it May Concern

M. Fernando R. Pereira, Full Professor at the Faculty of Engineering of the University of Porto (FEUP), Adrián M.T. Silva, Assistant Professor at FEUP, C. Marisa R. Almeida, Researcher at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), Ana R. Lado Ribeiro, Researcher at the Associate Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials (LSRE-LCM), and Marta O. Barbosa, Researcher at the Mountains of Research Collaborative Laboratory – Association (MORE) hereby authorize the PhD student Ana Margarida Gorito Gonçalves from the FEUP Doctoral Programme of Environmental Engineering, to include the following publication in her doctoral Thesis entitled "*Micropollutants in aquaculture: constructed wetlands and advanced oxidation technologies as prospective treatments*":

Quenchers in advanced oxidation technologies for analysis of micropollutants by liquid chromatography coupled to mass spectrometry: Sodium sulphite or catalase?, Science of the Total Environment, 692 (2019) 995-1004, doi: https://doi.org/10.1016/j.scitotenv.2019.07.278

(Authors: Ana M. Gorito., Marta O. Barbosa., C.M.R. Almeida, M.F.R. Pereira, Adrián M.T. Silva and Ana R.L. Ribeiro.)

Signatures

(M. Fernando R. Pereira)

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(Ana R. Lado Ribeiro)

(Marta O. Barbosa)

January 2022

To Whom it May Concern

M. Fernando R. Pereira, Full Professor at the Faculty of Engineering of the University of Porto (FEUP), Olga C. Nunes, Associate Professor at FEUP, Adrián M.T. Silva, Assistant Professor at FEUP, C. Marisa R. Almeida, Researcher at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), Ana R. Lado Ribeiro, Researcher at the Associate Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials (LSRE-LCM), Nuno F.F. Moreira, PhD, and Joana F.J.R. Pesqueira, PhD student at LSRE-LCM/FEUP hereby authorize the PhD student Ana Margarida Gorito Gonçalves from the FEUP Doctoral Programme of Environmental Engineering, to include the following publication in her doctoral Thesis entitled "*Micropollutants in aquaculture: constructed wetlands and advanced oxidation technologies as prospective treatments*":

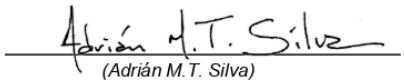
Ozone-based water treatment (O_3, O_3/UV, O_3/H_2O_2) for removal of organic micropollutants, bacteria inactivation and regrowth prevention, Journal of Environmental Chemical Engineering, 9 (2021) 105315, doi: https://doi.org/10.1016/j.jece.2021.105315

(Authors: Ana M. Gorito, Joana F.J.R. Pesqueira, Nuno F.F. Moreira, Ana R. Ribeiro, M. Fernando R. Pereira, Olga C. Nunes, C. Marisa R. Almeida, and Adrián M.T. Silva)

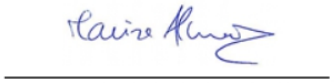
Signatures

(M. Fernando R. Pereira)

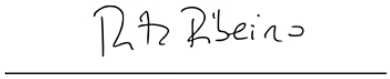
(Olga C. Nunes)



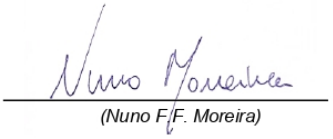
(Adrián M.T. Silva)



(C. Marisa R. Almeida)



(Ana R. Lado Ribeiro)



(Nuno F.F. Moreira)

(Joana F. J. R. Pesqueira)

January 2022

To Whom it May Concern

M. Fernando R. Pereira, Full Professor at the Faculty of Engineering of the University of Porto (FEUP), Adrián M.T. Silva, Assistant Professor at FEUP, C. Marisa R. Almeida and Laura Maria S.C. Guimarães, Researchers at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), Ana R. Lado Ribeiro, Researcher at the Associate Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials (LSRE-LCM), and Pedro Rodrigues, PhD student at CIIMAR hereby authorize the PhD student Ana Margarida Gorito Gonçalves from the FEUP Doctoral Programme of Environmental Engineering, to include the following publication in her doctoral Thesis entitled "*Micropollutants in aquaculture: constructed wetlands and advanced oxidation technologies as prospective treatments*":

Antibiotics removal from aquaculture effluents by ozonation: chemical and toxicity descriptors, under publication.
(Authors: Ana M. Gorito, Pedro Rodrigues, Ana R. L. Ribeiro, M. Fernando R. Pereira, Laura Guimarães, C. Marisa R. Almeida, and Adrián M.T. Silva)

Signatures

(M. Fernando R. Pereira)

(Adrián M.T. Silva)

(C. Marisa R. Almeida)

(Laura Maria S.C. Guimarães)

(Ana R. Lado Ribeiro)

(Pedro Rodrigues)

Appendix C

Ozone decomposition chain reactions

$O_3 + HO^- \rightarrow HO_2^\bullet + O_2^{\bullet-}$	**(1)**	**Initiation step**
$O_3 + HO_2^- \rightarrow HO_2^\bullet + O_3^{\bullet-}$	**(2)**	
$HO_2^\bullet \leftrightarrow O_2^{\bullet-} + H^+$	**(3)**	
$O_3 + O_2^{\bullet-} \rightarrow O_3^{\bullet-} + O_2$	**(4)**	
$O_3^{\bullet-} + H^+ \leftrightarrow HO_3^\bullet$	**(5)**	
$HO_3^\bullet \rightarrow HO^\bullet + O_2$	**(6)**	
$O_3 + HO^\bullet \rightarrow HO_4^\bullet$	**(7)**	**Propagation steps**
$HO_4^\bullet \rightarrow HO_2^\bullet + O_2$	**(8)**	
$HO_2^- + H^+ \leftrightarrow H_2O_2$	**(9)**	
$HO^\bullet + H_2O_2 \rightarrow HO_2^\bullet + H_2O$	**(10)**	
$HO^\bullet + HO_2^- \rightarrow HO_2^\bullet + HO^-$	**(11)**	
$HO^\bullet + O_3 \rightarrow O_3 + HO^-$	**(12)**	
$HO_4^\bullet + HO_4^\bullet \rightarrow H_2O_2^\bullet + 2\ O_3$	**(13)**	**Break**
$HO_4^\bullet + HO_3^\bullet \rightarrow H_2O_2^\bullet + O_3 + O_2$	**(14)**	

Eqs. C1-14: Ozone decomposition chain reactions in aqueous solution - based on Hoigné-Staehelin-Bader (HSB) and Gordon-Tomiyasu-Fukutomi (GTF) mechanisms (Barceló, D. and Petrovic, M., Emerging Contaminants from Industrial and Municipal Waste: Removal technologies, Vol. 5/S/1 of *The Handbook of Environmental Chemistry*, Springer (2008)).

Appendix D

Supplementary Material

D1.

Supplementary material of *Chapter 3*

Table of Contents

Tables

Table D1.1. Classification, chemical structure, CAS number, molecular weight, Log K_{ow} and pK_a of target micropollutants. *Priority substances (Directive 2013/39/EU); *[1,2,3] Contaminants of emerging concern ([1]Decision 2015/495/EU; [2]Decision 2018/840/EU; and [3]Decision 2020/1161/EU); **Other relevant contaminants of emerging concern. n.a. - not available.

Table D1.2. Method detection and quantification limits, respectively MDL and MQL, for each target analyte. *Priority substances (Directive 2013/39/EU); *[1,2,3] Contaminants of emerging concern ([1]Decision 2015/495/EU; [2]Decision 2018/840/EU; and [3]Decision 2020/1161/EU); **Other relevant contaminants of emerging concern.

Table D1.1. Classification, chemical structure, CAS number, molecular weight, Log K_{ow} and pK_a of target micropollutants. *Priority substances (Directive 2013/39/EU); *[1,2,3] Contaminants of emerging concern ([1]Decision 2015/495/EU; [2]Decision 2018/840/EU; and [3]Decision 2020/1161/EU); **Other relevant contaminants of emerging concern. n.a. - not available.

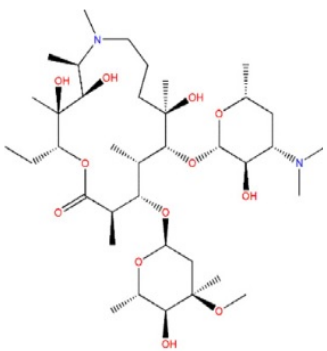
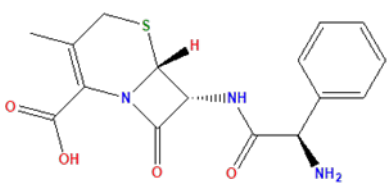
Micropollutants	Chemical Structure	CAS number	Molecular weight (g mol^{-1})	Log K_{ow}	pK_a
Pharmaceuticals					
Antibiotics					
Azithromycin *[1,2]		83905-01-5 [a]	748.996 [a]	4.02 [a]	8.74 [a]
Cefalexin **		15686-71-2 [a]	347.39 [a]	n.a.	4.50 [a]

Table D1.1. (Continued)

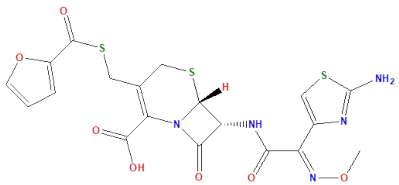
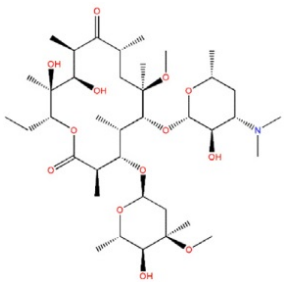
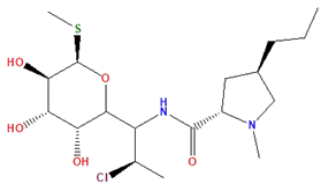
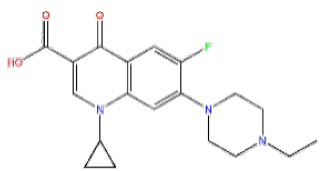
Ceftiofur **		80370-57-6 [a]	523.55 [a]	n.a.	n.a.
Clarithromycin *[1,2]		81103-11-9 [a]	747.96 [a]	3.16 [a]	8.99 [a]
Clindamycin **		18323-44-9 [a]	424.98 [a]	2.16 [a]	7.79 [a]
Enrofloxacin **		93106-60-6 [a]	359.40 [a]	n.a.	2.7-3.9 [b]

Table D1.1. (Continued)

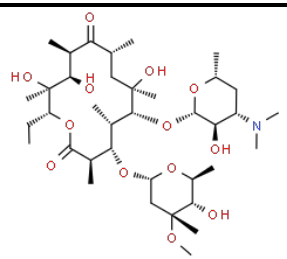
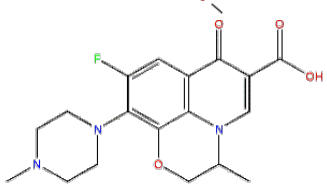
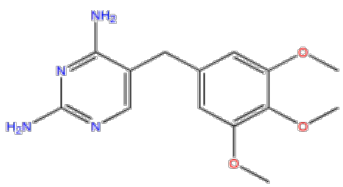
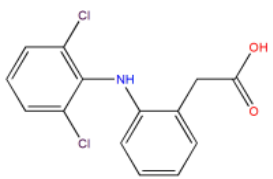
Erythromycin *[1,2]		114-07-8 [a]	733.94 [a]	3.06 [a]	8.90 [a]
Ofloxacin **		82419-36-1 [a]	361.37 [a]	-0.39 [a]	5.97 [a] 9.28 [a]
Trimethoprim *[3]		738-70-5 [a]	290.32 [a]	0.91 [a]	7.12 [a]
Anti-inflammatories					
Diclofenac *[1]		15307-86-5 [a]	296.15 [a]	4.51 [a]	4.15 [a]

Table D1.1. (Continued)

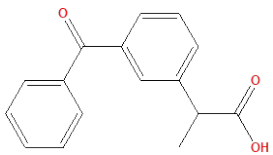
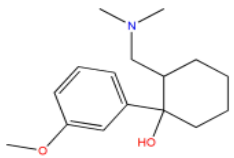
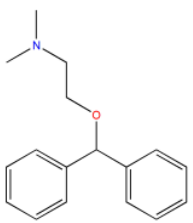
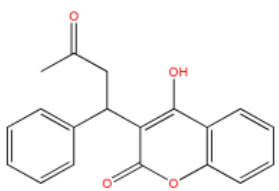
Ketoprofen **		22071-15-4 [a]	254.29 [a]	3.12 [c]	4.45 [a,c]
Tramadol **		27203-92-5 [a]	263.38 [a]	3.01 [a]	9.23 [a] 13.08 [a]
Antihistamine					
Diphenhydramine **		58-73-1 [a]	255.36 [a]	3.27 [a]	8.98 [a]
Anticoagulant					
Warfarin **		81-81-2 [a]	308.33 [a]	2.70 [a]	5.87 [a]

Table D1.1. (Continued)

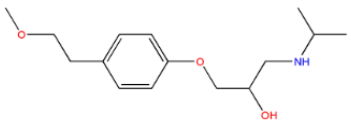
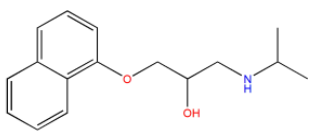
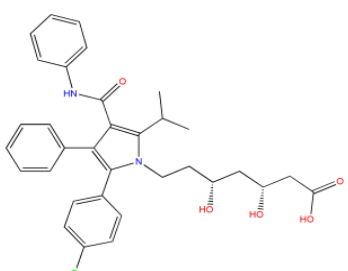
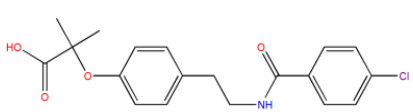
Beta-blockers					
Metoprolol **		37350-58-6 [a]	267.37 [a]	1.88 [a]	9.60 [a]
Propranolol **		525-66-6 [a]	259.35 [a]	2.81 [a]	9.19 [a]
Cholesterol lowering statin drugs					
Atorvastatin **		134523-00-5 [a]	558.65 [a]	6.36 [a]	4.30 [a] 14.90 [a]
Lipid regulator					
Bezafibrate **		41859-67-0 [a]	361.82 [a]	4.25 [d]	3.60 [d]

Table D1.1. (Continued)

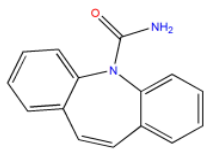
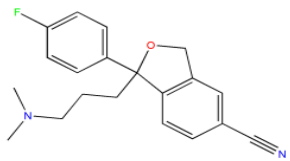
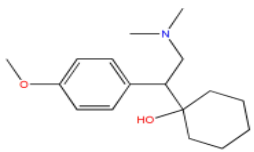
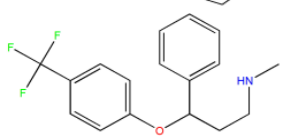
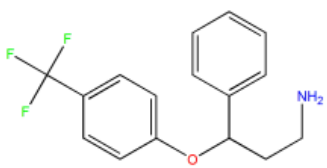
Psychiatric drugs					
Carbamazepine **		298-46-4 [a]	236.27 [a]	2.45 [a]	13.90 [a]
Citalopram **		59729-33-8 [a]	324.40 [a]	3.58 [a]	9.22 [e]
Venlafaxine *[3]		93413-69-5 [a]	277.41 [a]	3.20 [a]	10.09 [a]
Fluoxetine **		54910-89-3 [a]	309.33 [a]	4.05 [f]	10.05 [g]
Metabolite					
Norfluoxetine **		83891-03-6 [a]	295.31 [a]	3.80 [a]	9.05 [g]

Table D1.1. (Continued)

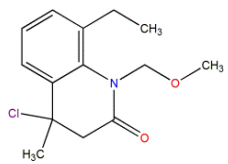
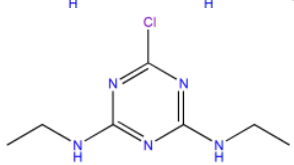
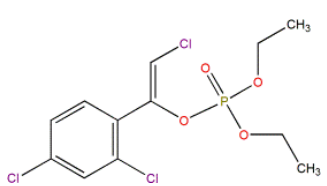
Pesticides					
Chloroacetanilide					
Alachlor *		15972-60-8 [a]	269.77 [a]	3.52 [a]	n.a.
Triazine					
Atrazine *		1912-24-9 [a]	215.69 [a]	2.61 [a]	1.60 [a]
Simazine *		122-34-9 [a]	201.66 [a]	2.18 [a]	1.62 [a]
Organophosphorus					
Chlorfenvinphos *		470-90-6 [a]	359.56 [a]	3.81 [a]	n.a.

Table D1.1. (Continued)

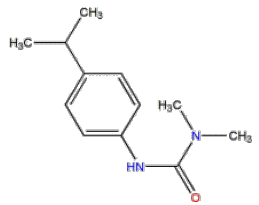
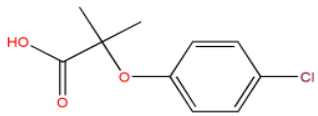
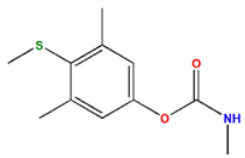
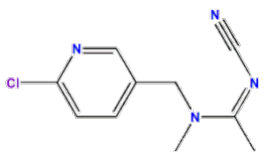
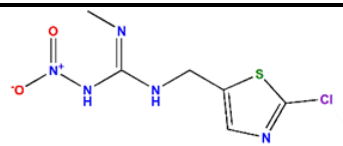
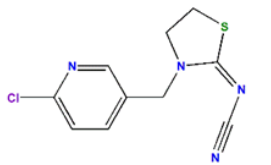
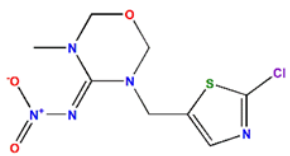
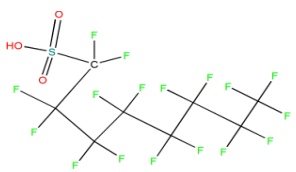
Phenylurea					
Isoproturon *		34123-59-6 [a]	206.29 [a]	2.54 [h]	0.85 [a]
Herbicide					
Clofibric acid **		882-09-7 [a]	214.65 [a]	2.57 [c]	3.18 [c]
Insecticide					
Methiocarb *[1,2]		2032-65-7 [a]	225.30 [a]	2.92 [a]	12.16 [i]
Neonicotinoids					
Acetamiprid *[1]		135410-20-7 [a]	222.68 [a]	0.80 [a]	0.70 [a]

Table D1.1. (Continued)

Clothianidin *[1]		210880-92-5 [a]	249.67 [a]	0.70 [a]	11.09 [a]
Thiacloprid *[1,2]		111988-49-9 [a]	252.72 [a]	1.26 [a]	1.62 [j]
Thiamethoxam *[1,2]		153719-23-4 [a]	291.71 [a]	-0.13 [a]	0.41 [j]
Industrial Compound					
Perfluorooctanesulfonic acid (PFOS) *		1763-23-1 [a]	500.13 [a]	4.49 [a]	< 1.00 [a]

Information from:

[a] National Centre of Biotechnology, US (available at https://pubchem.ncbi.nlm.nih.gov);

[b] Choi, Y-J., *et al.,* Removal characteristics and mechanism of antibiotics using constructed wetlands, Ecological Engineering 91, 85-92 (2016);

[c] Gao, Y., *et al.*, Adsorption of clofibric acid and ketoprofen onto powdered activated carbon: Effect of natural organic matter, Environmental Technology 32, 1719-1727 (2011);

[d] Ternes, T.A., Human pharmaceuticals, hormones and fragances : a challenge for urban water management, IWA Publ., London (2006);

[e] Russo, G., *et al.*, Determination of in Vitro and in Silico Indexes for the Modeling of Blood–Brain Barrier Partitioning of Drugs via Micellar and Immobilized Artificial Membrane Liquid Chromatography, Journal of Medicinal Chemistry 60, 3739-3754 (2017);

[f] Benotti, M.J., *et al.*, Removal of pharmaceuticals and endocrine disrupting compounds through pilot- and full-scale riverbank filtration, Water Science and Technology: Water Supply 12, 11-23 (2012);

[g] European Bioinformatics Institute (EBI), part of Molecular Biology Laboratory (EMBL), UK (available at https://www.ebi.ac.uk/chembl/);

[h] Evelyne, C., *et al*., Quantitative relationships between structure and penetration of phenylurea herbicides through isolated plant cuticles, Chemosphere 24, 189-200 (1992);

[i] Ware, G.W., *et al.*, Reviews of environmental contamination and toxicology, Springer 193, New York (2006);

[j] Toxic Exposome Databse, T_3DB (available at http://www.t3db.ca).

Table D1.2. Method detection and quantification limits, respectively MDL and MQL, for each target analyte. *Priority substances (Directive 2013/39/EU); *[1,2,3] Contaminants of emerging concern ([1]Decision 2015/495/EU; [2]Decision 2018/840/EU; and [3]Decision 2020/1161/EU); **Other relevant contaminants of emerging concern.

Analyte	MDL [a] (ng L^{-1})	MQL [b] (ng L^{-1})
Azithromycin *[1,2]	0.15	0.44
Cefalexin**	0.18	0.56
Ceftiofur **	0.13	0.39
Clarithromycin *[1,2]	0.15	0.39
Clindamycin **	0.16	0.48
Enrofloxacin **	1.58	4.79
Erythromycin *[1,2]	0.13	0.39
Ofloxacin **	7.12	21.58
Trimethoprim *[3]	3.61	10.94
Diclofenac *[1]	1.24	3.75
Ketoprofen **	3.26	9.88
Tramadol **	0.16	0.49
Diphenhydramine **	0.02	0.05
Warfarin **	0.37	1.13
Metoprolol **	0.30	0.90
Propranolol **	1.18	3.57
Atorvastatin **	0.21	0.65
Bezafibrate **	1.50	4.55
Carbamazepine **	0.03	0.08
Citalopram **	4.11	12.44
Venlafaxine *[3]	0.04	0.11
Fluoxetine **	0.10	0.30
Norfluoxetine **	1.61	4.88
Alachlor *	2.87	8.71
Atrazine *	0.05	0.09
Simazine *	0.66	2.01
Chlorfenvinphos *	1.06	3.22
Isoproturon *	0.04	0.13
Clofibric acid **	0.80	2.42
Methiocarb *[1,2]	0.41	1.25
Acetamiprid *[1]	1.09	3.30
Clothianidin *[1]	0.21	0.65
Thiacloprid *[1,2]	0.21	0.64
Thiamethoxam *[1,2]	1.68	5.10
Perfluorooctanesulfonic acid (PFOS) *	0.91	2.77

[a] MDL is method detection limit; [b] MQL is method quantification limit.

D2.

Supplementary material of *Chapter 4*

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Tables

Table D2.1. Class of the target micropollutants, chemical structure, relative molecular mass (M_r), Log $K_{ow,}$ pK_a, precursor ion (m/z), product ion (m/z) for quantification by selected reaction monitoring, and *presence or absence of suppression in the LC-MS/MS, after addition of Na_2SO_3 to quench H_2O_2.

Table D2.2 Retention time, range, method detection (MDL) and quantification (MQL) limits and recovery for each target analyte.* Suppression effect in LC-MS/MS.

Figures

Fig. D2.1. Selected ion chromatogram of metoprolol in a spiked (100 ng L^{-1}) surface water sample **(a)**, after adding the H_2O_2 quencher catalase **(b)**, without analytical interference, and after adding the H_2O_2 quencher Na_2SO_3 **(c)**, with analytical interference.

Table D2.1 Class of the target micropollutants, chemical structure, relative molecular mass (M_r), Log K_{ow}, pK_a, precursor ion (m/z), product ion (m/z) for quantification by selected reaction monitoring, and *presence or absence of suppression in the LC-MS/MS, after addition of Na_2SO_3 to quench H_2O_2.

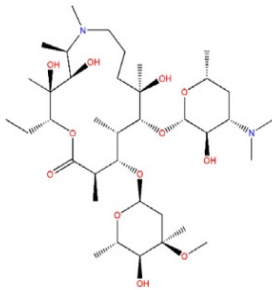
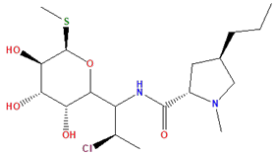
Micropollutant	Chemical Structure	M_r	Log K_{ow}	pK_a	Precursor ion (m/z)	1st product ion	Suppression effect in LC-MS/MS*
Pharmaceuticals							
Antibiotics							
Azithromycin		748.51[a]	4.02[a]	8.74[a]	749.50$^+$	83.15	Yes
Clindamycin		424.98[a]	2.16[a]	7.79[a]	425.00$^+$	126.15	Yes

Table D2.1. (Continued)

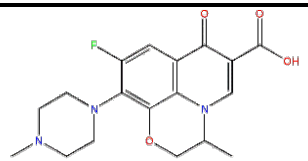
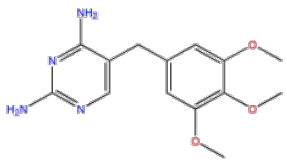
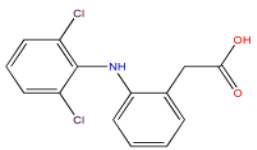
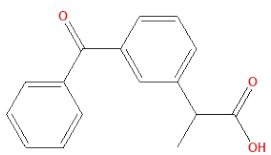
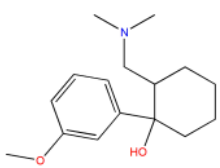
Ofloxacin		361.37 [a]	-0.39 [a]	5.97 [a] 9.28 [a]	361.90^{+}	318.20	Yes
Trimethoprim		290.32 [a]	0.91 [a]	7.12 [a]	290.80^{+}	230.00	Yes
Anti-inflammatories							
Diclofenac		296.15 [a]	4.51 [a]	4.15 [a]	294.00^{-}	250.00	No
Ketoprofen		254.29 [a]	3.12 [c]	4.45 [a,c]	253.20^{-}	209.15	No
Analgesic							
Tramadol		263.38 [a]	3.01 [a]	9.23 [a] 13.08 [a]	264.00^{+}	57.70	Yes

Table D2.1. (Continued)

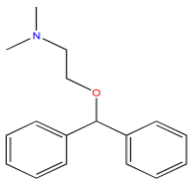
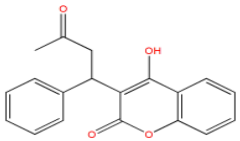
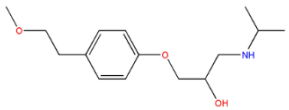
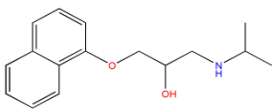
Antihistamine							
Diphenhydramine		255.36 [a]	3.27 [a]	8.98 [a]	255.80^{+}	167.00	Yes
Anticoagulant							
Warfarin		308.33 [a]	2.70 [a]	5.87 [a]	309.10^{+}	163.00	No
Beta-blockers							
Metoprolol		267.37 [a]	1.88 [a]	9.60 [a]	267.80^{+}	116.15	Yes
Propranolol		259.35 [a]	2.81 [a]	9.19 [a]	259.70^{+}	116.10	Yes

Table D2.1. (Continued)

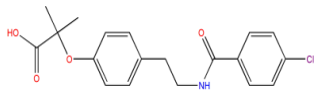
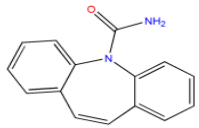
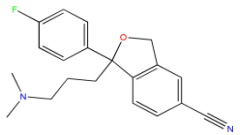
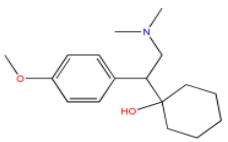
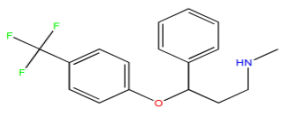
Lipid regulator							
Bezafibrate		361.82 [a]	4.25 [d]	3.60 [d]	360.20^{-}	274.15	No
Psychiatric drugs							
Carbamazepine		236.27 [a]	2.45 [a]	13.90 [a]	236.90^{+}	194.10	Yes
Citalopram		324.40 [a]	3.58 [a]	9.22 [e]	324.50^{+}	109.10	Yes
Venlafaxine		277.41 [a]	3.20 [a]	10.09 [a]	277.80^{+}	58.10	Yes
Fluoxetine		309.33 [a]	4.05 [f]	10.05 [g]	310.00^{+}	44.15	Yes

Table D2.1. (Continued)

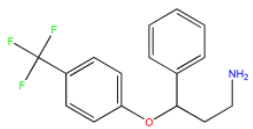
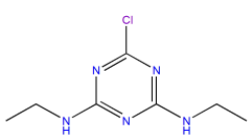
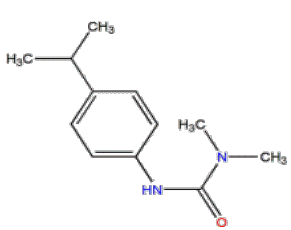
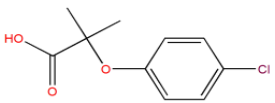
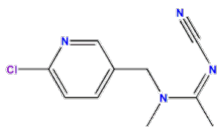
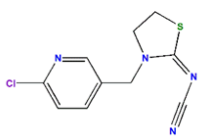
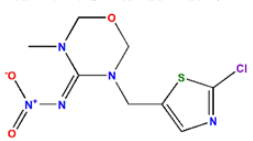
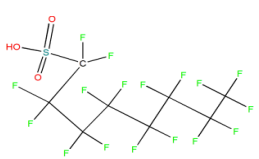
Metabolite							
Norfluoxetine		295.31 [a]	3.80 [a]	9.05 [g]	296.00^{+}	134.15	Yes
Pesticides							
Triazine							
Atrazine		215.69 [a]	2.61 [a]	1.60 [a]	215.90^{+}	174.05	No
Simazine		201.66 [a]	2.18 [a]	1.62 [a]	201.90^{+}	124.10	No
Phenylurea							
Isoproturon		206.29 [a]	2.54 [h]	0.85 [a]	206.90^{+}	72.10	No

Table D2.1. (Continued)

Herbicide							
Clofibric acid		214.65 [a]	2.57 [c]	3.18 [c]	213.10^{-}	127.00	No
Neonicotinoids							
Acetamiprid		222.68 [a]	0.80 [a]	0.70 [a]	222.70^{+}	126.00	No
Thiacloprid		252.72 [a]	1.26 [a]	1.62 [i]	252.90^{+}	126.00	No
Thiamethoxam		291.71 [a]	-0.13 [a]	0.41 [i]	291.90^{+}	211.10	No
Industrial Compound							
Perfluorooctanesulfonic acid (PFOS)		500.13 [a]	4.49 [a]	< 1.00 [a]	498.70^{-}	79.95	No

Information from:

[a] National Centre of Biotechnology, US (available at https://pubchem.ncbi.nlm.nih.gov);

[b] Choi, Y-J., *et al*., Removal characteristics and mechanism of antibiotics using constructed wetlands, Ecological Engineering 91, 85-92 (2016);

c Gao, Y., *et al.*, Adsorption of clofibric acid and ketoprofen onto powdered activated carbon: Effect of natural organic matter, Environmental Technology 32, 1719-1727 (2011);

d Ternes, T.A., Human pharmaceuticals, hormones and fragrances : a challenge for urban water management, IWA Publ., London (2006);

e Russo, G., *et al.*,Determination of in Vitro and in Silico Indexes for the Modelling of Blood–Brain Barrier Partitioning of Drugs via Micellar and Immobilized Artificial Membrane Liquid Chromatography, Journal of Medicinal Chemistry 60, 3739-3754 (2017);

f Benotti, M.J., *et al*., Removal of pharmaceuticals and endocrine disrupting compounds through pilot- and full-scale riverbank filtration, Water Science and Technology: Water Supply 12, 11-23 (2012);

g European Bioinformatics Institute (EBI), part of Molecular Biology Laboratory (EMBL), UK (available at https://www.ebi.ac.uk/chembl/);

hEvelyne, C., *et al.*, Quantitative relationships between structure and penetration of phenylurea herbicides through isolated plant cuticles, Chemosphere 24, 189-200 (1992);

i Toxic Exposome Databse, T3DB (available at http://www.t3db.ca).

Table D2.2. Retention time, range, method detection (MDL) and quantification (MQL) limits and recovery for each target analyte.* Suppression effect in LC-MS/MS.

Analyte	Retention time (min)	Range (ng L^{-1})	MDL[a] (ng L^{-1})	MQL[b] (ng L^{-1})	Recovery (%)
Acetamiprid	0.921	3.30 – 100	1.09	3.30	n.d.
Atrazine	1.470	0.14 – 100	0.05	0.14	67
Azithromycin *	0.945	0.44 – 100	0.15	0.44	45
Bezafibrate	1.795	4.55 – 100	1.50	4.55	40
Carbamazepine *	1.240	0.08 – 100	0.03	0.08	102
Citalopram *	0.774	12.44 – 100	4.11	12.44	42
Clarithromycin *	1.047	0.39 – 100	0.15	0.39	67
Clindamycin *	0.876	0.48 – 100	0.16	0.48	67
Clofibric acid	1.825	2.42 – 100	0.80	2.42	68
Diclofenac	2.716	3.75 – 100	1.24	3.75	77
Diphenhydramine *	0.836	0.05 – 100	0.02	0.05	42
Fluoxetine *	0.876	0.30 – 100	0.10	0.30	35
Isoproturon	1.472	0.13 – 100	0.04	0.13	72
Ketoprofen	1.642	9.88 – 100	3.26	9.88	50
Metoprolol *	0.700	0.90 – 100	0.30	0.90	56
Norfluoxetine *	0.838	4.88 – 100	1.61	4.88	22
Ofloxacin *	0.834	21.58 – 100	7.12	21.58	11
PFOS	3.941	2.77 – 100	0.91	2.77	82
Propranolol *	0.829	3.57 – 100	1.18	3.57	52
Simazine	1.217	2.01 – 100	0.66	2.01	70
Thiacloprid	0.918	0.64 – 100	0.21	0.64	48
Thiamethoxam	0.862	5.10 – 100	1.68	5.10	59
Tramadol *	0.698	0.49 – 100	0.16	0.49	82
Trimethoprim *	0.801	10.94 – 100	3.61	10.94	72
Venlafaxine *	0.788	0.11 – 100	0.04	0.11	93
Warfarin	1.875	1.13 – 100	0.37	1.13	105

[a] MDL is method detection limit; [b] MQL is method quantification limit.

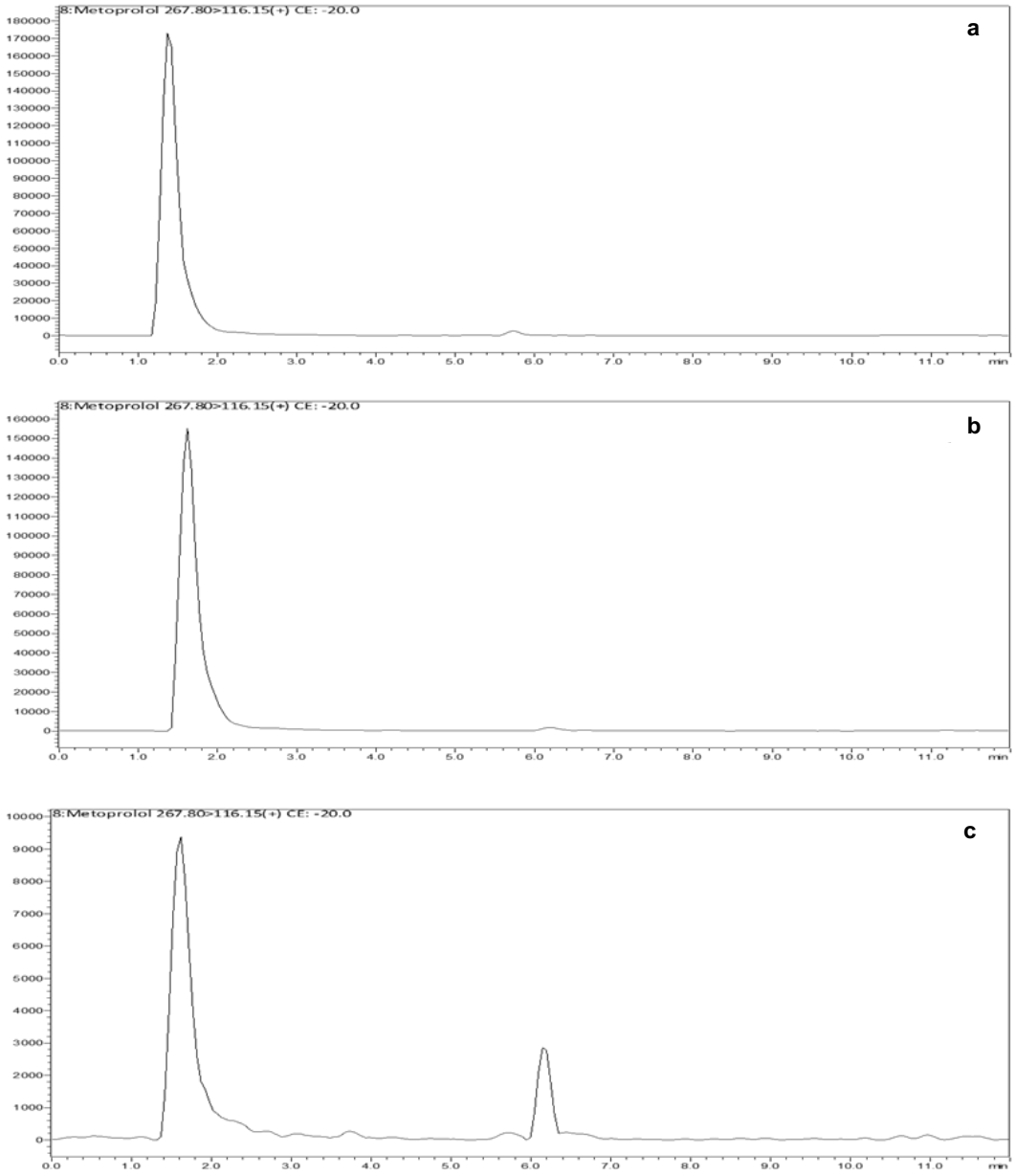


Figure C2.1. Selected ion chromatogram of metoprolol in a spiked (100 ng L^{-1}) surface water sample **(a)**, after adding the H_2O_2 quencher catalase **(b)**, without analytical interference, and after adding the H_2O_2 quencher Na_2SO_3 **(c)**, with analytical interference.

D3.

Supplementary material of *Chapter 5*

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Tables

Table D3.1. Target compounds, class and sub class, chemical structure, molecular weight, Log K_{ow} and pK_a. *Priority substances (Directive 2013/39/EU); *[1,2,3] Contaminants of emerging concern ([1]Decision 2015/495/EU; [2]Decision 2018/840/EU; and [3]Decision 2020/1161/EU); **Other relevant contaminants of emerging concern. n.a. - not available.

Table D3.2. Physico-chemical characterization of the collected SW (values of pH, dissolved organic/inorganic carbon (DOC/DIC), ions and iron dissolved concentrations, as the average of 5 independent samples).

Table D3.3. Retention time, range, method detection and quantification limits (MDL and MQL, respectively) for each target analyte. *Priority substances (Directive 2013/39/EU); *[1,2,3] Contaminants of emerging concern ([1]Decision 2015/495/EU; [2]Decision 2018/840/EU; and [3]Decision 2020/1161/EU); **Other relevant contaminants of emerging concern.

Table D3.1. Target compounds, class and sub class, chemical structure, molecular weight, Log K_{ow} and pK_a. *Priority substances (Directive 2013/39/EU); *[1,2,3] Contaminants of emerging concern ([1]Decision 2015/495/EU; [2]Decision 2018/840/EU; and [3]Decision 2020/1161/EU); **Other relevant contaminants of emerging concern. n.a. - not available.

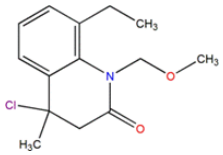
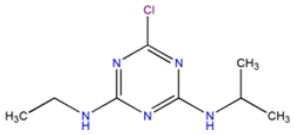
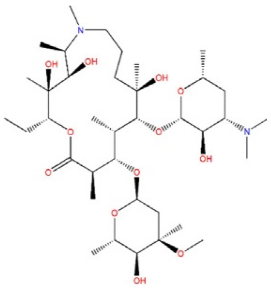
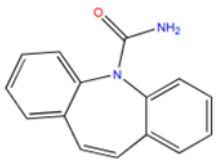
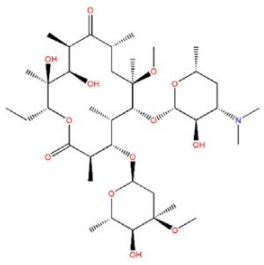
Compound	Class and sub class	Chemical Structure	Molecular weight (g mol^{-1})	Log K_{ow}	pK_a
Alachlor *	Pesticide *Chloroacetanilide*		269.77 [a]	3.52 [a]	n.a.
Atrazine *	Pesticide *Triazine*		215.69 [a]	2.61 [a]	1.60 [a]
Azithromycin *[1,2]	Pharmaceutical *Antibiotic*		748.51 [a]	4.02 [a]	8.74 [a]
Carbamazepine **	Pharmaceutical *Psychiatric drug*		236.27 [a]	2.45 [a]	13.90 [a]
Clarithromycin *[1,2]	Pharmaceutical *Antibiotic*		747.96 [a]	3.16 [a]	8.99 [a]

Table D3.1. (Continued)

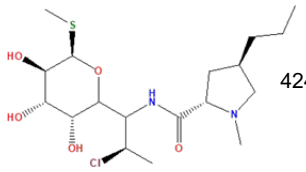
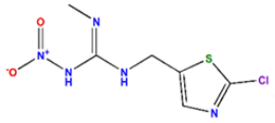
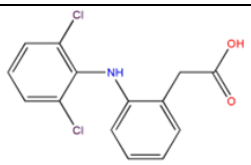
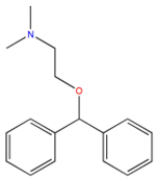
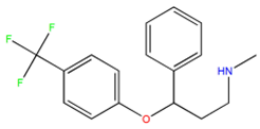
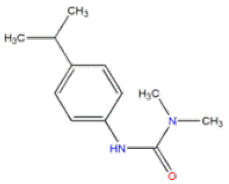
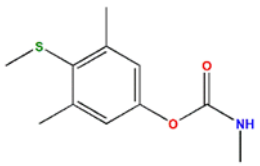
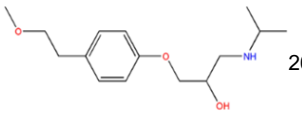
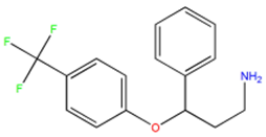
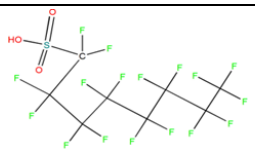
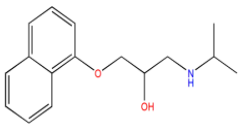
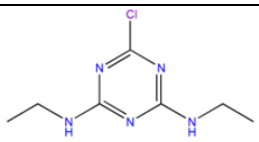
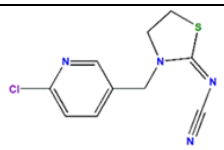
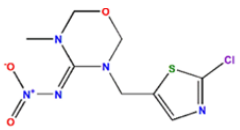
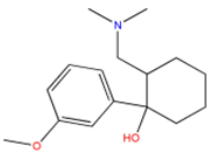
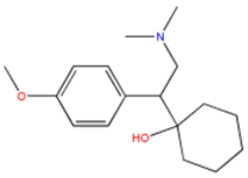
Clindamycin **	Pharmaceutical *Antibiotic*		424.98 [a]	2.16 [a]	7.79 [a]
Clothianidin *[1]	Pesticide *Neonicotinoid*		249.67 [a]	0.70 [a]	11.09 [a]
Diclofenac *[1]	Pharmaceutical *Anti-inflammatory*		296.15 [a]	4.51 [a]	4.15 [a]
Diphenhydramine **	Pharmaceutical *Antihistamine*		255.36 [a]	3.27 [a]	8.98 [a]
Fluoxetine **	Pharmaceutical *Psychiatric drug*		309.33 [a]	4.05 [b]	10.05 [c]
Isoproturon *	Pesticide *Phenylurea*		206.29 [a]	2.54 [e]	0.85 [a]
Methiocarb *[1,2]	Pesticide *Insecticide*		225.30 [a]	2.92 [a]	12.16 [e]
Metoprolol **	Pharmaceutical *Beta-blocker*		267.37 [a]	1.88 [a]	9.60 [a]

Table D3.1. (Continued)

Norfluoxetine **	Pharmaceutical *Metabolite*		295.31 [a]	3.80 [a]	9.05 [c]
Perfluorooctanesulfonic acid (PFOS) *	Industrial compound		500.13 [a]	4.49 [a]	<1.00 [a]
Propranolol **	Pharmaceutical *Beta-blocker*		259.35 [a]	2.81 [a]	9.19 [a]
Simazine *	Pesticide *Triazine*		201.66 [a]	2.18 [a]	1.62 [a]
Thiacloprid *[1,2]	Pesticide *Neonicotinoid*		525.72 [a]	1.26 [a]	1.62 [a]
Thiamethoxam*[1,2]	Pesticide *Neonicotinoid*		291.71 [a]	-0.13 [a]	0.41 [f]
Tramadol **	Pharmaceutical *Analgesic*		263.38 [a]	3.01 [a]	9.23 [a] 13.08 [a]
Venlafaxine *[3]	Pharmaceutical *Psychiatric drug*		277.41 [a]	3.20 [a]	10.09 [a]

Information from:

[a] National Centre of Biotechnology, US (available at https://pubchem.ncbi.nlm.nih.gov);

[b] Benotti, M.J., *et al*., Removal of pharmaceuticals and endocrine disrupting compounds through pilot- and full-scale riverbank filtration, Water Science and Technology: Water Supply 12, 11-23 (2012);

[c] European Bioinformatics Institute (EBI), part of Molecular Biology Laboratory (EMBL), UK (available at https://www.ebi.ac.uk/chembl/);

[d] Evelyne, C., *et al.*, Quantitative relationships between structure and penetration of phenylurea herbicides through isolated plant cuticles, Chemosphere 24, 189-200 (1992);

[e] Ware, G.W., *et al*., Reviews of environmental contamination and toxicology. Volume 193. Springer, New York (2006);

[f] Toxic Exposome Databse, T_3DB (available at http://www.t3db.ca).

Table D3.2. Physico-chemical characterization of the collected SW (values of pH, dissolved organic/inorganic carbon (DOC/DIC), ions and iron dissolved concentrations, as the average of 5 independent samples).

Parameters	
pH	8.3 ± 0.3
DOC (mg L^{-1})	3.52 ± 0.94
DIC (mg L^{-1})	14.88 ± 3.97
Chlorides (mg Cl^- L^{-1})	20.13 ± 3.83
Sulfates (mg SO_4^{2-} L^{-1})	20.34 ± 4.41
Phosphates (mg PO_4^{3-} L^{-1})	< MDL (0.32) [a]
Nitrites (mg NO_2^- L^{-1})	< MDL (0.3) [a]
Nitrates (mg NO_3^- L^{-1})	2.45 ± 0.71
Bromides (mg Br^- L^{-1})	0.29 ± 0.07
Bromates (mg BrO_3^- L^{-1})	< MDL (7.6×10^{-2}) [a]
Sodium (mg Na^+ L^{-1})	17.67 ± 3.21
Ammonium (mg NH_4^+ L^{-1})	< MDL (1.5×10^{-2}) [a]
Potassium (mg K^+ L^{-1})	2.42 ± 0.42
Magnesium (mg Mg^{2+} L^{-1})	5.93 ± 1.98
Calcium (mg Ca^{2+} L^{-1})	31.5 ± 3.3

[a] MDL is method detection limit

Table D3.3. Retention time, range, method detection and quantification limits (MDL and MQL, respectively) for each target analyte. *Priority substances (Directive 2013/39/EU); *[1,2,3] Contaminants of emerging concern ([1]Decision 2015/495/EU; [2]Decision 2018/840/EU; and [3]Decision 2020/1161/EU); **Other relevant contaminants of emerging concern.

Analyte	Retention time (min)	Range (ng L^{-1})	MDL [a] (ng L^{-1})	MQL [b] (ng L^{-1})
Alachlor *	15.88	0.40 – 150	0.13	0.40
Atrazine *	11.43	0.51 – 200	0.17	0.51
Azithromycin *[1,2]	10.28	1.55 – 100	0.51	1.55
Carbamazepine **	10.42	0.13 – 200	0.04	0.13
Clarithromycin *[1,2]	10.27	0.68 – 75	0.23	0.68
Clindamycin **	6.66	0.27 – 75	0.09	0.27
Clothianidin *[1]	5.10	9.50 – 75	3.13	9.50
Diclofenac *[1]	15.37	2.10 – 75	0.69	2.10
Diphenhydramine **	8.03	0.15 – 75	0.05	0.15
Fluoxetine **	10.14	0.11 – 20	0.04	0.11
Isoproturon *	11.97	0.24 – 75	0.08	0.24
Methiocarb *[1,2]	13.86	15.6 – 75	5.15	15.6
Metoprolol **	2.92	6.15 – 75	2.03	6.15
Norfluoxetine **	9.90	3.62 – 30	1.19	3.62
PFOS *	22.34	0.89 – 75	0.29	0.89
Propranolol **	7.28	0.40 – 75	0.13	0.4
Simazine *	9.30	0.15 – 75	0.05	0.15
Thiacloprid *[1,2]	8.52	2.91 – 75	0.96	2.91
Thiamethoxam *[1,2]	3.54	15.06 – 50	4.97	15.06
Tramadol **	2.97	5.32 – 75	1.76	5.32
Venlafaxine *[3]	6.33	0.48 – 75	0.16	0.48

[a] MDL is method detection limit; [b] MQL is method quantification limit.

D4.

Supplementary material of *Chapter 6*

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Table D4.1. Physico-chemical characterization of the collected aquaculture effluent: pH, conductivity, chemical (COD) and biochemical (BOD) oxygen demand, total suspended (TSS) and dissolved (TDS) solids, dissolved organic carbon (DOC), and ions concentrations.

Table D4.2. Concentrations of target antibiotics in collected aquaculture effluent.

Table D4.3. Recovery, accuracy, precision (intra- and inter-batch) and matrix effect for each target analyte.

Table D4.4. Selected reaction monitoring (SRM) instrument parameters for tandem mass spectrometry analysis of target analytes.

Table D4.5. Retention time, range, linearity, instrument and method detection and quantification limits for each target analyte.

Figures

Fig. D4.1. Normalized concentration of florfenicol (FF) **(a)** and sulfadimethoxine (SDM) **(b)** during ozonation in the presence of other antibiotics (batch experiments; UPW; without scavenger; 10

mg L^{-1} of each antibiotic). OTC (oxytetracycline); SMX (sulfamethoxazole); and TMP (trimethoprim).

Fig. D4.2. Concentrations of bromides and bromates in aquaculture effluent before and after ozonation.

Fig. D4.3. Chromatograms suggesting the formation of florfenicol by-products during ozonation (red circles): **(1)** initial samples; **(2)** treated samples; **(a)** continuous-flow experiments; and **(b, c)** batch experiments. In continuous-flow experiments **(a)**, all antibiotics were contained in spiked aquaculture effluents, while in batch experiments florfenicol was analysed alone **(b)** and in the presence of all other analytes **(c)** in spiked UPW.

Table D4.1. Physico-chemical characterization of the collected aquaculture effluent: pH, conductivity, chemical (COD) and biochemical (BOD) oxygen demand, total suspended (TSS) and dissolved (TDS) solids, dissolved organic carbon (DOC), and ions concentrations.

Parameter	
pH	6.4
Conductivity (μS/cm)	80
COD (mg/L)	< MDL (3.5) [a]
BOD (mg/L)	0.0
TSS (mg/L)	6.0
TDS (mg/L)	52.4
DOC (mg L^{-1})	1.9
Chlorides (mg Cl^{-} L^{-1})	6.3 ± 0.9
Sulfates (mg SO_4^{2-} L^{-1})	1.1 ± 0.1
Phosphates (mg PO_4^{3-} L^{-1})	3.2 ± 1.6
Nitrites (mg NO_2^{-} L^{-1})	< MDL (0.3) [a]
Nitrates (mg NO_3^{-} L^{-1})	3.8 ± 0.3
Bromides (mg Br^{-} L^{-1})	(5.0 ± 0.5) x 10^{-3}
Bromates (mg BrO_3^{-} L^{-1})	< MDL (0.3 x 10^{-3}) [a]
Sodium (mg Na^{+} L^{-1})	6.3 ± 0.5
Ammonium (mg NH_4^{+} L^{-1})	< MDL (1.5 x 10^{-2}) [a]
Potassium (mg K^{+} L^{-1})	1.3 ± 0.9
Magnesium (mg Mg^{2+} L^{-1})	< MQL (0.4) [b]
Calcium (mg Ca^{2+} L^{-1})	0.2 ± 0.1

[a] MDL is method detection limit; [b] MQL is method quantification limit.

Table D4.2. Concentration of target antibiotics in collected aquaculture effluent.

Antibiotic	Concentration (ng/L)
Trimethoprim	2.92 ± 0.02
Sulfadimethoxine	< MQL (2.71) [b]
Oxytetracycline	< MDL (2.26) [a]
Florfenicol	57.5 ± 0.2
Sulfamethoxazole	< MDL (5.08) [a]

[a] MDL is method detection limit; [b] MQL is method quantification limit.

Table D4 3. Recovery, accuracy, precision (intra- and inter-batch) and matrix effect for each target analyte.

Analyte	Recovery (%)	Accuracy (%)	Intra-batch precision RSD (%)	Inter-batch precision RSD (%)	Matrix effect (%)	Process efficiency (%)
Florfenicol	76.7	99.4 ± 3.1	3.5 – 4.0	5.6	-72.0	21.5
Oxytetracycline	129.3	98.3 ± 9.8	15.3 – 16.0	18.0	-42.2	74.8
Sulfadimethoxine	73.1	88.8 ± 5.4	4.6 – 5.8	7.5	-9.7	66.0
Sulfamethoxazole	96.4	96.1 ± 8.0	7.1 – 8.3	11.0	-57.7	40.8
Trimethoprim	80.6	91.8 ± 4.3	4.3 – 5.3	6.8	-18.6	65.7

Table D4.4. Selected reaction monitoring (SRM) instrument parameters for tandem mass spectrometry analysis of target analytes.

Analyte	IS set [a]	ESI mode (NI [b] or PI [c])	Precursor ion (m/z)	Quantification (SRM1)				Confirmation (SRM2)			
				Product Ion	DP [d] (V)	CE [e] (V)	CXP [f] (V)	Product Ion	DP [d] (V)	CE [e] (V)	CXP [f] (V)
Florfenicol	1	NI	356.00	336.05	13.	10	24	185.10	13	10	24
Oxytetracycline	1	PI	461.15	426.05	-22	-21	-29	200.90	-22	-41	-18
Sulfadimethoxine	1	PI	331.10	156.00	-15	-23	-29	91.95	-15	-35	-15
Sulfamethoxazole	1	NI	251.95	156.05	18	15	28	-	-	-	-
Trimethoprim	1	PI	290.80	230.10	-24	-25	-23	123.10	-24	-30	-20
Sulfadimethoxine-*d6* (1)	-	PI	317.07	92.05	-16	-36	-30	-	-	-	-

[a] IS is internal standard; [b] NI is negative ionization mode; [c] PI is positive ionization mode; [d] DP is the declustering potential; [e] CE is the collision energy; [f] CXP is the collision cell exit potential.

Table D4.5. Retention time, range, linearity, instrument and method detection and quantification limits for each target analyte.

Analyte	Retention time (min)	Range (ng L^{-1})	r^2	IDL[a] (µg L^{-1})	IQL[b] (µg L^{-1})	MDL[c] (ng L^{-1})	MQL[d] (ng L^{-1})
Florfenicol	7.754	3.05 – 500	0.997	18.72	56.71	1.01	3.05
Oxytetracycline	6.358	6.84 – 500	0.995	12.06	36.57	2.26	6.84
Sulfadimethoxine	12.512	2.71-200	0.999	5.41	16.41	0.89	2.71
Sulfamethoxazole	8.045	15.41 – 200	0.996	49.90	151.28	5.08	15.41
Trimethoprim	4.140	0.87 – 200	0.994	0.57	5.28	0.29	0.87

[a] IDL is instrument detection limit; [b] IQL is instrument quantification limit; [c] MDL is method detection limit; [d] MQL is method quantification limit.

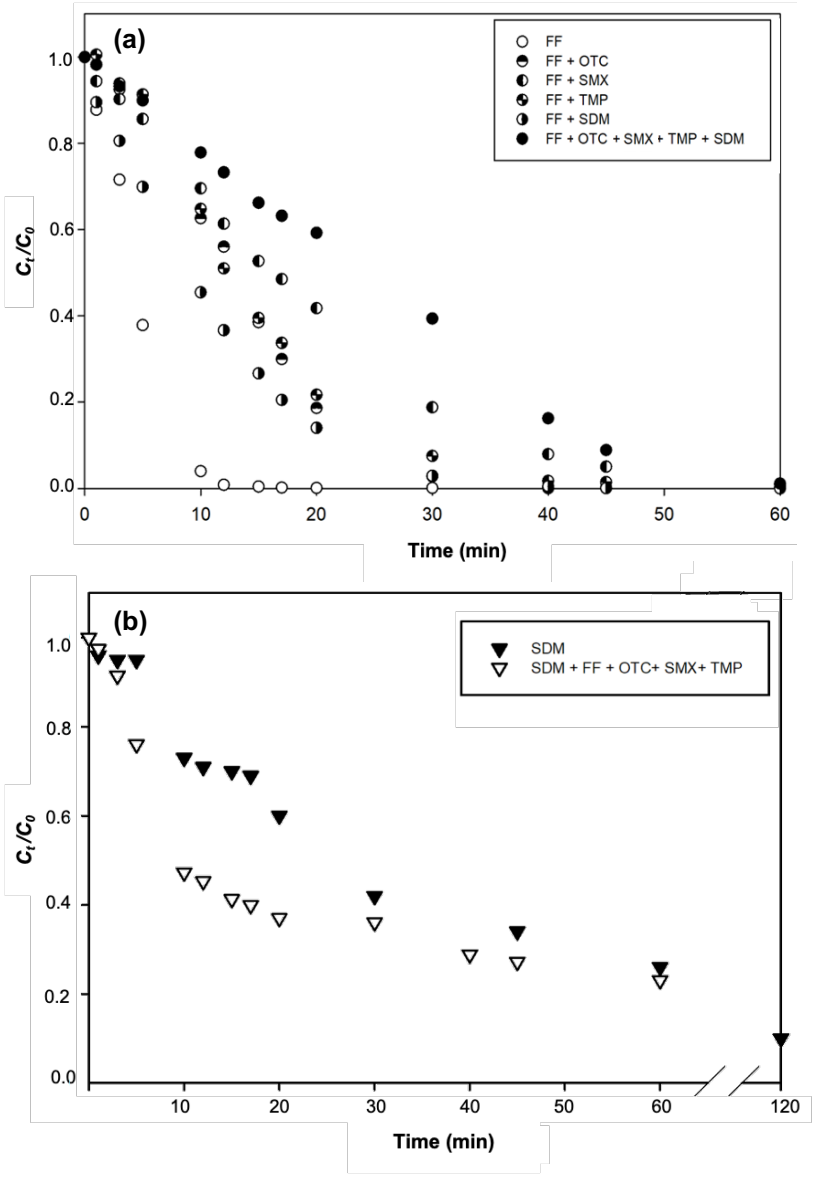

Fig D4.1. Normalized concentration of florfenicol (FF) **(a)** and sulfadimethoxine (SDM) **(b)** during ozonation in the presence of other antibiotics (batch experiments; UPW; without scavenger; 10 mg L^{-1} of each antibiotic). OTC (oxytetracycline); SMX (sulfamethoxazole); and TMP (trimethoprim).

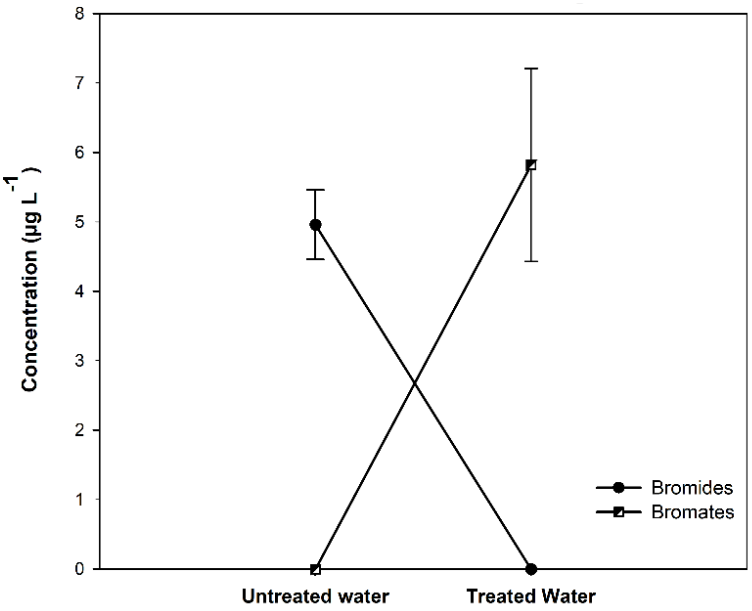

Fig D4.2. Concentrations of bromides and bromates in aquaculture effluent before and after ozonation.

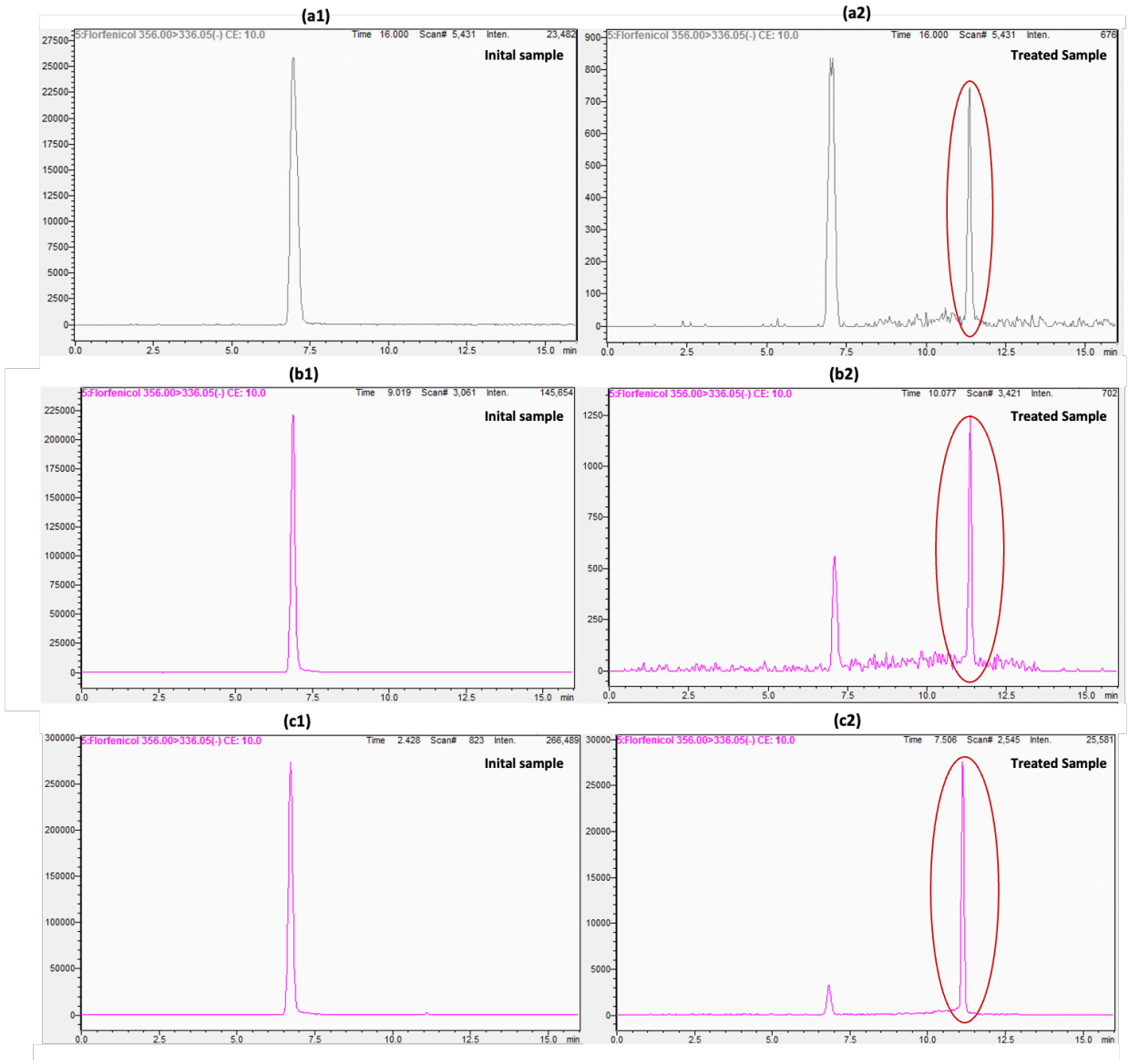

Fig D4.3. Chromatograms suggesting the formation of florfenicol by-products during ozonation (red circles): **(1)** initial sample; **(2)** treated sample; **(a)** continuous-flow experiments; and **(b, c)** batch experiments. In continuous-flow experiments **(a),** all antibiotics were contained in spiked aquaculture effluents, while in batch experiments florfenicol was analysed alone **(b)** and in the presence of all other analytes **(c)** in spiked UPW.