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ADVANCED WASTEWATER TREATMENT: ORGANIC MICROPOLLUTANTS, HUMAN PATHOGENS AND ANTIBIOTIC RESISTANCE GENES

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Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes

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Some additional acknowledgments are included in the respective PDF version of each research article (Appendix A – D) and respective chapter.

Statement of originality

I certify that this work does not contain any material that has been used nor will be 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.

Nuno Filipe Figueiredo Moreira

Nuno Filipe Figueiredo Moreira

Statement

In order to fulfil the Rules of Ethics of the Doctoral Program of Chemical and Biological Engineering (PDEQB), we hereby declare that all the contents of the thesis presented by Nuno Filipe Figueiredo Moreira, entitled “Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes”, are exclusively from the author 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 F.

“Só sei que nada sei por completo

Só sei que nada sei que só eu saiba

Só sei que nada sei que eu não possa vir a saber

Só sei que nada sei que outra pessoa não saiba

Só sei que nada sei que eu e outra pessoa não saibamos juntos”

- Mário Sergio Cortella -

À Marta
À minha irmã
À minha família

Preface

It should come as no surprise that the growing use of the expression *water safety* reflects the growing concerns over how society is getting enlightened regarding the risks of poor water quality, due to increasing levels of chemical and biological pollution, drinking water scarcity and limited sanitation coverage.

As a young researcher, during the last six years, I had the opportunity to contribute with new knowledge on methods to improve water quality, which might help future researchers and water professionals. Everything started during my Master thesis, on the subject of urban wastewater treatment for the removal of chemical and biological pollutants by advanced oxidation technologies. Finished this period, I was involved in the project “NEPCAT” that was focused on the development of new catalytic advanced technologies for water treatment, which led to the promising preliminary results that worked as the driving force of the hereby presented results.

This PhD thesis was carried out under the Doctoral Program in Chemical and Biological Engineering (PDEQB - *Programa Doutoral em Engenharia Química e Biológica*) at the Faculty of Engineering of the University of Porto (FEUP), and under the supervision of Professor Adrián M.T. Silva, Professor Olga C.P. Nunes and Professor Manuel F.R. Pereira, from February 2016 to February 2020. Most of the experimental work was performed at the Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials (Associate Laboratory LSRE-LCM) and the Laboratory for Process Engineering, Environment, Biotechnology and Energy (LEPABE), both located at the Department of Chemical Engineering at FEUP. Part of the work was performed at the Associate Laboratory Centre of Biotechnology and Fine Chemistry - Catholic University of Portugal, and at *Plataforma Solar de Almeria* (CIEMAT), Spain.

It is urgent to make people aware of the repercussions that our actions will have in the future, therefore I am not writing just for the present, but also for the future. That said, the writing took time, reflection, apprehension and concern, but left written a work that should be shared with everyone.

Nuno Moreira

Porto, July 2020

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Agradeço à minha família, em particular aos meus pais e avós, que direta ou indiretamente foram cruciais no meu percurso académico e sem os quais não me tornaria na pessoa que sou hoje.

Um agradecimento muito especial à minha irmã, que me apoiou e ajudou ao longo destes anos e que esteve sempre ao meu lado nos momentos mais difíceis.

Marta, não tenho como te agradecer. Fizeste este percurso sempre a meu lado, usando todas as forças que tinhas para me ajudar a erguer sempre que eu caía. Obrigado por toda a paciência e pela resiliência perante a minha ausência em muitos momentos ao longos destes anos. És a minha inspiração.

Esta caminhada foi dura, mas teria sido muito pior sem a ajuda de todos vocês. Um muito obrigado a todos. Não há nada mais belo do que ajudar e ser ajudado, e espero que o futuro continue assim, pois somos capazes de ir muito longe se pensarmos sempre, nem que seja por um momento, no próximo.

Abstract

Water scarcity and water quality are current concerns in numerous regions around the globe, and water/wastewater treatment and/or reuse practices are of major relevance nowadays. A single water source can serve many people, and bad control of the water quality may lead to serious public health problems and negative effects on the equilibrium of the ecosystems.

National and European legislations, such as the Portuguese Decree-Law No. 152/97 of 19 June 1997 and the European Council Directive 91/271/EEC of 21 May 1991, states that urban wastewater (UWW) entering the collecting systems must be subject to secondary treatment or an equivalent treatment before discharge to the environment. Limits have been established for some parameters such as biochemical oxygen demand without nitrification, chemical oxygen demand and total suspended solids. Other contaminants, including organic micropollutants (OMPs) and potentially harmful microorganisms (e.g., human pathogens, antibiotic resistant bacteria and antibiotic resistance genes - ARB&ARGs), are not yet considered in part due to analytical limitations. However, the low efficiency on the removal of these pollutants by conventional processes urges the development and implementation of more advanced technologies for proper wastewater reuse or discharge to the environment, and to fulfil the quality standards that are expected to be included in future legislations.

This PhD thesis is focused on the simultaneous removal of OMPs and potentially harmful microorganisms (such as human pathogens and ARB&ARGs) from effluents collected after the secondary treatment of UWW treatment plants (UWWTPs) by advanced oxidation technologies (AOTs), including ozone-based and light-driven processes, considered promising tertiary treatment options. The disturbance on the bacterial communities that may occur by the selective pressure posed by these treatments was also addressed in this research.

Preliminary studies were conducted in batch mode for the removal of organic pollutants by ozonation, photolysis, photolytic ozonation, photocatalysis and photocatalytic ozonation (using the TiO_2 benchmark photocatalyst Degussa P25, Evonik). The obtained results showed that photocatalytic ozonation performed extremely well on the removal of spiked parent compounds (amoxicillin and diclofenac) and naturally occurring OMPs in UWW effluents. However, the oxamic acid formed was very refractory to oxidation.

These preliminary results were the driving force to better investigate photocatalytic ozonation in continuous rather than in batch mode, for the first time with TiO_2-P25 coated Raschig rings and eco-friendly high-energy efficient light emitting diodes (LEDs, λ_{max} = 382 nm) to treat UWW. Surface water (SW) collected from the supply area of a drinking water treatment plant was also included in this particular study. Photocatalytic ozonation showed a high efficiency removal of detected OMPs, potentially harmful microorganisms and ARGs. However, regrowth of total heterotrophs (in both matrices) and ARB (in SW only) was observed after 3-days storage, as was also the case for the genes 16S rRNA and *intI*1 in opposite to the ARGs (*bla*$_{TEM}$, *qnr*S and *sul*1). From biological assays, it follows that compounds with estrogenic effects were not formed and that differences concerning cell viability were not statistically significant for both UWW and SW. In parallel, collaborators from an ongoing project (NEPCAT - NORTE-07-0202-FEDER-038900) analysed the bacterial community composition of these treated samples and concluded that photocatalytic ozonation can lead to the selection of some microbial groups, namely *Proteobacteria*, with genera such as *Pseudomonas*, *Acinetobacter* or *Rheinheimera*, which might compromise the microbiological quality of the treated water.

Considering the complexity (and cost) of photocatalytic ozonation, and the observed regrowth of biological contaminants after 3-days storage of the UWW and SW treated by this process, the next treatment alternatives studied were those that can take advantage from locations with an average annual solar radiation reaching high levels.

Different solar-driven oxidation processes (H_2O_2, TiO_2-P25 and graphene oxide (GO)-TiO_2 photocatalysis, photo-Fenton) were performed in a pilot-plant compound parabolic collector during a short-term mission for advanced training at *Plataforma Solar de Almeria*, Spain. H_2O_2-assisted heterogeneous photocatalysis applying TiO_2-P25 showed the best compromise regarding the removal of both chemical and biological pollutants. However, the reactivation of ARGs occurred in stored treated water and the potential disturbance of the water bacterial communities was once again verified regardless of the oxidation technology studied. Moreover, the higher relative abundance of the phylum *Proteobacteria* was again observed, namely of genera *Pseudomonas*, *Rheinheimera* and *Methylotenera*.

Owing to these drawbacks, and because solar radiation is not always available, suffering discrepancies around the globe, further studies proceeded with a more flexible approach, *i.e.* applying LEDs with λ_{max} = 417 nm (instead of solar light or more expensive UV LEDs) and metal-free exfoliated carbon nitride (g-C_3N_4) as photocatalyst (instead of TiO_2-P25, GO-TiO_2, and non-active GO) for the first time in the degradation of OMPs in UWW. Removal of targeted OMPs to values below the detection limits was found in the slurry photocatalytic treatment. As expected, lower efficiencies were observed for the removal of these OMPs when the photocatalytic treatment of UWW was performed under continuous mode employing g-C_3N_4 immobilized on glass rings. However, further studies are needed to optimize the process parameters and to investigate the removal of biological pollutants, in particular using the continuous treatment system.

The knowledge on the simultaneous removal of chemical and biological pollutants with consolidated (ozonation) and new perspective processes (solar H_2O_2, photo-Fenton, photolytic ozonation, heterogeneous photocatalysis and photocatalytic ozonation) for the advanced treatment of UWW, is the main contribution of this PhD thesis. All these studies are fundamental to understand if we are in the right direction regarding UWW treatment or if we should re-think water treatment targets, towards a more enlightened design and application of advanced full-scale UWW treatments.

Resumo

A escassez e a qualidade da água são preocupações atuais em várias regiões do mundo, sendo o tratamento e a reutilização de águas e águas residuais urbanas de relevância nos dias de hoje. Uma única fonte de água pode servir um elevado número de pessoas, e um controlo inadequado da sua qualidade pode resultar em sérios problemas de saúde pública, assim como provocar efeitos nefastos no equilíbrio dos ecossistemas.

Atualmente, legislações nacionais e europeias, como o Decreto-Lei Nº 152/97 de 19 de junho de 1997 da República Portuguesa, e a Diretiva Nº 91/271/EEC do Conselho Europeu, de 21 de maio de 1991, decretam que as águas residuais urbanas que entram nos sistemas coletores devem ser sujeitas a tratamento secundário ou tratamento equivalente antes da sua descarga para o meio ambiente. Foram estabelecidos limites para alguns parâmetros, tais como a carência bioquímica de oxigénio sem nitrificação, a carência química de oxigénio e os sólidos suspensos totais. Outros contaminantes, incluindo micropoluentes orgânicos e microrganismos potencialmente perigosos (nomeadamente, microrganismos patogénicos, bactérias resistentes a antibióticos e genes de resistência a antibióticos), ainda não são considerados, em parte devido a limitações analíticas. No entanto, a baixa eficiência na remoção desses poluentes pelos processos convencionais de tratamento, revela que existe alguma urgência no desenvolvimento e implementação de tecnologias mais avançadas para uma reutilização ou descarga adequada de água residual urbana no meio ambiente, e desta forma o cumprimento dos padrões de qualidade que se espera virem a ser incluídos em legislações futuras.

O trabalho descrito nesta tese compreende o estudo da remoção simultânea de micropoluentes orgânicos e microrganismos potencialmente perigosos, presentes em efluentes recolhidos após o tratamento secundário convencional aplicado nas estações de tratamento água residual. Para tal, são utilizadas tecnologias avançadas de oxidação,

que incluem processos aplicando ozono e radiação, alguns já sendo considerados opções promissoras como tratamento terciário. O possível distúrbio nas comunidades bacterianas que pode ocorrer devido à pressão seletiva exercida por esses tratamentos foi também objeto de análise.

Os estudos preliminares consistiram na remoção de poluentes orgânicos por ozonização, fotólise, ozonização fotolítica, fotocatálise e ozonização fotocatalítica (usando o catalisador de referência TiO_2 Degussa P25, Evonik) em modo descontínuo. Os resultados obtidos demonstraram que o processo de ozonização fotocatalítica foi extremamente eficiente na remoção de dois compostos (amoxicilina e diclofenac) adicionados a uma água residual e também na remoção de micropoluentes orgânicos que ocorrem naturalmente em águas residuais resultantes do tratamento secundário em estações de tratamento de águas residuais urbanas. No entanto, verificou-se que o ácido oxâmico formado foi bastante resistente à oxidação.

Estes resultados preliminares constituíram a força motriz para investigar pela primeira vez o processo de ozonização fotocatalítica em modo contínuo utilizando anéis Raschig revestidos com TiO_2-P25 e díodos emissores de luz de alta eficiência energética (LEDs, λ_{max} = 382 nm), mais ecológicos que outras fontes de radiação habitualmente utilizadas, para o tratamento de água residual urbana. Amostras de água de superfície recolhidas na área circundante de uma estação de tratamento de águas foram também analisadas neste estudo. Foram obtidos valores elevados de remoção dos micropoluentes orgânicos detetados, de microrganismos potencialmente perigosos e de genes de resistência a antibióticos após o tratamento de ozonização fotocatalítica. No entanto, observou-se um recrescimento dos heterotróficos totais (em ambas as matrizes) e de bactérias resistentes a antibióticos (apenas em água de superfície) após 3 dias de armazenamento, assim como dos genes 16S rRNA e *intI*1 em oposição aos genes de resistência a antibióticos (*bla*$_{TEM}$, *qnr*S e *sul*1). A partir de ensaios biológicos, conclui-se que não foram formados compostos com efeitos estrogénicos e que as diferenças em relação à viabilidade celular não foram estatisticamente significativas para ambas as matrizes. Paralelamente, outros colaboradores de um projeto em curso (NEPCAT - NORTE-07-0202-FEDER-038900) analisaram a composição da comunidade bacteriana das amostras tratadas e concluíram que a ozonização fotocatalítica provocou a seleção de determinados grupos microbianos, nomeadamente *Proteobacteria*, e principalmente dos géneros como *Pseudomonas*, *Acinetobacter* ou *Rheinheimera*, que podem comprometer a qualidade microbiológica da água tratada.

Considerando a complexidade (e o custo) do processo de ozonização fotocatalítica e o facto de se ter observado crescimento bacteriano após três dias de armazenamento, tanto em água residual urbana como em água de superfície, as seguintes alternativas de tratamento estudadas consistiram em tecnologias que podem beneficiar de locais com elevados níveis de radiação solar. Diversos processos de oxidação que utilizam a energia solar (H_2O_2, fotocatálise com TiO_2-P25 ou com um material compósito de TiO_2 e óxido de grafeno (*graphene oxide* - GO), e o processo de foto-Fenton) foram estudados num coletor solar parabólico à escala piloto, durante uma missão de formação avançada de curta duração à Plataforma Solar de Almeria, localizada em Espanha. A fotocatálise heterogénea com a adição de H_2O_2 e aplicando TiO_2-P25 como catalisador obteve o melhor desempenho relativamente à remoção simultânea de poluentes químicos e biológicos. No entanto, a reativação de genes de resistência a antibióticos após o armazenamento da água tratada e o potencial distúrbio nas comunidades bacterianas da água foram novamente observados (independentemente da tecnologia de tratamento aplicada). Uma vez mais, foi verificada uma maior abundância relativa do filo *Proteobacteria*, principalmente dos géneros *Pseudomonas*, *Rheinheimera* e *Methylotenera*.

Devido a essas desvantagens, e considerando que a radiação solar não está disponível de forma contínua e uniforme ao redor do mundo, os próximos estudos foram realizados adotando uma abordagem mais flexível, ou seja, através da aplicação de LEDs com λ_{max}

= 417 nm (em vez de radiação solar ou LEDs UV mais caros) e de nitreto de carbono grafítico esfoliado sem metais ($g-C_3N_4$) como catalisador (em vez de TiO_2-P25, GO-TiO_2 e GO não ativo), o qual foi investigado pela primeira vez na degradação de micropoluentes orgânicos em água residual urbana. Remoções de micropoluentes orgânicos para valores inferiores ao limite de deteção foram obtidos para o sistema fotocatalítico utilizando o catalisador em suspensão e operando em modo descontínuo. Como esperado, foram observadas eficiências mais baixas para a remoção desses micropoluentes orgânicos quando o tratamento fotocatalítico da água residual urbana foi realizado em modo contínuo utilizando $g-C_3N_4$ imobilizado em anéis de vidro. No entanto, são necessários mais estudos para a otimização de vários parâmetros experimentais, assim como a investigação da remoção de poluentes biológicos, preferencialmente utilizando o sistema de tratamento em modo contínuo.

Portanto, o conhecimento sobre a remoção simultânea de poluentes químicos e biológicos por processos consolidados (ozonização) e por novos processos em perspetiva de aplicação futura (H_2O_2 solar, foto-Fenton, ozonização fotolítica, fotocatálise heterogénea e ozonização fotocatalítica) para o tratamento avançado de água residual urbana, é a principal contribuição deste trabalho. Todos estes estudos são fundamentais para compreender se estamos na direção correta relativamente ao tratamento de água residual urbana, ou se, por outro lado, devemos repensar os parâmetros chave do seu tratamento, desenvolvendo e aplicando tratamentos à escala real de uma forma cada vez mais cuidada.

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List of abbreviations

UWW	Urban wastewater
AOP	Advanced oxidation processes
AOT	Advanced oxidation technologies
OMP	Organic micropollutants
ARB&ARG	Antibiotic resistant bacteria and antibiotic resistance genes
TiO_2-P25	Titanium dioxide Degussa P25, Evonik
GO	Graphene oxide
LED	Light emitting diodes
UWWTP	Urban wastewater treatment plant
SW	Surface water
GW	Groundwater
DW	Drinking water
CPC	Compound parabolic collector
BOD_5	5-day Biochemical oxygen demand
COD	Chemical oxygen demand
p.e.	Population equivalent
PS	Priority substances
EQS	Environmental quality standards
CEC	Contaminants of emerging concern
COT	Chemical oxidation technologies
ROS	Reactive oxygen species
DOM	Dissolved organic matter
EfOM	Effluent organic matter
NOM	Natural organic matter
LCA	Life cycle assessment
DOC	Dissolved organic carbon
AMX	Amoxicillin

DFC	Diclofenac
TOC	Total organic carbon
HPLC	High performance liquid chromatography
SPE	Solid phase extraction
UHPLC – MS/MS	Ultra-high performance liquid chromatography with tandem mass spectrometry
DWTP	Drinking water treatment plant
16S rRNA	16S Ribosomal ribonucleic acid
YES	Yeast estrogen screen
MTT	Thiazolyl blue tetrazolium
LDH	Lactate dehydrogenase
HRT	Hydraulic retention time
SEM	Scanning electron microscopy
EDS	Energy-dispersive X-ray spectroscopy
TG	Thermogravimetric analysis
PCA	Plate count agar
qPCR	Quantitative polymerase chain reaction
IR	Induction ratio
LOD	Limit of detection
LOQ	Limit of quantification
CFU	Colony forming units
CBZ	Carbamazepine
SMX	Sulfamethoxazole
DIC	Dissolved inorganic carbon
TSS	Total suspended solids
Q_{UV}	Accumulated solar UVA energy received in the solar reactor per unit of treated water volume
m-FC	membrane faecal coliforms agar
S&B	Slanetz & Bartley agar
QIIME	Quantitative insights into microbiological ecology
OTU	Operational taxonomic units

PD	Phylogenetic diversity
CSS	Cumulative sum scaling
PCoA	Principal Coordinates Analysis
$g\text{-}C_3N_4$	Graphitic carbon nitride
gCN_T	Metal-free exfoliated graphitic carbon nitride
PVA	Polyvinyl alcohol
S_{BET}	Specific surface area
BET	Brunauer-Emmett-Teller
DRUV-Vis	Diffuse reflectance UV–Vis
TEM	Transmission electron microscopy
FWHM	Full-Width Half-Maximum
kapp	Apparent first order reaction rate constant

PART I

Introduction

CONTENT

Chapter 1

Road map for the thesis

Water, a vital resource for life on earth, is being compromised due to an immeasurable consumption, withdrawal, pollution and waste. Despite all the progress that is being done in terms of developing new strategies to mitigate the above-mentioned problems, including water resources management and access to water supply and sanitation services, mankind still has a long way to pursue to assure that *"no one is left behind"* [1].

The PhD project addresses the problematic of water pollution, namely the simultaneous removal of chemical and biological pollutants from urban wastewater (UWW) effluents by using advanced oxidation processes (AOPs) - conceptually based on the production of hydroxyl radicals ($HO^{\bullet}$), or advanced oxidation technologies (AOTs) - a classification including hydroxyl radicals and other reactive species.

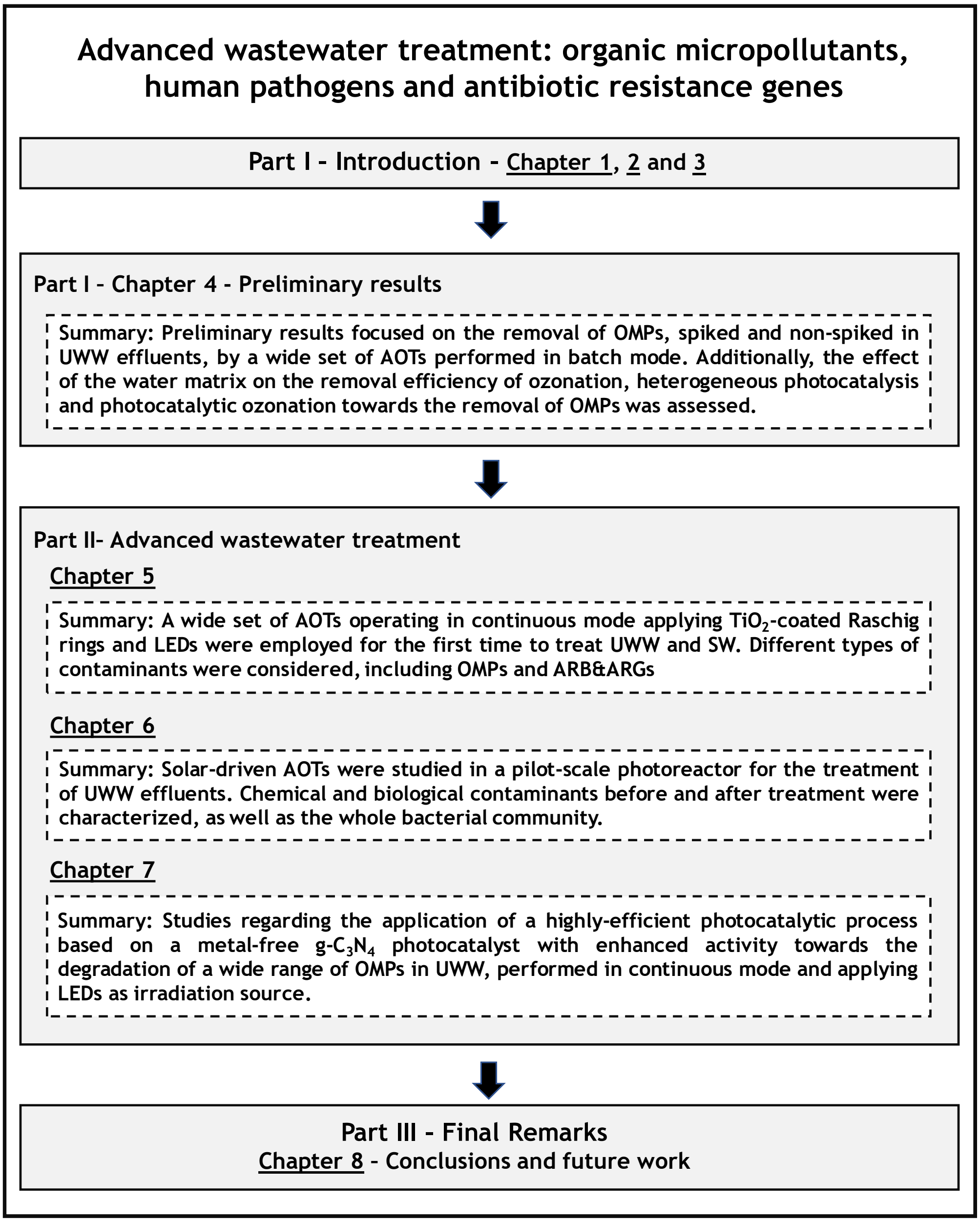
This PhD thesis is divided into three parts:

Part I – **Introduction** – provides the **Road map for the thesis (Chapter 1) -** the thesis outline and its major goals are represented in Figure 1.1. The **Relevance and Motivation (Chapter 2)** includes the state-of-the-art and a wide overview of the environmental problem associated with water pollution and scarcity. This chapter also discusses the promising technologies which might be able of mitigating the proliferation of a wide set of organic micropollutants (OMPs) and potentially harmful microorganisms (including human pathogens, antibiotic resistant bacteria and antibiotic resistance genes (ARB&ARGs)). Some parts of this chapter are based on a scientific publication that I co-authored: Ribeiro, A.R., **Moreira, N.F.F.**, Li Puma, G., Silva, A.M.T. *Impact of water matrix on the removal of micropollutants by advanced oxidation technologies,* Chemical Engineering Journal, 363 (2019) 155-173. In PART I the main **Objectives (Chapter 3)** of the present work and some **Preliminary results (Chapter 4)** are also included. The preliminary results herein presented were obtained in a certain extent during my Master thesis, while the results related to the removal of OMPs present in real UWW effluents were attained during my participation in the NEPCAT project (New EquiPment involving integrated CATalytic processes for treatment of organic pollutants and disinfection of water, NORTE-07-0202-FEDER-038900). These findings were the main driving force for the following studies in the frame of this PhD thesis and for this reason were herein discussed, which resulted in the following publication: **Moreira, N.F.F.**, Orge, C.A., Ribeiro, A.R., Faria, J.L., Nunes, O.C., Pereira, M.F.R., Silva, A.M.T. *Fast mineralization and detoxification of amoxicillin and diclofenac by photocatalytic ozonation and application to an urban wastewater,* Water Research, 87 (2015) 87-96.

Part II – **Advanced wastewater treatment** – comprises a compilation of three scientific contributions published in international peer-reviewed ISI indexed journals, each one corresponding to a chapter. Motivated by the preliminary results, a wide set of AOTs operating in continuous mode and applying TiO_2-P25 coated Raschig rings and high intensity light emitting diodes (LEDs) were employed for the first time to treat a secondary effluent of an urban wastewater treatment plant (UWWTP, located in Northern Portugal) and surface water (SW, collected from the supply area of a drinking water treatment plant). Different types of contaminants were considered, including OMPs and ARB&ARGs. Moreover, possible biological effects due to the formation of reaction by-products were evaluated by cytotoxicity and cellular viability assays – **Photocatalytic ozonation of urban wastewater and surface water using immobilized TiO_2 with LEDs: Micropollutants, antibiotic resistance genes and estrogenic activity (Chapter 5).** Afterwards, considering other treatment processes that can take advantage from geographical areas with high yearly average solar irradiation, the efficiency of different solar-driven AOTs was compared for the treatment of a secondary effluent of a UWWTP located in Almeria, Spain. The experiments were performed in a pilot-plant compound parabolic collector (CPC) reactor under a short-term mission for advanced training in the framework of the project: “Removal of emerging contaminants and pathogens from wastewaters by using advanced technologies” (Ref.P1404290052 EConPath), a collaboration between LSRE-LCM, LEPABE and Plataforma Solar de Almeria, funded by SFERA (European Commission). The treatment technologies were evaluated in terms of the simultaneous removal of chemical and biological pollutants, as well as the changes in the bacterial communities – **Solar treatment (H_2O_2, TiO_2-P25 and GO-TiO_2 photocatalysis, photo-Fenton) of organic micropollutants, human pathogen indicators, antibiotic resistant bacteria and related genes in urban wastewater (Chapter 6)**. However, since solar radiation has its own drawbacks (is not a continuous light source and suffer discrepancies around the globe), the application of visible light LEDs as irradiation source and a metal-free g-C_3N_4 photocatalyst was also investigated in slurry and continuous mode of operation – **Metal-free g-C_3N_4 photocatalysis of organic micropollutants in urban wastewater under visible light (Chapter 7).**

Part III - Final remarks - the main findings and conclusions of these PhD studies are discussed, followed by the suggestions for future work **(Chapter 8)**.

Appendices A, B, C and D correspond to the copies of the articles shown in chapters 4, 5, 6 and 7, respectively (the supplementary information materials are also provided). The lists of scientific contributions resulting from this PhD thesis are given in **Appendix E**: articles published in international peer-reviewed ISI indexed journals (**E1**), oral (**E2**) and poster (**E3**) communications in scientific meetings. The full agreement of the co-authors on the inclusion of the manuscripts in the present thesis can be found in Appendix F.



Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes

Part I - Introduction - Chapter 1, 2 and 3

Part I – Chapter 4 - Preliminary results

Summary: Preliminary results focused on the removal of OMPs, spiked and non-spiked in UWW effluents, by a wide set of AOTs performed in batch mode. Additionally, the effect of the water matrix on the removal efficiency of ozonation, heterogeneous photocatalysis and photocatalytic ozonation towards the removal of OMPs was assessed.

Part II- Advanced wastewater treatment

Chapter 5

Summary: A wide set of AOTs operating in continuous mode applying TiO_2-coated Raschig rings and LEDs were employed for the first time to treat UWW and SW. Different types of contaminants were considered, including OMPs and ARB&ARGs

Chapter 6

Summary: Solar-driven AOTs were studied in a pilot-scale photoreactor for the treatment of UWW effluents. Chemical and biological contaminants before and after treatment were characterized, as well as the whole bacterial community.

Chapter 7

Summary: Studies regarding the application of a highly-efficient photocatalytic process based on a metal-free $g\text{-}C_3N_4$ photocatalyst with enhanced activity towards the degradation of a wide range of OMPs in UWW, performed in continuous mode and applying LEDs as irradiation source.

Part III - Final Remarks

Chapter 8 - Conclusions and future work

Figure 1.1. Scheme of the thesis outline and the major goals in the present study.

Chapter 2

Relevance and motivation

2.1. Water: An increasingly stressed resource

Water is one of the most essential resources for life in its whole. This natural resource is being squandered, and it has been estimated that its use increases 1% per year since the 1980s [1]. Despite the progress made across several sectors since the turn of the millennium, much remains to be done in order to achieve a sustainable development in terms of both water resources management and water supply and sanitation services. The number of countries experiencing from average to high water stress is continuously increasing (Figure 2.1a). Agriculture is the largest activity in terms of both water withdrawal and water consumption (Figure 2.2c and d) [1].

Nowadays, it is estimated that 4.5 billion (Figure 2.2a) and 2.1 billion people (Figure 2.2b) lack access to safely managed sanitation and drinking water services, respectively, which are recognized as basic human rights since they are essential to maintain health livelihoods [1, 2]. Moreover, public health is strongly affected by the quality of drinking water, which should not pose a significant risk to health over a lifetime consumption. However, the number of people killed every year by inadequate water and sanitation surpasses largely those killed by drought, flooding, earthquakes and epidemics and conflicts (Figure 2.1b) [1].

The current water crisis is strictly related to the lack of integrated water management, population growth, socio-economic development, continuously increasing temperatures (due to the greenhouse gas emissions), water pollution in large scale (by numerous sources), massive water withdrawal and consumption (Figure 2.2c and d), lack of investment in advanced technologies and poor regulation [1, 3]. Regarding water pollution, suspended solids, biodegradable organics, heavy metals, pathogens, nutrients, priority pollutants, refractory organics and dissolved inorganics are considered as major water contaminants [3]. Bearing this in mind, one of the main challenges of the 21st century is ensuring universal access to potable water, as well as increasing water reuse practices even in countries considered as water-rich [3-6].

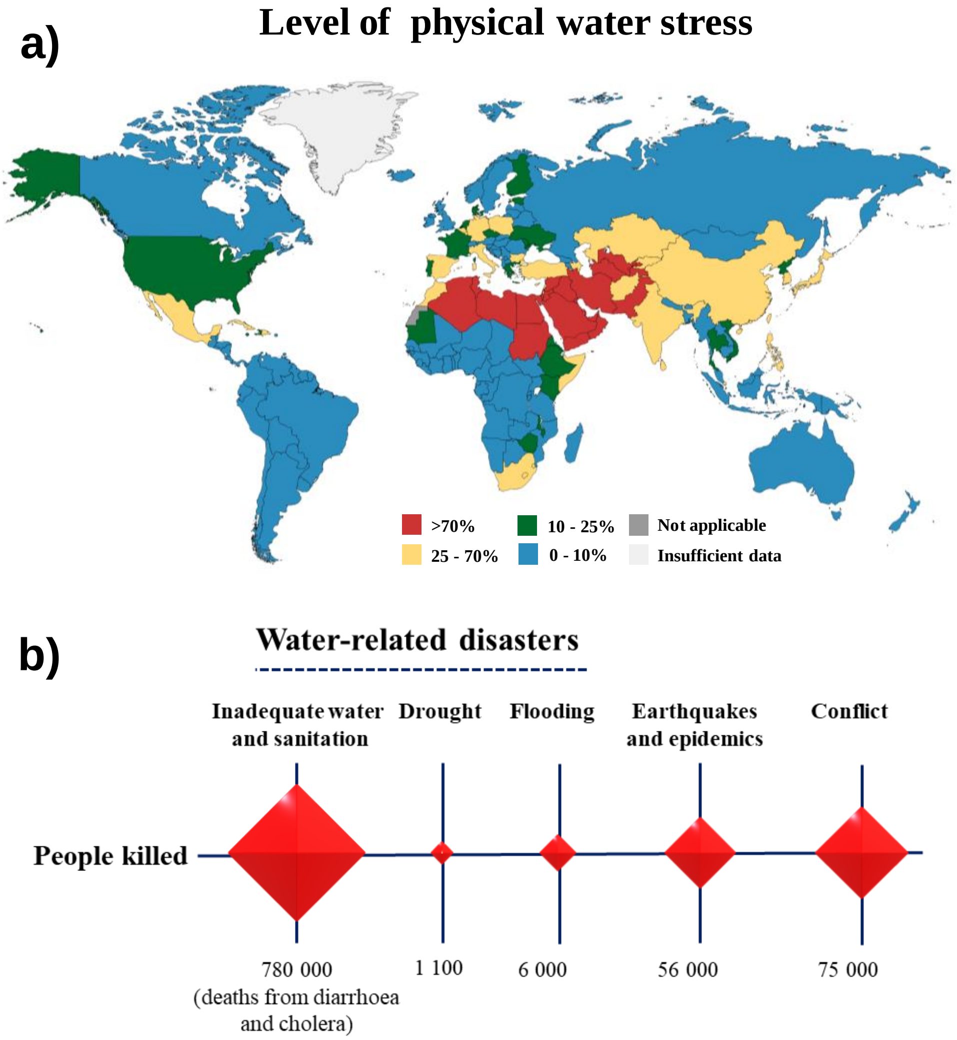


Figure 2.1. A global overview of countries experiencing different levels of water stress (a) and people killed by water related disaster (b) (adapted from [1]).

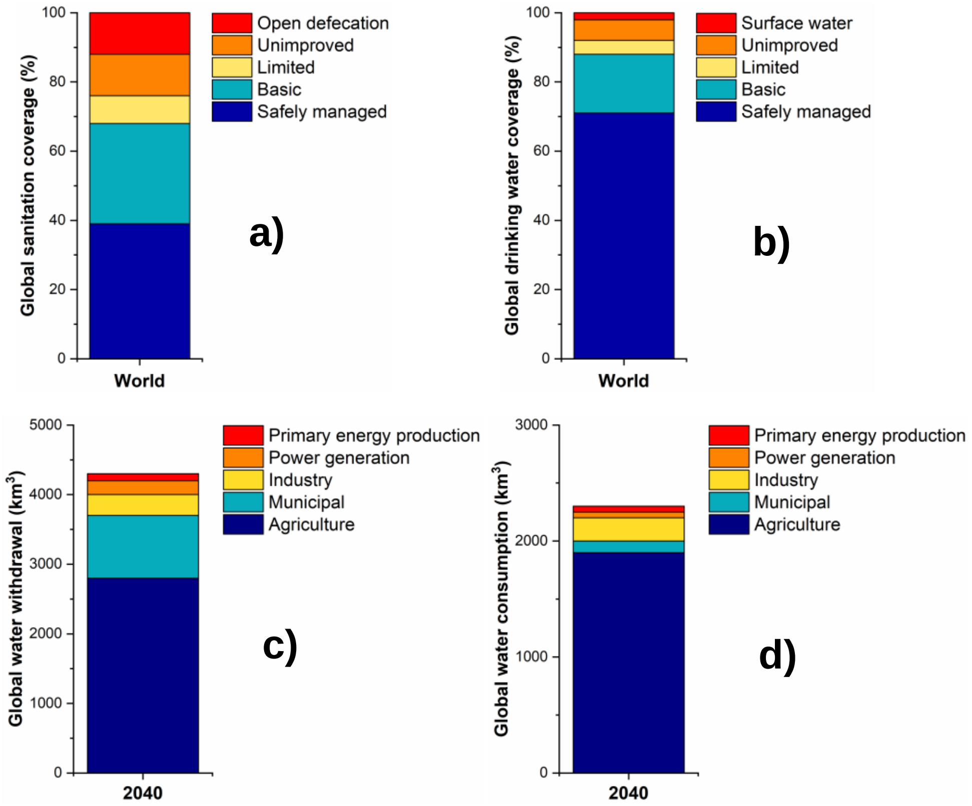


Figure 2.2. Levels of global sanitation (a) and global drinking water coverage (b) and levels of global water withdrawal (c) and consumption (d) (adapted from [1]).

The treatment of UWW consists in the partial reduction or complete removal of excessive impurities, which are present in concentrations much higher than those acceptable for final disposal or for wastewater reuse. The most common physical unit operations are screening, coarse solids reduction, flow equalization, mixing and flocculation, grit removal, sedimentation, high-rate clarification, accelerated gravity separation, flotation, oxygen transfer, aeration and volatilization. Chemical unit process includes chemical coagulation, chemical precipitation, chemical disinfection, chemical oxidation, advanced oxidation, ion exchange and chemical neutralization, scale control and stabilization. The biological treatment comprises suspended-growth and attached-growth processes and can be classified with respect to their metabolic function as aerobic, anaerobic, anoxic, facultative and combined processes [7-10].

Conventional treatment technologies applied in UWWTPs comprise the combination of physical, chemical and biological operations/processes for the removal of solids, organic matter and nutrients from urban wastewater. Preliminary, primary and secondary, are the main treatment stages applied in conventional UWWTPs (Figure 2.3). In some plants, disinfection and removal of refractory compounds can be assessed following the last treatment step [3, 7].

The treatment degree will depend mostly on the nature of the effluent and the discharge goal, ensuring the protection of public health and the environment. The preliminary treatment consists of the removal of large objects, rags, sticks, floatables and grease that may cause maintenance or operational problems on the downstream treatment process. Following the preliminary treatment, the removal of organic and inert particulate matter, that readily separates as settled sludge and floating scum, will be achieved at the primary treatment, which can be enhanced by the addition of chemicals or filtration (advanced primary) [7]. The secondary treatment involves the removal of biodegradable organics, suspended solids and nutrients (nitrogen and/or phosphorus) [7, 11]. Finally, wastewater constituents that are not significantly reduced by the secondary treatment (conventional biological treatment) can be removed by advanced or tertiary treatment [12]. Disinfection is usually included as part of tertiary treatment [7].

The Portuguese Decree-Law Nº. 152/97 of 19 June 1997 [13], that transposes the Council Directive 91/271/EEC of 21 May 1991 of the Official Journal of the European Communities [14], states that UWW entering collecting systems before discharge shall be subject to secondary treatment (or an equivalent treatment) to accomplish the following parameters: 5-day Biochemical oxygen demand (BOD_5 at 20 °C) without nitrification (25 mg L^{-1} O_2); Chemical oxygen demand (COD) (125 mg L^{-1} O_2); and Total suspended solids (35 mg L^{-1} or 60 mg L^{-1} depending on the number of population equivalents (p.e.)). Regarding the requirements for discharge of effluents from UWWTPs to sensitive areas to eutrophication, the following maximum concentrations were defined: Total phosphorus (2 or 1 mg L^{-1} P depending on the p.e.); and Total nitrogen (15 or 10 mg L^{-1} N depending on the p.e.). In sensitive areas, or when required, anaerobic and anoxic processes may be implemented for the removal of phosphorous and nitrogen (Figure 2.3a).

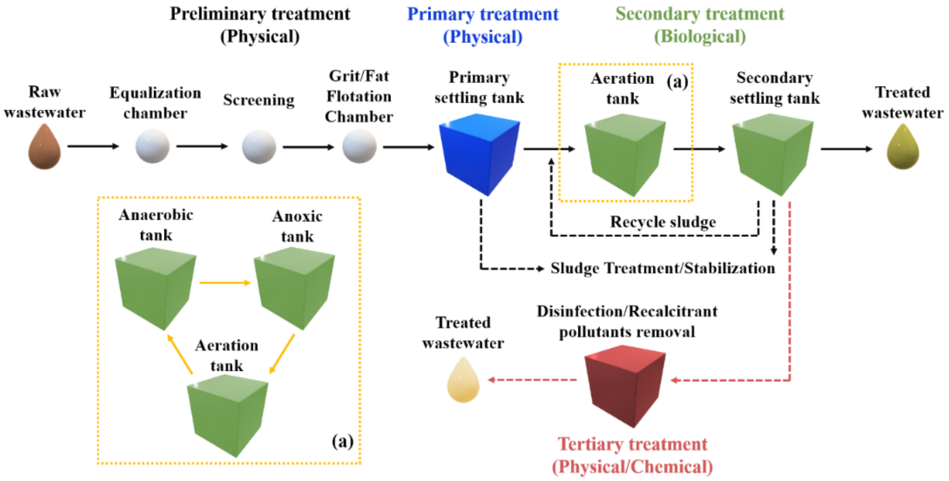


Figure 2.3. Flow diagram of a conventional UWWTP applying different stages, including physical, biological and chemical treatment. Possible implementation of anaerobic and anoxic processes for phosphorous and nitrogen removal in secondary treatment (a) (adapted from [7, 15]).

Despite the great improvement over the years concerning the management of environmental pollution accomplished by the conventional wastewater treatment plants, the occurrence of chemical and biological pollutants in water compartments urges the development and implementation of more advanced technologies to fulfil the quality standards needed to prevent negative effects that can be posed to mankind and the environment [16]. Over the years, more OMPs, including natural or anthropogenic substances, and increasing concentrations, have been detected in different water matrices, including UWW, SW, drinking water (DW) and groundwater (GW) [16-20]. Besides chemical contamination, biological pollution is also a matter of concern in aquatic environments, particularly in human-impacted areas [21, 22]. These contaminants might follow numerous pathways in the environment and UWWTPs are considered particular hot spots, with influents resulting from the combination of the water-carried wastes collected from residences, institutions, commercial and industrial establishments, together with GW, SW and stormwater [18, 23, 24] (Figure 2.4). Consequently, a large number of potentially harmful microorganisms or disease-causing microorganisms are present in untreated wastewater [25].

The commonly implemented wastewater treatments have not been designed to remove most of the OMPs and potentially harmful microorganisms (including human pathogens and ARB&ARGs). Even those with UV-C post-treatment disinfection are not entirely efficient in eliminating OMPs and do not contribute to significant reductions of antibiotic resistance prevalence as in some cases, can even contribute to its increase [18]. Furthermore, when the wastewater is not properly treated, its reuse in activities such as irrigation in agriculture is compromised [26, 27].

The continuous disposal of antibiotics and related products into the environment can lead to the development and proliferation of ARB and related genes, decreasing the efficiency of these antibiotics when supplied to humans and animals, conveying mankind back to a pre-antibiotic era as aftermath [28-30]. Moreover, the contamination of interrelated compartments (such as SW, GW and soils) may cause cumulative negative effects along with multigenerational exposure in aquatic organisms, and/or may affect the human's health by DW contamination (Figure 2.4) [16].

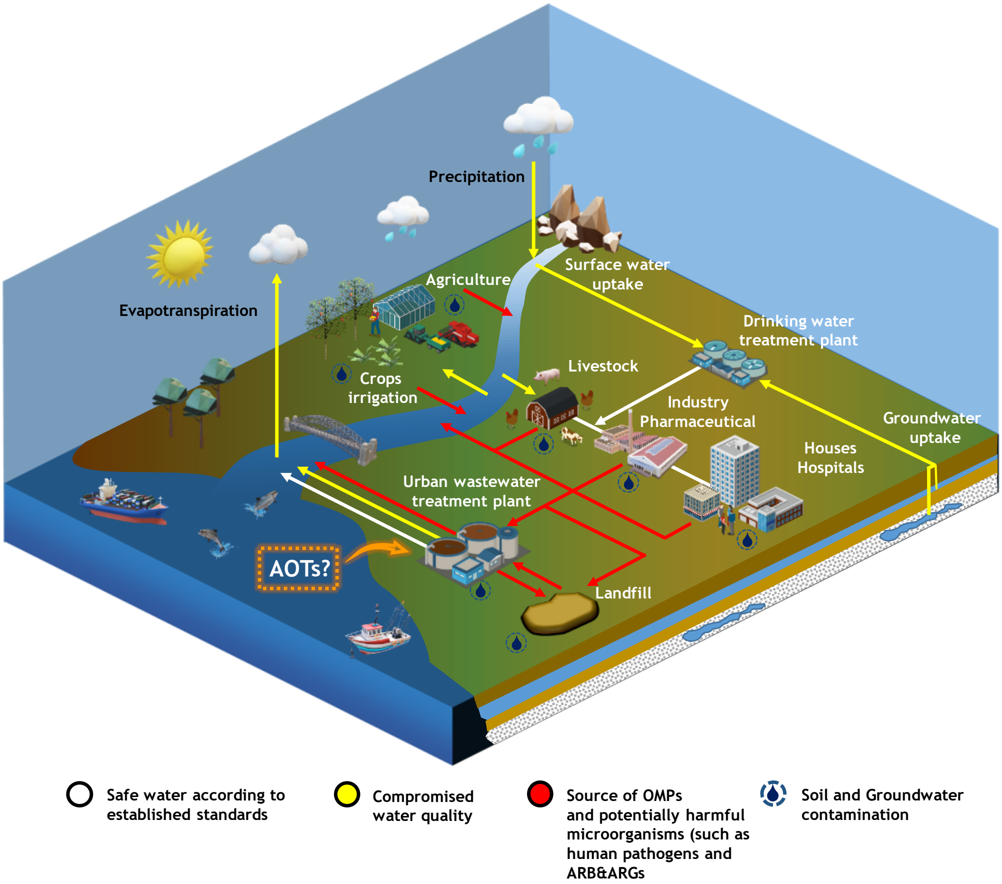


Figure 2.4. Major sources and routes of OMPs and potentially harmful microorganisms (such as human pathogens and ARB&ARGs) in the environment. Safe water according to established standards (white line), compromised water quality (risk of dissemination - yellow line), source of OMPs and ARB&ARGs (red line) and possible sources of soil and groundwater contamination (blue water drop).

Bearing this in mind, the European Union has been publishing guidelines since the year of 2000, when the Directive 2000/60/EC was launched to establish the framework for Community action in the field of water policy, representing a major improvement towards achieving adequate ecological and chemical status of inland surface waters, transitional waters, coastal waters and groundwater [31].

The dissemination of all these types of contaminants urges the development of new technologies able to remove, simultaneously, OMPs and potentially harmful microorganisms to levels posing no risk for human and environmental health [32, 33].

Innovative AOTs have been widely studied and are considered as a viable solution for the removal of organic compounds. Some AOTs have already been applied for disinfection [34-38]. The installation of AOTs after secondary biological treatment has been triggering a huge interest among the water industry to improve the effluent quality and promote the reuse of reclaimed water [39, 40]. However, it should be realized that the implementation of new technologies in UWWTPs is not the only solution to reduce chemical and biological contamination in the environment, but is part of a broader mitigation strategy, including source controls, best management practices and end-of-pipe solutions [33].

Despite all the promising developments, further research is still needed to ensure that the protection of the environment is accomplished in a commensurate way between public health, economic, civil society, political concerns and human rights.

2.1.1. Organic micropollutants in the aquatic environment

Through the years, the concern of the scientific community on the occurrence, fate, and adverse effects of a wide set of OMPs (natural or anthropogenic substances), that were undetected previously, increased mainly due to the development of new and more sensitive analytical instrumentation and methods capable of detecting those contaminants at trace levels [20, 41]. However, the detection of these OMPs is still a challenge considering the very low concentrations in which they are present (usually between ng L^{-1} and µg L^{-1} levels), their diverse chemical properties and the complexity of the water matrices [42, 43]. The number of reports regarding this subject has increased. Different OMPs, such as pharmaceuticals, pesticides, steroid hormones, personal care products, industrial compounds in various water matrices (GW, SW, wastewater and oceans), have been detected [16, 17]. However, a lack of knowledge regarding the consequences of the presence of OMPs in the environment (such as longterm exposure to very low-doses), and in irrigation practices and GW replenishment, still exists [42]. In some cases, different compounds may have synergistic interactions leading to unexpected adverse effects to humans and other organisms [44].

Industrial wastewater, runoff from agriculture, livestock and aquaculture, landfill leachates and domestic and hospital effluents, are considered the main anthropogenic sources of OMPs. Many of these micropollutants (including antibiotics) are purposely made to be stable and to remain biologically active with the aim of being effective in health care [16, 45-48].

The treated effluents released by UWWTPs are consensually recognised as a significant origin of OMPs, since UWWTPs applying conventional physicochemical and biological treatments are not able to fully eliminate refractory organic compounds occurring at trace concentrations. The removal extent of OMPs in a giving process usually depends on features such as the compound physico-chemical properties, process-specific factors, seasonal parameters, among others [11, 16, 49]. OMPs in domestic and hospital wastewaters reach UWWTPs after metabolism and excretion as parent compounds and/or metabolites, although the release by direct discharge of unused or expired drugs may also be a significant contributing factor. OMPs in industrial effluents discharged into UWWTPs are generally poorly removed, even when these have been previously treated at industrial level. Therefore, the uninterrupted discharge of OMPs and their metabolites into UWWTPs can be considered a continuous source of toxicity, which harms the receiving aqueous compartments, such as surface, ground and drinking waters [46].

Since the year 2000, the European Union published several guidelines to improve the water quality and define high risk substances to be prioritized. The Directive 2000/60/EC, transposed to the Portuguese law by Law N.° 58/2005 of 29 December 2005 [50], is considered as one of the most important achievements regarding water policy, implementing strategies against water pollution. The identification of priority substances/group of substances (PSs), with high risk to or via the aquatic environment, and the determination of EU Environmental Quality Standards (EQS) was also demanded by this directive [31, 46].

In 2001, 33 PSs (11 substances were identified as priority hazardous) that should be monitored at the community level were introduced by Decision 2455/2001/EC [51]. Only in Directive 2008/105/EC, amending the Directive 2000/60/EC and transposed to the Portuguese law by Decree-Law N.º 103/2010 of 24 September 2010 [52], the EQS of these PSs were established, along with other 8 certain pollutants [53]. Later, the Directive 2013/39/EU [54], transposed to the Constitution of the Portuguese Republic Decree-Law N.º 218/2015 of 7 October 2015 [55], updated the previous documents and proposed a first Watch List of substances for Union-wide monitoring, which was then published in Decision 2015/495/EU [56]. The Watch List defined 10 substances/group of substances, considered contaminants of emerging concern (CECs), *i.e.* unregulated pollutants, which monitoring data needs to be collected to support prioritization exercises. In 2018, the Commission Implementing Decision (EU) 2018/840 updated this Watch List of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council and repealing Commission Implementing Decision (EU) 2015/495 [57].

The continuous disposal of OMPs (PSs and CECs) into the aquatic environment turns them pseudo-persistent (*i.e.* those pollutants that despite their half-lives are short, they are continuously introduced into the aquatic environment [58]), which may increase the potential to trigger off harmful effects and urging the need for the development of new mitigation strategies such as the implementation of AOTs [16, 46].

2.1.2. Biological contaminants in the aquatic environment

The majority of microorganism are harmless and play essential roles in ecosystems and in human life sustaining. For instance, humans and animals are colonized by a wide diversity of commensal bacteria, most of them being innocuous, many are beneficial, and some are even necessary [59]. However, a wide variety of diseases in humans and animals can be caused by some microorganisms considered as pathogens, *i.e.* parasitic organism with selected mechanisms that promote the invasion and further colonization of a host organism and can be distinguished as primary or opportunistic pathogens [60]. The biological contamination of water bodies by pathogenic microorganisms with the potential of causing illness represents one of the most significant risks for human health on a global scale (Figure 2.1b), with numerous cases of disease outbreaks been directly related to untreated (or poorly treated) water [60, 61]. Therefore, water matrices such as SW, GW, UWW and DW, which can be used for direct consumption or reuse practices, should be monitored in a daily basis and properly treated to improve water quality [62]. Waterborne pathogens include bacteria (such as diarrhoeagenic and enterohaemorrhagic *Escherichia coli*, *Shigella dysenteriae, Salmonella typhi*, *Salmonella enterica*, *Vibrio cholerae*, *Legionella pneumophila*, *Burkholderia pseudomallei*), viruses (such as *Adenoviruses, Astroviruses, Enteroviruses, Rotaviruses, Hepatitis E virus and Hepatitis A virus*), protozoa (such as *Cryptosporidium hominis/parvum, Acanthamoeba culbertsoni, Cyclospora cayetanensis, Entamoeba histolytica and Giardia intestinalis*) and helminths (*Dracunculus medinensis*). These waterborne pathogens can have different routes of infection (sepsis and generalized infection may occur), such as ingestion (gastrointestinal), inhalation and aspiration (respiratory) and contact (skin, mucous membranes, wounds, eyes) [63].

Easy to use and effective monitoring methods are crucial to ensure the microbiological quality of water. However, the detection of pathogenic microorganisms occurring in water matrices is a challenging task, mainly due to their wide variety and lower abundance when compared to that of innocuous environmental bacteria, requiring very specific, sophisticated and expensive methods, which could be of difficult implementation for routine analysis.

As a consequence, pollution indicators such as faecal indicator bacteria (e.g. *E. coli* and enterococci) are widely used to evaluate water contamination by faecal material [60]. However, these indicators have limited predictive efficiency and an important modern challenge is the rapid, specific and sensitive detection of reliable microbial indicators and specific waterborne pathogens [60].

Hosts and bacteria have coevolved over millions of years and pathogenic bacteria have modified their virulence mechanisms to be adapted to host defence systems. Although the discovery and widespread use of antimicrobial agents hindered the spread of pathogens, antimicrobial resistance has increased globally. The emergence of resistant bacteria has accelerated in recent years, mainly as a result of increased selective pressure [59]. Antibiotic resistance exists in three interlocking sectors: human health, animal health and environmental health and, therefore, it is important to understand the relative importance of each sector in the evolution of resistant bacteria. We should also understand the genetic determinants of resistance, their interactions and transmission routes [59, 64].

2.1.2.1. Antibiotic resistance

Antibiotics are the first line substances for fighting bacterial infections and can be categorized as bacteriostatic (growth inhibition) or bactericidal (cell death). There are several classes of antibiotics, which are grouped according to their chemical structures and mechanism of action. There is a diversity of mechanisms of action such as damaging or inhibiting the synthesis of bacterial cell walls (e.g. penicillins, cephalosporins, vancomycin), inhibiting the bacterial DNA synthesis (e.g. fluoroquinolones), the RNA synthesis (rifamycins) or proteins synthesis (e.g. chloramphenicol, streptomycin and tetracyclines), or metabolic pathways (sulphonamides) [65].

However, the gains achieved with antibiotics are being jeopardized by the spread of antibiotic-resistant bacteria [65]. The sensitivity of bacteria towards antibiotics differs from bacteria to bacteria, with some exhibiting a high level of intrinsic resistance. Additionally, gene mutation and acquirement of new genes contribute to resistance towards new antibiotics, or enhanced resistance towards an antibiotic. Bacteria are very efficient at disseminating the effects of resistance due to their ability to multiply very rapidly, passing on resistance genes to other bacteria by replication and through horizontal transfer mechanisms (conjugation, transduction and transformation) [60, 65, 66].

Usually, the genes conferring antibiotic resistance are located on mobile genetic elements, including plasmids, integrons and transposons [67]. Furthermore, the combination of acquired genes with chromosomally encoded resistance genes frequently results in bacteria that are resistant to most of the main classes of antimicrobials available. The genes conferring resistance will be expressed in mechanisms of resistance such as changes in surface permeability, enzymatic inactivation of the antibiotic before it reaches its target site, alteration of the antibiotic target, an increase of efflux pumps that rapidly extrude de antibiotic, or acquisition of metabolic pathways alternative to those inhibited by the drug [65, 68]. The selective pressure exerted by the use of antibiotics, mainly overuse or misuse in humans and livestock production, has been considered the main factor in the rapid emergence of bacterial resistance to antimicrobials [65].

Over the last decades, a worldwide increase of antimicrobial resistance (even to drugs of last resort) has been found in diverse environments (including UWW, sludge, soil, SW, DW), transforming impacted environments into reservoirs of ARGs and driving the resistance problem by killing susceptible strains and selecting those that are resistant [69-71].

The spread of antibiotic resistance was identified by the World Health Organization as one of the major risks to human health, in which the urban water cycle is considered an important route [72]. Moreover, the United Nations Environment Programme points out antibiotic resistance as one of the top six emerging issues of environmental concern [73]. The preliminary studies regarding antibiotic resistance were mainly focused on clinical microorganisms. However, the public and medical awareness increased with the appearance of superbugs (multi-drug resistant), recognising antibiotic resistance as an interdisciplinary problem involving human health, animal agriculture and the environment [74, 75]. UWWTPs, aquaculture and animal farming (using antibiotics in growth promotion and therapeutic), and pharmaceutical manufacturing effluents (Figure 2.4) are considered major environmental point sources of antibiotic resistance [32].

The incapacity of the implemented conventional wastewater treatments to remove ARB, ARGs and antibiotic substances (potential contributors to the environmental selection of ARB) from domestic and clinical sources, leads to a continuous disposal of these pollutants into the environment, high concentrations being detected in not only in raw wastewater but also in treated effluents and in the surplus sludge of the biological treatment [28, 29, 60, 71, 76, 77]. In addition to the contribution from point sources, the natural environment also receives microorganisms by diffuse sources of contamination, such as runoff and soil leaching from animal origin, leakage from sanitation systems and leaks in networks defaulters, among others [60]. Until now, the impact of high loads of ARB and ARGs is still not fully understood [78, 79].

As previously mentioned, the continuous disposal of potentially harmful microorganisms (including human pathogens, ARB and associated genes) into environment compartments urges the need for the development of new mitigation strategies such as the implementation of AOTs, which already have shown promising results on the inactivation of these biological contaminants [80]. However, some studies have demonstrated that inactivation can be a transient effect and when the stress status is relieved the reactivation and regrowth of bacteria can occur during water storage, together with changes on the diversity of the bacterial communities, suggesting that more interdisciplinary studies are needed [81].

Antibiotics should never be considered mere commodities, and must not be overvalued as we all are entirely dependent on them for the treatment of infectious diseases and for the success of advanced surgical procedures, including organ and prosthetic transplants. The current concern is that, although efforts have been made in order to monitor antibiotics usage, there is no doubt that the situation of antibiotic resistance is grim and there are no straightforward solutions to this problem. Furthermore, a point that must be highlighted is that it is crucial to search for new antimicrobial agents. Indeed, the microbial parvome is close to being exhausted in the pursuit of new antimicrobials, even though the careless attitude of big pharma [82]. Systems biology approaches are now revealing new types of metabolic interactions, and are giving explanations for many aspects of antibiotic modes of action and resistance. With the increasing application of such interactive genome-associated studies, it can be anticipated that new and valid targets will be identified, the onus on academia being to furnish evidence and material and not in most of the pharmaceutical companies that are now shrinking the responsibilities of their own business missions [82].

In summary, the biological risk assessment of the emergence and propagation of ARB in the environment is a priority, and national and international guidelines should be developed given the public health threat posed by this type of pollution [32]. This risk of transmission of antibiotic resistance to humans and other animals should consider the environmental ARB (considered as ARG reservoirs) and the contaminant ARB (considered as ARG carriers), some of which can colonize and sometimes invade the human body and may have a role in the transmission of ARG to pathogens (considered as ARG vectors) [64]. Given the capacity of vectors to colonize humans, they should be pivotal for assessing the risks of transmission of antibiotic resistance from the environment to humans. However, it should be highlighted that most vectors are not pathogens, since they may lack crucial virulence genes [64]. The pathways and modes of transmission of antibiotic resistance to humans are still poorly understood. The current knowledge suggests that a complex combination of variables referring to different environmental compartments and ubiquitous- and human–bacteria interactions may rule the risks of transmission to humans [64].

2.1.3. Advanced oxidation technologies for water treatment

The incapability of conventional water treatment processes for OMPs abatement, neither to act as a barrier to the proliferation of ARB&ARGs, which may pose ecological risks and jeopardise human health, urges the development of new technologies for possible upgrade of UWWTPs at full scale. To fight the abovementioned problems, AOTs including AOPs and chemical oxidation technologies (COTs) are widely studied for the oxidation of OMPs and disinfection through highly reactive species generated in sufficient quantity to improve the effluent quality and promote the reuse of reclaimed water (Table 2.1) [17, 83-86]. AOTs can be classified as chemical, photochemical, electrochemical, sonochemical and hydrochemical processes, depending on the way the highly reactive species are involved, or as homogeneous and heterogeneous processes, depending on the number of phases involved during the transport and reaction of the species. Homogeneous AOTs include processes that utilize UV, H_2O_2, the Fenton reagent, dissolved O_3, wet oxidation and wet peroxide oxidation; whereas heterogeneous AOTs comprise heterogeneous photocatalysis, heterogeneous Fenton-like processes, catalytic and photocatalytic ozonation, catalytic wet oxidation and catalytic wet peroxide oxidation [84].

Hydroxyl radicals are the most powerful oxidizing species after fluorine, with an $E^0 = 2.80$ V. These unselective radicals might mineralize the parent compounds with no generation of secondary waste, yielding CO_2, H_2O and inorganic ions as final products Recently, sulphate radical mediated AOTs gained attention due to the high potential redox of $SO_4^{\bullet-}$ which is comparable with that of $HO^\bullet$ [17, 87-89].

Until now, UV, UV/H_2O_2 and ozone-based processes have been successfully implemented in full-scale UWWTPs to degrade OMPs (Table 2.1). AOTs not yet applied at full scale include UV/O_3, UV/O_3/H_2O_2, UV/H_2O_2/Fe (mild photo-Fenton) and other advanced processes such as heterogeneous photocatalysis, heterogeneous photo-Fenton, photocatalytic ozonation, photocatalytic membrane processes, electrochemical oxidation and sonolysis [17, 34]. The use of AOTs in hybrid processes has been envisaged through integration with membrane technologies and through the coupling of two or more AOTs. This last strategy can result: (i) in a synergistic effect due to the positive interactions among the single processes; (ii) in a cumulative effect caused by the increased generation of reactive oxygen species (ROS); and less commonly (iii) in an antagonistic effect, due to excessive formation of ROS, which may act as self-scavengers [90]. For instance, some organic compounds can be very difficult to be degraded by a process alone, as well as the products resulting from the reaction in one single process can be more toxic than the parent pollutants, while the combination of two processes can be more efficient in the oxidation of both parent and intermediate compounds [20, 91, 92]. In fact, the scientific community interest in the identification of reaction intermediates has increased from an ecotoxicological point of view [20, 93]. The adverse effects of substances on different organisms can be studied by using toxicity bioassays. In this way, it is possible to assess the combined toxicity of mixtures of known and unknown compounds (with similar inhibitory mode of action), while by common chemical analysis it is only possible to quantify targeted chemical compounds without identification of their effect on the environment. Microorganisms, plants, algae, invertebrates and fish are the principal test organisms used in the toxicity bioassays [94].

Regarding potentially harmful microorganisms, the free radicals that are formed can also act as disinfectant species leading to high bacterial loads reduction [4, 5, 95-99]. In a recent review article, authors investigated the fate of ARB&ARGs during AOTs, the key operating conditions affecting their efficiency to inactivate ARB and remove ARGs, and the main oxidative damage pathways involved in these processes. The article suggests that besides the operating conditions, the variable behaviour observed on bacterial genetic constituents of the microbial community under study may be associated to the oxidative damage mechanisms in place during the application of each treatment technology [100].

One major disadvantage of the highly reactive species is the absence of residual effect (*i.e.* effect after a certain period) possibly allowing the reactivation of injured microorganisms. When performing microbiological analysis, it is necessary to consider the fact that some of the AOTs may only inhibit these microorganisms, and the damage can be reversible [101]. In addition, recent studies [81, 102-105], some resulting from this PhD project, showed that AOTs might have the potential to select some bacterial groups. Regardless of the treatment applied (UV_{254nm} radiation, ozonation or photocatalytic ozonation) the bacterial community was characterized by higher proportions of *Proteobacteria* (*Gamma*- and *Betaproteobacteria*) after 3-days storage in comparison with the non-treated wastewater after the same storage period [81]. Thus, when developing and designing new technologies, the potential to disturb the bacterial communities after treatment should be assessed.

The elimination of OMPs by AOTs is largely influenced by the quality of the water matrix that needs to be treated, and the process effectiveness relies on the impact of the dissolved components, which can have neutral, inhibitory or promoting effects. The composition of the water matrix can also have a significant impact on the inactivation of bacteria in water disinfection processes, as shown in the literature [106, 107]. The occurrence of scavengers in the matrix may also hinder the removal of dissolved organic matter (DOM), representing the main fraction of the effluent organic matter (EfOM) (*i.e.* a heterogeneous mixture of recalcitrant organic compounds comprising natural organic matter (NOM), soluble microbial products and trace OMPs) present in biologically treated urban wastewater [108].

In fact, most studies on AOTs for UWW treatment have been performed using aqueous solutions spiked with model contaminants at higher concentration levels than those actually detected in real matrices. This methodology allows: the application of simple analytical techniques; to exclude interactions between the contaminants or the oxidizing species and the matrix components; to study the degradation pathways of specific compounds; and to evaluate the performance of the studied treatment for a target contaminant [84]. However, the quality of the aqueous matrix for which the treatment is proposed is crucial to draw accurate conclusions about the effectiveness of a certain AOT, its suitability and practicability for the treatment of real contaminated waters. Bearing this in mind, there is an urgent need for additional studies regarding the treatment of realistic water matrices, considering the actual concentrations of detected OMPs and naturally occurring potentially harmful microorganisms.

Table 2.1 compiles the available information regarding the application of advanced technologies for the removal of chemical and/or biological contaminants, the scale of the study (lab, pilot or full scale) and the country where the UWW was collected. For the present survey only studies dealing with actual concentrations of both pollutants were included (Table 2.1), while spiked matrices were excluded (*except when the study assessed the simultaneous removal of both pollutants). The search comprised publications in Scopus database since 2010 until January 2020, using the following keywords (abstract, title, keywords): “ozonation” or “ozonolysis” or “ozone” or “UV/O_3” or “peroxone” or “photocatalysis” or “Fenton” or “UV/H_2O_2” or “photocatalytic ozonation" or "photo-Fenton" or "solar/H_2O_2" or "photolysis" or "UV_{254}" and "urban wastewater" or "municipal wastewater" (Table 2.1).

Despite the great attention given to AOTs (Figure 2.5a), only ca.15% (94 publications) of the 616 published papers refers to the treatment of at least one non-spiked type of pollutant (chemical, biological or both) [37, 40, 43, 62, 80, 91, 98, 102, 106, 109-193]. Moreover, from these 94 studies, only ca. 20% (19 publications [37, 91, 102, 121, 127, 130, 140-143, 146-149, 159, 162, 164-166, 174]) deals with the simultaneous removal of both chemical and biological contaminants (Figure 2.5b); however, at least one of the pollutant types (chemical or biological) were spiked in more than half of these 19 publications [102, 127, 130, 143, 146-148, 164, 166, 174]. It is worth to mention that studies neglecting the simultaneous removal of chemical and biological pollutants can lead the scientific community to less accurate conclusions, which may invalidate the conclusions and future application of certain technologies. Surprisingly, only 4 publications [80, 102, 116, 137] out of 94 assessed the bacterial community composition or preformed metagenome analysis after treatment. Additionally, only 12 studies (*i.e.* 13%) were conducted with full scale reactors (most of those with ozone-based processes), two studies performing bacterial community diversity or metagenome analyses [80, 110, 117, 119, 132, 136, 137, 139, 140, 182, 191].

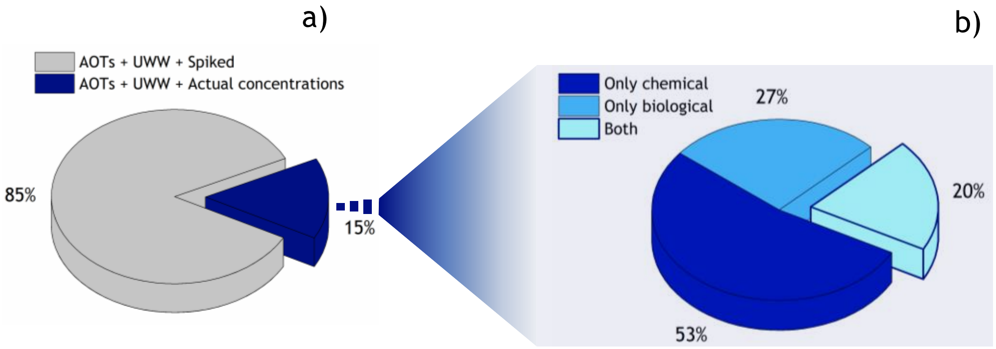


Figure 2.5. Relative frequency of publications since 2010 until January 2020 dealing with the application of AOTs for UWW treatment in both spiked (when both chemical and biological pollutants are considered) and non-spiked conditions (actual concentrations when at least chemical or biological pollutants are

considered) (a); and the frequency of publications that considered chemical, biological or both contaminants with actual concentrations in UWW (b).

In most of the cases, the full-scale application of perspective and more efficient AOTs has inherent some restrictions such as the lack of explicit regulations for the elimination of OMPs from UWW [34]. The Swiss Federal Office for the Environment was pioneer in the development of a legal framework for OMPs emission in effluents of certain UWWTP, by applying a new Water Protection Act aiming at 80% average removal of some target OMPs [34, 48]. In this context, some UWWTP are being upgraded with advanced treatment based on ozone and/or activated carbon in Switzerland, as well as in Germany (carried on a voluntary basis). Still, one of the major drawbacks of these technologies is their installation and maintenance costs, limiting the wide full-scale implementation of these processes, increasing the need to perform cost effectiveness analysis for each of them under a common based comparison [16, 33, 34].

Advanced technologies will be inevitable for the removal of specific chemical and biological pollutants from UWW effluents, and the application of robust tools such as life cycle assessment (LCA) could be of a major value for the identification of the “breaking-even” points (*i.e.* when the additional environmental impacts generated by the processes are surpassed by the reduction in environmental impacts generated by effluent discharge), for process optimization and for helping on decision making since it provides an overview of the environmental impacts of these processes [194].

In this sense, the results of this PhD thesis are expected to offer an integrated perspective of the simultaneous removal of chemical and biological pollutants (including the analysis of the bacterial community diversity) by advanced technologies, which might be important to conclude about the most suitable treatment options for real case scenarios and full-scale application.

2.1.3.1. Ozone-based processes

Ozonation is a consolidated AOT widely applied for the purification of drinking water [195, 196], which later drew the attention of the scientific community as a feasible water treatment technology of either raw wastewaters, as pre-treatment (increasing the biodegradability of the effluent), or secondarily treated wastewater effluents, as post-treatment, to minimize the release of recalcitrant OMPs and potentially harmful microorganisms (including human pathogens and ARB&ARGs) into the receiving waters (commonly SW) (Figure 2.6) [8, 37, 91, 98, 197-204]. Over the last years, several studies conducted in lab-, pilot- and full-scale applications over real matrices (UWW) were published [34, 40, 43, 83, 109-113, 117, 119, 120] (Table 2.1), achieving high efficiencies with respect to the removal of chemical or biological contaminants, a residual percentage of studies examining the simultaneous removal of both. Still, most of the studies are performed under unrealistic conditions, including the spiking of water matrices (such as ultrapure/distilled water or synthetic wastewater) with concentrations of OMPs higher than those naturally found in UWW effluents, and with considerably high ozone concentrations [16, 17, 34, 205]. Regarding biological contamination, the knowledge on the efficiency of ozonation to eliminate ARB&ARGs is still limited since most studies performed so far only focused on the effect of ozone on cultivable bacterial populations, with the effect of the process on ARB or ARGs being still unclear, and even less studied regarding the disturbance on the bacterial communities [37, 81, 100].

A recent study evaluates the environmental impact of ozonation and solar photo-Fenton by LCA, showing that both tertiary processes reduce water stress locally. Ozonation had a better overall environmental performance than the photo-Fenton process. The environmental impact of the required ozone is smaller than that of the reactants for solar photo-Fenton, and ozonation can be operated both day and night and there is no need to collect the nightly generated secondary effluent (*i.e.* resulting in lower infrastructure related impacts). This study concludes that the implementation of ozonation may diminish the environmental damage by 37% compared to the baseline scenario [206]. Switzerland was the first country controlling the discharge of OMPs from UWWTPs, with intensive studies indicating the effectivity of ozonation and adsorption using activated carbon on the abatement of OMPs [34]. Ozonation implemented at full scale in an existing UWWTP was considered feasible both technically and economically [39].

Ozone is a strong oxidant, usually produced by electric discharge in the presence of air or oxygen, with the ability to degrade OMPs and disinfect, directly and selectively at low pH, or to undergo decomposition via a chain reaction mechanism that produces $HO^{\bullet}$ (non-selective radicals, more reactive than ozone), at neutral or alkaline pH (indirect reaction) [207]. In many cases, high pH values favour the degradation efficiency due to the decomposition of ozone into $HO^{\bullet}$ [195]. Molecular ozone attacks preferentially electron-rich organic moieties (phenols, anilines, olefins, sulphur, and amine moieties). The half-life of molecular ozone depends on several operating parameters, such as pH and temperature. Furthermore, the stability of ozone in different matrices (e.g. SW and UWW) is determined by the nature of the EfOM [17]. Ozone decomposition proceeds through the following chain reactions (Eqs. 1-8) [195, 208]:

$$\mathrm{O_3 + HO^- \rightarrow HO_2^- + O_2} \qquad (1)$$

$$\mathrm{O_3 + HO_2^- \rightarrow HO^{\bullet} + O_2^{\bullet -} + O_2} \qquad (2)$$

$$\mathrm{O_3 + O_2^{\bullet -} \rightarrow O_3^{\bullet -} + O_2} \qquad (3)$$

$$\mathrm{O_3^{\bullet -} + H^+ \leftrightarrow HO_3^{\bullet}} \qquad (4)$$

$$\mathrm{HO_3^{\bullet} \rightarrow HO^{\bullet} + O_2} \qquad (5)$$

$$\mathrm{O_3^{\bullet -} \leftrightarrow O^{\bullet -} + O_2} \qquad (6)$$

$$\mathrm{O^{\bullet -} + H_2O \rightarrow HO^{\bullet} + HO^-} \qquad (7)$$

$$HO^{\bullet} + O_3 \rightarrow HO_2^{\bullet} + O_2 \qquad (8)$$

Ozonation is an oxidation process that can take place at both the gas-liquid interface and in the bulk liquid, depending on the concentration of the reactants and on the Hatta number [209]. Therefore, the polarity of the molecules can play an important role in the reaction rate, since non-polar organic compounds tend to accumulate at the interface and be more reactive, while more polar or dissociated substances remain in the bulk liquid [210].

Ozone decomposition is fast during the initial stage ($t < 20$ s) of natural water ozonation, known as "instantaneous ozone demand'', while during the second stage ($t > 20$ s) it follows an apparent first-order rate attributed to radical-type chain reactions, during which $HO^{\bullet}$ radicals are generated [211]. EfOM and carbonate/bicarbonate ions interfere with the rate of ozone decomposition depending on their concentrations and pH, affecting the yield of $HO^{\bullet}$ [212]. Besides the scavenging effect of $HO^{\bullet}$ by EfOM [211, 213-220] and carbonate/bicarbonate [213, 217, 218, 221, 222], other anions (e.g., chloride, phosphate, sulphate) can also act as ozonation inhibitors [221, 222].

The structural and functional complexity of EfOM turns difficult its full characterization; therefore, the dissolved organic carbon (DOC) is commonly used as a surrogate parameter for its quantification, providing a general assessment and lacking on structural elucidation [108]. Both the content and the composition of DOC determine the rate of decomposition of aqueous ozone and, thus, DOC is a crucial water quality parameter that is monitored to normalize the dosing of ozone [211].

The main drawback of the ozonation process is the generally limited rate of DOC mineralization and consequent accumulation of unknown reaction by-products, resulting from the incomplete oxidation of the parent compounds and from the reaction with the water matrix constituents [115]. Undesirable toxic oxidation by-products, resulting from the reaction with bromide or DOM, include nitrosamines (e.g., N-Nitrosodimethylamine, NDMA), bromate, aldehydes, ketones and carboxylic acids [83, 115, 223]. Although these by-products may increase the toxicity of the treated wastewater, they are typically biodegradable and therefore a biological post-filtration is usually applied [115, 224]. The limited extent of DOC mineralization can be overcome by adding catalysts [225, 226] or H_2O_2 [92, 222], though, the process costs also increase.

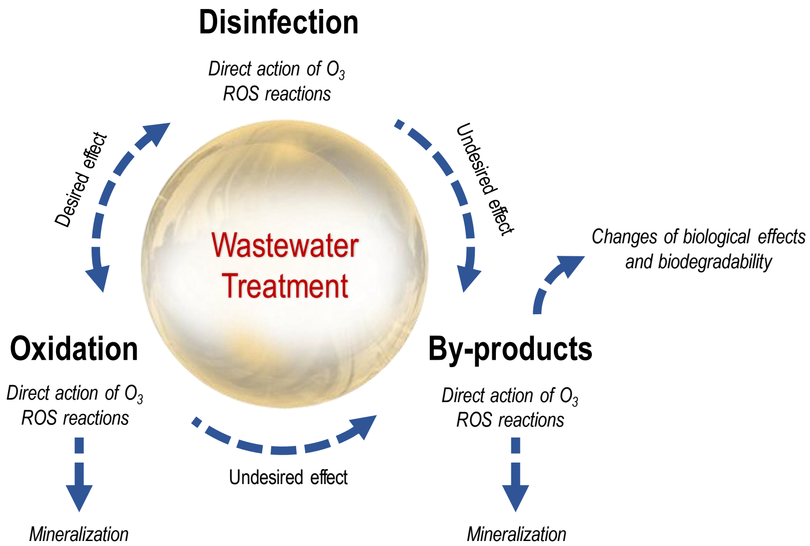


Figure 2.6. Main goals for the application of ozone. Both processes are accompanied by the undesired formation of by-products (adapted from [195, 227]).

The use of heterogeneous catalysts or photocatalysts is an attractive solution in ozonation, since the combination of two or more AOTs can lead to higher removal rates in comparison to the single processes. The most widely used catalysts include transition metal oxides (TiO_2, MnO_2, Al_2O_3, and CeO_2), metal oxyhydroxide (FeOOH), metals (Cu, Ru, Pt and Co) on supports (SiO_2, TiO_2, Al_2O_3, CeO_2 and activated carbon), zeolites modified with metals, activated carbon and natural minerals [228].

Recently, photocatalytic ozonation has been studied as an alternative to photocatalysis and ozonation due to the increased production of $HO^{\bullet}$ through the formation of an ozonide radical ($O_3^{\bullet -}$) in the adsorption layer of a semiconductor (e.g., TiO_2) and lower electron-hole recombination, in comparison to ozonation and photocatalysis, respectively [17, 229]. Specifically, the $O_3^{\bullet-}$ species (Eq. 9) generated from the reaction of ozone with the semiconductor electrons react with H^+ in the solution, forming $HO_3^{\bullet}$ radicals (Eq. 10) which then evolve to produce oxygen and HO• (Eq. 11). In this scenario, it must be also considered that HO• can react with ozone (Eq. 12). When ozone is completely consumed, dissolved O_2 can accept the semiconductor conduction band electrons, generating $O_2^{\bullet-}$ (Eq. 13), which in turn can be protonated (Eq. 14). $HO_2^{\bullet}$ radicals can also originate HO•, according to the pathway shown in Eqs. 15-16 [230].

$$O_3 + e^- \rightarrow O_3^{\bullet-} \tag{9}$$

$$O_3^{\bullet-} + H^+ \rightarrow HO_3^{\bullet} \tag{10}$$

$$HO_3^{\bullet} \rightarrow O_2 + HO^{\bullet} \tag{11}$$

$$HO^{\bullet} + O_3 \leftrightarrow O_2 + HO_2^{\bullet} \tag{12}$$

$$O_2 + e^- \rightarrow O_2^{-} \tag{13}$$

$$O_2^{\bullet-} + H^+ \leftrightarrow HO_2^{\bullet} \tag{14}$$

$$2HO_2^{\bullet} \rightarrow H_2O_2 + O_2 \tag{15}$$

$$H_2O_2 + O_2^{\bullet-} \rightarrow HO^{\bullet} + HO^- + O_2 \tag{16}$$

Another possible reaction pathway has been proposed by Kopf et al. [231]. These authors suggested that the photocatalytic ozonation reactions are initiated by an electron transfer from TiO_2 to O_2 (Eqs. 20-22, 25), followed by reaction of $O_2^{\bullet-}$ with O_3 (Eq. 17), giving place to Eqs. 10-11, as well as to Eqs. 18-19.

$$O_3 + O_2^{\bullet-} \rightarrow O_3^{\bullet-} + O_2 \tag{17}$$

$$HO^{\bullet} + R - H \rightarrow R^{\bullet} + H_2O \tag{18}$$

$$HO^{\bullet} + R \rightarrow R^{\bullet} - HO \tag{19}$$

There are some factors that can affect the photocatalytic ozonation process, some of them similar to those influencing the single processes, such as the ozone dosage, pH and nature of the photocatalyst. The DOC removal can increase with the ozone dosage, but low ozone dosages are economically more attractive [232]. The pH of the wastewater has a stronger influence on the ozonation reactions and may impact the degradation pathways and the kinetics in the reaction process, which can also occur in the photocatalytic ozonation process [233, 234]. The synergistic effect between both processes can reduce the treatment costs and reaction time under optimum operating conditions (e.g., ozone concentration, the catalyst properties, the loading in the reactor and the usage of more cost-efficient and long-lasting UV sources, such as UV LEDs) [228]. Photocatalytic ozonation has been studied for the removal of pharmaceuticals in effluents from UWWTPs [95, 204, 235-239]; however, only a few studies were performed under realistic scenarios (real water matrices and OMPs at real concentrations) and not much is known about the potential of photocatalytic ozonation to remove ARB&ARGs from urban wastewater (Table 2.1) [118, 228, 240]. Moreover, despite the high efficiency of ozone-based processes on the inactivation of bacteria and genes, some studies showed that if the inactivation is not effective there is the possibility of bacteria regrowth after the stress status is relieved followed by changes on the bacterial communities which may have implications on the microbiological quality of the disinfected water [81, 116].

2.1.3.2. Photocatalysis

Heterogeneous photocatalysis has been extensively studied in the last decades, not only for water/wastewater and air treatment, but also for production of fuels [17]. However, due to technological implications and costs, it is still considered a perspective method and it was not yet applied at full scale as advanced treatment of UWW [34]. The degradation of a wide variety of OMPs and removal of potentially harmful microorganisms has been explored by numerous authors with very promising results, at both lab and pilot scale, mostly using TiO_2-based materials as catalyst [34]. Still, only few studies have been performed with realistic and non-spiked matrices (Table 2.1). Once again, despite the high efficiency of these processes on the inactivation of bacteria and genes, if the inactivation is not effective there is the possibility of bacteria regrowth after the stress status is relieved followed by changes on the bacterial communities [81, 102].

Heterogeneous photocatalysis is based on the use of wide band-gap semiconductors which generate conduction band electrons and valence band holes (e^-/h^+), and subsequent chain reactions, when irradiated with photons of energy higher than the semiconductor band-gap energy. Electrons are excited from the valence band to the conduction band of the semiconductor, creating an electronic vacancy at the valence edge. These electrons and holes are responsible for the formation of free radicals (such as $HO^\bullet$ and $O_2^{\bullet-}$) and consequent oxidation of the organic compounds or by direct oxidation of the pollutants by photogenerated holes (Figure 2.7) [45, 241, 242]. Transfer of the reactants from the fluid phase to the catalyst surface, adsorption of at least one of the reactants, reaction in the adsorbed phase (photocatalytic reaction), desorption of the products and finally the removal of the products from the interface region are the main independent steps of heterogeneous photocatalysis. In heterogeneous photocatalysis the activation of the catalyst occurs by photonic activation instead of thermal activation [243].

An ideal photocatalyst should be chemically and photochemically stable, should have a high surface area for the adsorption of the reacting species, a high photon absorption coefficient, a small scattering albedo, be easily available and of low cost whenever possible. Generally, the photodegradation rate of OMPs increases with catalyst loading up to an optimum concentration corresponding to the maximum amount of catalyst at which all particles are efficiently irradiated, beyond which the efficiency drops off due to the lower penetration of activating photons through the turbid water [244]. Such process is dependent on reactor geometry, the design of which has been rationalised in terms of optimum dimensionless optical thickness of photoreactors [245]. In the last decades, the most common, commercially available photocatalyst has been titanium dioxide from Degussa-Evonik (TiO_2-P25). This photocatalyst consists of two crystalline TiO_2 phases (ca. 80% anatase and 20% rutile) and it has been tested either in bulk or supported on a substrate, due to its relatively high photoactivity, high mineralization efficiency, low cost and toxicity, high photochemical stability and suitable band-gap energy [246]. The following reactions (Eqs. 20-28) can occur at the surface of a TiO_2 particle [45, 247-250].

$$\mathrm{TiO_2} + hv \rightarrow \mathrm{e^-} + \mathrm{h^+} \tag{20}$$

$$\mathrm{e^-} + \mathrm{O_2} \rightarrow \mathrm{O_2^{\bullet -}} \tag{21}$$

$$\mathrm{h^+} + \mathrm{H_2O} \rightarrow \mathrm{HO^\bullet} + \mathrm{H^+} \tag{22}$$

$$\mathrm{e^-} + \mathrm{O_2} + \mathrm{H^+} \rightarrow \mathrm{HO_2^\bullet} \tag{23}$$

$$\mathrm{h^+} + \mathrm{HO^-} \rightarrow \mathrm{HO^\bullet} \tag{24}$$

$$\mathrm{O_2^{\bullet -}} + \mathrm{H^+} \rightarrow \mathrm{HO_2^{\bullet -}} \tag{25}$$

$$\mathrm{HO^\bullet} + \mathrm{RH} \rightarrow \mathrm{R^\bullet HOH} \tag{26}$$

$$\mathrm{HO^\bullet} + \mathrm{RH} \rightarrow \mathrm{R^\bullet} + \mathrm{H_2O} \tag{27}$$

$$\mathrm{h^+} + \mathrm{RH} \rightarrow \mathrm{RH^{\bullet +}} \tag{28}$$

The first step involves the generation of excited electrons and holes through the absorption of photons by the solid catalyst (Eq. 20). Then, $HO^\bullet$ and $O_2^{\bullet -}$ radicals are produced on the catalyst surface in aerated conditions and in the presence of water molecules and hydroxyl anions (Eqs. 21-24). Superoxide radicals can further react with protons to produce peroxide radicals (Eq. 25). In this way, the organic compound can be oxidized by the radicals or even by the generated holes (Eqs. 26-28).

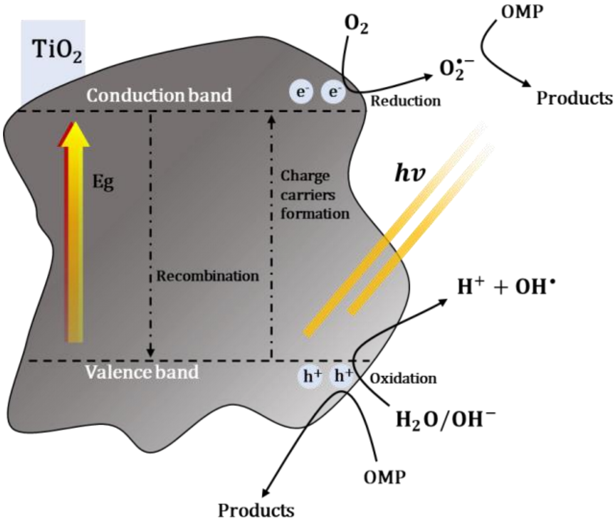


Figure 2.7. Schematic representation of a possible photocatalytic mechanism for the degradation of OMPs using TiO_2 (adapted from [4]).

ZnO has also been largely used mainly due to its higher electronic conductivity and near band-gap energy [251]. However, its susceptibility to photo-corrosion limits its use and thus TiO_2-P25 is still the most used photocatalyst. Although it has higher performance in an aqueous slurry suspension, the separation of TiO_2-P25 from the treated water may in some cases present a major challenge in industrial scale applications [114]. In consequence, a range of support materials has been used for its immobilization, including polymers, metals, silica, carbon materials or ceramics [252]. Ideally, a proper support must also promote the interaction between the photocatalyst and the contaminants, without hindering the absorption of active photons, and should be resistant to the oxidizing conditions [252].

The two major shortcomings of TiO_2-heterogeneous photocatalysis are: (i) the limited absorption of natural sunlight since near-UV radiation is needed for photo-activation and this part represents only ca. 3-5% of the solar radiation reaching the earth surface; and (ii) the relatively high rate of electron-hole pairs recombination which reduces the available charges for the redox reactions. Several studies have been published using different approaches to develop photocatalysts that can address the above shortcoming [90], by creating defect structures on the catalyst [253], by metal deposition on the catalyst surface [254], by semiconductor coupling of the energy bands [255] and by doping with metals/non-metal ions [256]. TiO_2 combinations with noble metals (e.g., silver, gold and platinum) [257] or with carbon-based materials such as graphene-based materials [102, 258] have also been examined. Other carbon materials have been widely studied, including activated carbons [259], carbon xerogels [260], carbon nanotubes [261, 262], graphite [263], and more recently graphitic carbon nitride [264]. Additionally, the electron-hole pairs recombination can be reduced by adding a strong oxidant such as H_2O_2, by accepting an electron from de conduction band [5, 265].

The contaminants concentration and the physical-chemical properties of the water matrix are some aspects affecting the treatment efficiency [266]. The detrimental effect of components co-existing in the water matrix is attributed to the organic and inorganic species intrinsically present in the wastewater [267], including: (i) light attenuation by suspended particles and dissolved species [96]; (ii) the EfOM partially consuming $HO^{\bullet}$ and other oxidizing species, thus competing with the degradation of OMPs [267-273]; (iii) the anions present in wastewater (e.g., bicarbonate, sulphate and chloride) which scavenge $HO^{\bullet}$ to form the respective radicals with lower oxidation potential [244, 267, 268, 270-275]; and (iv) both organics and inorganics adsorbing onto the catalyst surface, fouling and/or competing for the active reaction sites [257, 276-279].

Most of the studies in the literature were performed with mercury vapour or xenon lamps, which overheat and have high implementation and maintenance costs, or environmental implications [17, 121, 129, 280]. To overcome this problem, research in the last years has been focused on the development of photocatalytic processes implementing more energy and cost-efficient light sources, such as LEDs that can emit light at different wavelengths (infrared, visible and UV) [280]. In terms of converting electricity into UV/Vis light, LEDs are far more efficient than commonly applied lamps, and are smaller and more robust. Furthermore, LEDs are non-toxic, inexpensive and have a long lifespan (ca. 100,000 h) [281, 282].

Directly harvesting solar light is an inexpensive and ecological alternative solution that will save the installation and energy consumption costs of an artificial light source [4]. However, sunlight is a not continuously and constantly available light source and the applied commercial semiconductors display poor efficiency under solar light irradiation. Taking this into account, the preparation and characterization of photocatalysts with an absorption spectrum near UV-VIS has been a target of many authors in order to increase the applicability of this technology [283, 284].

Water treatment can also be performed by direct or indirect photolysis. The direct photolysis of organic substances by UV radiation (typically at $\lambda = 254$ nm) promotes the electronic excitation of the molecules, leading to: (i) electron transfer from an excited state of the organic compounds to molecular oxygen; or (ii) homolysis of the organic substance, producing organic radicals that further react with oxygen [155, 172, 249]. Once again, the water matrix can play an essential role in the yield of photodegradation reactions, depending on the presence of promoting and inhibitory substances. Generally, lower contaminants removal rates are expected in wastewater effluents in comparison to an ultrapure water matrix, which can be enlightened by the presence of EfOM absorbing a fraction of the incident radiation [214]. Nevertheless, the wastewater components might enhance the removal rate of certain OMPs by mean of indirect photolysis, resulting from the action of ROS originated by the irradiation of photosensitizers dissolved in the water matrix [285]. The anion NO_3^- may also play an important role in the UV treatment. This inorganic species can generate $HO^\bullet$ and $NO_2^\bullet$

under direct UV photolysis, consequently increasing the degradation of some compounds [286].

2.1.3.3. Fenton-based and H_2O_2-based processes

The conventional Fenton process employs the "Fenton's reagent" [287], a mixture of Fe^{2+} and H_2O_2 that yields $HO^\bullet$ and Fe^{3+} as reaction products (Eqs. 29–30). This reagent combines the use of the fourth most abundant element in the earth's crust, which has environmental compatibility and low-toxicity [288], with the oxidant H_2O_2, which self-decomposition leads to non-toxic products (H_2O and O_2) [289]. The low cost of the Fenton's reagent and the negligible activation energy, coupled to the simplicity of the process, are attractive advantages. However, the regeneration of Fe^{2+} under darkness in the conventional Fenton process is very slow and governed by the following reactions (Eqs. 31-33) [155].

$$\mathrm{Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^\bullet} \tag{29}$$

$$\mathrm{Fe^{2+} + HO^\bullet \rightarrow Fe^{3+} + OH^-} \tag{30}$$

$$\mathrm{HO^\bullet + RH \rightarrow H_2O + R^\bullet} \tag{31}$$

$$\mathrm{R^\bullet + Fe^{3+} \rightarrow R^+ + Fe^{2+}} \tag{32}$$

$$\mathrm{Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2^\bullet + H^+} \tag{33}$$

The main shortcomings of the Fenton process include: (i) the fast depletion of Fe^{2+} and the slow regeneration rate; (ii) the amount of sludge produced needing additional treatment; (iii) the complexation of some iron species; (iv) the potential loss of oxidants by scavenging effect or auto-degradation; and (v) the requirement of a pH between 2.5 and 3.0 to achieve optimal performance and below 4.0 to avoid the iron precipitation [290]. The latter shortcoming originates the need to neutralize the wastewater after treatment, and the consequently raised salt concentration would be deleterious for specific reuses of the treated water, such as for irrigation [156].

Photo-Fenton based processes are assisted by UV-Vis irradiation, accelerating the regeneration rate of Fe^{2+} from Fe^{3+} complexes (Eqs. 34–35). The most photoactive complex $[Fe(OH)]^{2+}$ is predominant at acidic pH, limiting the use of the photo-Fenton process. Temperature is also an important parameter that has an impact on both Fenton and photo-Fenton reaction rates, since raising the temperature accelerates the oxidation of Fe^{2+} by H_2O_2 (thermal Fenton) and enhances the light absorption coefficient of Fe^{3+} [291]. However, the increase of temperature may lead to a higher leaching of iron and consequent deactivation of catalyst in successive cycles, as well as to the thermal decomposition of H_2O_2 to water and oxygen above 40°C [292].

$$[\mathrm{Fe(H_2O)}]^{3+} + h\nu \rightarrow \mathrm{Fe}^{2+} + \mathrm{HO}^{\bullet} + \mathrm{H}^{+} \qquad (34)$$

$$[\mathrm{Fe(HO)}]^{2+} + h\nu \rightarrow \mathrm{Fe}^{2+} + \mathrm{HO}^{\bullet} \qquad (35)$$

The water matrix components may raise a negative and/or a positive impact on the effectiveness of Fenton-based processes and the overall result depends on their balance [293-295]. In complex water matrices containing many species, the degradation of OMPs is generally lower than in pure water when Fenton-based processes are employed, implying the use of higher doses of reactants (iron species and H_2O_2) [296].

UV/H_2O_2 is a simpler AOT combining the instantaneous UV photolytic effect (either direct or indirect) and the reaction with $HO^{\bullet}$ originated from the homolytic disruption of H_2O_2 (Eqs. 36-40) [249, 297].

$$\mathrm{H_2O_2} + h\nu \rightarrow 2\mathrm{HO}^{\bullet} \qquad (36)$$

$$\mathrm{H_2O_2} + \mathrm{HO}^{\bullet} \rightarrow \mathrm{HO}_2^{\bullet} + \mathrm{H_2O} \qquad (37)$$

$$H_2O_2 + HO_2^{\bullet} \rightarrow HO^{\bullet} + O_2 + H_2O \quad (38)$$

$$2HO_2^{\bullet} \rightarrow H_2O_2 + O_2 \quad (39)$$

$$HO^{\bullet} + HO_2^{\bullet} \rightarrow O_2 + H_2O \quad (40)$$

Although H_2O_2 produces $HO^{\bullet}$ under UV irradiation, at elevated concentrations it also scavenges $HO^{\bullet}$ (Eqs. 37-40), hindering the oxidation of the target organic OMPs; therefore, the dose of H_2O_2 needs to be controlled very carefully in order to maximize the removal rate of OMPs [298]. Furthermore, H_2O_2 is a major contributor to the operating cost of the UV/H_2O_2 process [298].

In the H_2O_2-assisted photocatalysis, it is likely that H_2O_2 promotes the degradation of target water contaminants by reacting with conduction band electrons (Eq. 41) and the superoxide radical (O_2^{-}) (Eq. 42) to produce $HO^{\bullet}$ and anions [269]. The formation of additional oxidizing species and the suppression of the recombination of e^{-}/h^{+} pairs, in the case of photocatalysis, enhance the effectiveness of this AOT [269].

$$H_2O_2 + e^{-} \rightarrow HO^{-} + HO^{\bullet} \quad (41)$$

$$HO^{\bullet} + HO_2^{\bullet} \rightarrow O_2 + H_2O \quad (42)$$

Another important factor that may affect the performance of H_2O_2-assisted processes is the concentration of inorganic carbon in the water sample [299]. A high carbonate/ bicarbonate content may scavenge $HO^{\bullet}$, according to the following reactions (Eqs. 43-44) [249].

$$HO^{\bullet} + HCO_3^{-} \rightarrow H_2O + CO_3^{-} \quad (43)$$

$$HO^{\bullet} + CO_3^{2-} \rightarrow HO^{-} + CO_3^{-} \quad (44)$$

The degradation of a wide variety of OMPs and disinfection studies have been explored by numerous authors mostly at lab and pilot scale by using Fenton-based processes with very promising results, even at near neutral pH [106, 151, 161, 162, 172, 173]. Only a small percentage of total published works were performed under realistic conditions (Table 2.1) [102, 106, 149-151, 153, 161, 169].

Table 2.1. Studies dealing with AOTs for UWW treatment, considering only chemical or biological contaminants, or their simultaneous removal. For the present survey only studies dealing with actual concentrations of both pollutants were included, while spiked matrices were excluded (*****except when the study assessed the simultaneous removal of both pollutants). The search comprised publications in Scopus database since 2010 until January 2020, using the following keywords (abstract, title, keywords): “ozonation” or “ozonolysis” or “ozone” or “UV/O_3” or “peroxone” or “photocatalysis” or “Fenton” or “UV/H_2O_2” or “photocatalytic ozonation” or “photo-Fenton” or “solar/H_2O_2” or “photolysis” or “UV_{254}” and “urban wastewater” or “municipal wastewater”.

AOT	Chemical contamination	Biological contamination	Scale of study	Country	Reference
Ozonation Peroxone	8 OMPs [ng - μg L^{-1}]	*E. coli* and antibiotic resistant counterparts to trimethoprim and sulfamethoxazole 16S rRNA, *intI*1, *qacE*Δ1, *sul*1, *aad*A1, *dfr*A1	Lab scale	Portugal	[37]
Ozonation	43 OMPs [ng - μg L^{-1}]	—	Full scale	Switzerland	[40]
Ozonation Photolysis	22 OMPs [ng - μg L^{-1}]	—	Lab scale	Portugal	[43]
Ozonation Photolysis Photolytic ozonation Photocatalysis Photocatalytic ozonation	37 OMPs [ng - μg L^{-1}]	—	Lab scale	Portugal	[62]
Ozonation Photolysis Photolytic ozonation Photocatalysis Photocatalytic ozonation	37 OMPs [ng - μg L^{-1}]	Total heterotrophs, enterobacteria, enterococci, fungi 16S rRNA, *intI*1, *bla*$_{TEM}$, *qnr*S, *sul*1, *van*A	Lab scale	Portugal	[91]
Ozonation Photolysis	—	Total heterotrophs, enterobacteria, enterococci, fungi 16S rRNA, *intI*1, *bla*$_{TEM}$, *qnr*S, *sul*1, *van*A	Lab scale	Portugal	[98]
Photolysis Solar/H_2O_2 Photocatalysis/H_2O_2 Photo-Fenton	*****3 OMPs [Spiked]	Faecal coliforms, enterococci and antibiotic resistant counterparts to tetracycline and ciprofloxacin	Pilot scale	Spain	[102]

		16S rRNA, *intI*1, *qnr*S, *bla*$_{\text{CTX-M}}$, *sul*1, *bla*$_{\text{TEM}}$ Bacterial community analysis			
Photo-Fenton	—	*E. coli*, *E. faecalis*	Lab scale	Spain	[106]
Ozonation	3 OMPs [ng - µg L^{-1}]	—	Full scale	Switzerland	[110]
Ozonation Photolytic ozonation Photocatalytic ozonation	12 OMPs [ng - µg L^{-1}]	—	Lab scale	Portugal	[112]
Ozonation	18 OMPs [ng - µg L^{-1}]	—	Lab scale	Belgium	[111]
Ozonation	95 OMPs [ng - µg L^{-1}]	—	Pilot scale	Sweden	[109]
Ozonation	7 OMPs [ng - µg L^{-1}]	—	Full scale	Germany	[117]
Ozonation	72 OMPs [ng - µg L^{-1}]	—	Lab scale	Spain	[113]
Ozonation	6 OMPs [ng - µg L^{-1}]	—	Full scale	China	[119]
Ozonation	13 OMPs [ng - µg L^{-1}]	—	Pilot scale	USA	[120]
Ozonation	40 OMPs [ng - µg L^{-1}]	—	Pilot scale	Switzerland	[115]
Photocatalytic ozonation	—	*E. coli*, *Salmonella* spp., *Shigella* spp., *Vibrio cholerae*	Lab scale	South Africa	[118]
Ozonation	—	23S rDNA, *ecf*X, *amp*C, *mec*A, 16S rRNA, *erm*B, *van*A, *bla*$_{\text{VIM}}$ Bacterial community analysis	Pilot scale	Germany	[116]
Photocatalysis	52 OMPs [ng L^{-1}]	—	Pilot scale	Spain	[114]
Photocatalysis Photolysis	4 OMPs [ng - µg L^{-1}]	Total heterotrophs, *E. coli*, enterococci and antibiotic resistant counterparts to sulfamethoxazole, azithromycin and ofloxacin, respectively	Lab scale	Portugal	[121]
Photocatalysis	9 OMPs [ng - µg L^{-1}]	—	Lab scale	Portugal	[129]
Catalytic ozonation	*3 OMPs [Spiked]	*E. coli*, *Pseudomonas* spp.	Lab scale	Spain	[127]

Ozonation	–	Enterococci, *Acinetobacter* spp., *E. coli* and extended spectrum beta lactamase producing Gram-negative bacteria *yccT*, *secE*, 23S rRNA, 16S rRNA, *sul1*, *intI*1, *bla*$_{\text{TEM}}$, *tetM*, *CTX-M*, *CTX-M-32*, *bla*$_{\text{OXA}}$-48, *bla*$_{\text{VIM}}$, *CMY-2*, *vanA*, *mcr-1*, *bla*$_{\text{NDM}}$ Metagenome analysis	Full scale	Germany	[80]
Ozonation	–	Flow cytometry with total cell count and intact cell count and intracellular adenosine tri-phosphate analysis	Lab scale	Australia	[126]
Ozonation	15 OMPs [ng - µg L^{-1}]	–	Lab scale	China	[123]
Ozone Photolysis	–	*E. coli*	Lab scale	USA	[122]
Ozonation Photolysis	–	16S rRNA, *intI*1, *sul*1 and *tet*G	Lab scale	China	[134]
Ozonation	41 OMPs [ng - µg L^{-1}]	–	Pilot scale	Canada	[133]
Ozonation	37 OMPs [ng L^{-1}]	–	Pilot scale	Japan	[131]
Ozonation	13 OMPs [ng - µg L^{-1}]	–	Pilot scale	Belgium	[124]
Ozonation	*16 OMPs [Spiked]	Total coliforms	Lab scale	Canada	[130]
Ozonation	22 OMPs [ng - µg L^{-1}]	–	Pilot scale	Spain	[128]
Ozonation Catalytic ozonation	–	*E. coli*	Lab scale	Portugal	[125]
Ozonation	Non-Target screening	–	Full scale	Switzerland	[132]

Ozonation	—	*E. coli*, enterococci and *Pseudomonas aeruginosa* *ycc*T; *ecf*X *23S*-ent Live-dead discrimination analysis Metagenomic analysis	Full scale	UWWTP	[137]
Photolysis Ozonation	13 OMPs [ng L^{-1}]	—	Full scale	China	[139]
Ozonation	15 OMPs [ng - µg L^{-1}]	—	Full scale	France	[136]
Ozonation	22 OMPs [ng L^{-1}]	Total cell counts, *E. coli*	Lab scale Full scale	Switzerland	[140]
Ozonation	10 OMPs [ng - µg L^{-1}]	—	Pilot scale	France	[135]
Ozonation	13 OMPs [ng - µg L^{-1}]	—	Pilot scale	Canada	[138]
Ozonation	2 OMPs [ng L^{-1}]	Total coliforms, *E. coli*	Pilot scale	Italy	[141]
UV/H_2O_2 Ozonation	4 OMPs [µg L^{-1}]	Total coliforms, *E. coli*	Pilot scale	Italy	[142]
Photocatalysis	*3 OMPs [Spiked]	*E. coli* (spiked) *amp*C, *sul*1, *erm*B, *ecf*X, 23S rRNA specific for enterococci	Pilot scale	Cyprus	[143]
Photocatalysis	*2 OMPs [Spiked]	*E. coli*, other coliforms and antibiotic resistant counterparts to azithromycin and ofloxacin; *P. aeruginosa* and antibiotic resistant counterparts to ciprofloxacin and ofloxacin	Lab scale	Portugal	[148]
Photocatalysis	—	Total coliforms, enterococci	Lab scale	Greece	[144]
Photocatalysis	*1 OMP [Spiked]	*E. coli*	Lab scale	Italy	[146]
Photocatalysis UV/H_2O_2	—	*E. coli* and other coliforms	Pilot scale	Spain	[145]

UV/H_2O_2	*1 OMP [Spiked]	*E. coli*, spores of sulphite-reducing clostridia, somatic coliphages and phages infecting the CB390 strain	Lab scale	Spain	[147]
Solar/H_2O_2 Photocatalysis Photo-Fenton Photolysis	—	*E coli*, spores of sulphite-reducing clostridia, somatic coliphages and F-specific RNA bacteriophages	Pilot scale	Spain	[150]
UV/H_2O_2 Photolysis Fenton Photo-Fenton	32 OMPs [ng - µg L^{-1}]	—	Lab scale	Switzerland	[151]
UV/H_2O_2	—	Total coliforms, *E. coli* and antibiotic resistant counterparts to ampicillin, ciprofloxacin and tetracycline 16S rRNA, *bla*$_{TEM}$, *qnr*S, *tet*W,	Lab scale	Italy	[154]
Fenton	51 OMPs [ng L^{-1}]	—	Lab scale	Mexico	[153]
Fenton UV/H_2O_2 Photolysis Photo-Fenton	—	16S rRNA, *sul*1, *tet*X, *tet*G, and *intI*1	Lab scale	China	[160]
Photolysis Fenton UV/H_2O_2 Photo-Fenton	6 OMPs [ng - µg L^{-1}]	—	Lab scale	Switzerland	[155]
Photo-Fenton	28 OMPs [ng - µg L^{-1}]	Total heterotrophs, *E. coli*	Lab scale	Switzerland	[159]
Photo-Fenton	62 OMPs [ng - µg L^{-1}]	—	Pilot scale	Spain	[156]
Photo-Fenton	76 OMPs [ng - µg L^{-1}]	—	Pilot scale	Spain	[157]
Solar H_2O_2 Photo-Fenton	74 OMPs [ng - µg L^{-1}]	Total coliforms, *E. coli*, *Salmonella* spp., and *Enterococcus* spp.	Pilot scale	Spain	[149]
Photo-Fenton	—	Total coliforms, *E. coli*, *Enterococcus* sp. and antibiotic resistant counterparts to cefotaxime	Lab scale	Spain	[152]
Photo-Fenton	17 OMPs [ng - µg L^{-1}]	—	Pilot scale	Spain	[158]
Photolysis UV/H_2O_2 Fenton Photo-Fenton	Venlafaxine [ng L^{-1}]	—	Lab scale	Switzerland	[163]

Photo-Fenton Ozonation	35 OMPs [ng - µg L^{-1}]	—	Pilot scale	Spain	[167]
Photo-Fenton	—	Total coliforms, *E. coli*	Pilot scale	Spain	[168]
Photo-Fenton	10 OMPs [ng - µg L^{-1}]	Total coliforms, *E. coli*, *Enterococcus* sp. and antibiotic resistant counterparts to cefotaxime 16S rRNA, *intI*1, *qnr*, *bla*$_{\text{CTX-M}}$, *sul*1, *bla*$_{\text{TEM}}$.	Pilot scale	Spain	[162]
Photo-Fenton	—	*E. coli*	Lab scale	Italy	[161]
Photo-Fenton	*1 OMP [Spiked]	*E. coli* and resistant counterparts to ampicillin	Lab scale	Italy	[164]
Photolysis Photocatalysis	1 OMP [µg L^{-1}]	—	Lab scale	Italy	[165]
Solar Photo-Fenton	11 OMPs [ng - µg L^{-1}]	—	Pilot scale	Spain	[169]
Photo-Fenton	*7 OMPs [Spiked]	Total heterotrophs, faecal coliforms, *Enterococcus* spp., *P. aeruginosa* and antibiotic resistant counterparts to ofloxacin, erythromycin and trimethoprim 16S rRNA, *qnr*S, *bla*$_{\text{CTX-M}}$, *sul*1, *bla*$_{\text{TEM}}$. *bla*$_{\text{OXA}}$, *tet*M.	Pilot scale	Cyprus	[166]
Photolysis UV/H_2O_2 Photo-Fenton	22 OMPs [ng - µg L^{-1}]	—	Pilot scale	Switzerland	[172]
Photo-Fenton	43 OMPs [ng - µg L^{-1}]	—	Pilot scale	Spain	[175]
Heterogeneous Fenton's catalytic process	6 OMPs [ng - µg L^{-1}]	—	Pilot scale	United Kingdom	[171]
Photocatalysis	—	*E. coli*, *E. faecalis* and spores of *F. solani* (spiked)	Pilot scale	Spain	[170]
UV/H_2O_2 Photolysis	38 OMPs [ng - µg L^{-1}]	—	Lab scale	Japan	[180]

Photo-Fenton	*3 OMPs [Spiked]	*E. coli*, *Klebsiella* spp. and *P. aeruginosa*; *amp*C, *sul*1, *erm*B, *ecf*X, *Enc*	Pilot scale	Cyprus	[174]
UV/H_2O_2 UV/Chlorination	16 OMPs [ng - µg L^{-1}]	—	Pilot scale	Germany	[179]
Photo-Fenton	47 OMPs [ng - µg L^{-1}]	—	Pilot scale	Spain	[178]
Photo-Fenton	52 OMPs [ng - µg L^{-1}]	—	Pilot scale	Spain	[176]
Photo-Fenton	—	Total coliforms, *E. coli* and *Enteroccocus* sp.	Pilot scale	Spain	[173]
UV/H_2O_2	15 OMPs [ng - µg L^{-1}]	—	Lab scale Pilot scale	Germany	[177]
Photo-Fenton	35 OMPs [ng - µg L^{-1}]	—	Pilot scale	Spain	[186]
Ozonation Photo-Fenton Photocatalysis	66 OMPs [ng - µg L^{-1}]	—	Pilot scale	Spain	[189]
Photo-Fenton	35 OMPs [ng - µg L^{-1}]	—	Pilot scale	Spain	[187]
Ozonation	5 OMPs [ng - µg L^{-1}]	—	Pilot scale	South Korea	[181]
UV/H_2O_2 Solar/H_2O_2 Photolysis Ozone Peroxone Photolytic ozonation	—	Total coliforms, *E.coli*, Faecal coliforms	Lab scale	—	[190]
Ozonation	6 OMPs [ng - µg L^{-1}]	—	Pilot scale	USA	[188]
Ozonation	31 OMPs [ng - µg L^{-1}]	—	Pilot scale	USA	[192]
Ozonation	30 OMPs [ng - µg L^{-1}]	—	Pilot scale	Spain	[184]
Ozonation	—	Infectious prions	Lab scale	Canada	[183]
Ozonation Photolysis	—	Total heterotrophs and antibiotic resistant counterparts to tetracycline and sulfamethoxazole	Lab scale	China	[193]

		16S rRNA, *intI*1 *tet*A, *tet*M, *tet*O, *tet*Q, *tet*W, *sul*I, *sul*II			
Ozonation	—	*E. coli,* Total heterotrophs AR towards counterparts to sulfamethoxazole, trimethoprim tetracycline, norfloxacin and ceftazidime 16S rRNA *sul*1 *dfrB2*	Full scale Lab scale	Switzerland	[182]
Ozonation	—	*E. coli*, enterococci staphylococci and antibiotic resistant counterparts to ampicillin, chloramphenicol, ciprofloxacin, erythromycin, vancomycin	Pilot scale	Germany	[185]
Photolysis UV/H_2O_2	25 OMPs [ng - µg L^{-1}]	—	Full scale	Spain	[191]

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Chapter 3

Objectives

Water treatment is a continuous challenge considering the daily input of new contaminants in water bodies. The implemented conventional processes are only able to solve part of the problem, while not being designed for the removal of recalcitrant OMPs neither potentially harmful microorganisms (such as human pathogens and ARB&ARGs). Bearing this in mind, the urge for innovative advanced technologies towards the removal of a wide set of water chemical and biological contaminants has increased in the last years. However, despite the high number of studies dealing with the removal of those pollutants by AOTs, a lack of knowledge still exists in what concerns: (i) actual concentrations of OMPs and naturally occurring potentially harmful microorganisms in UWW effluents (not spiked matrices); (ii) the potential matrix effects on the processes efficiency; and (iii) the potential disturbance of the water bacterial communities which may have relevant ecology implications and should be considered in the design of AOTs. Thus, the present PhD studies aimed:

- To identify and quantify OMPs (some of them considered as CECs and others as PSs), potentially harmful microorganisms (human pathogens, including antibiotic resistance counterparts and their ARGs) naturally occurring in UWW effluents by validated analytical methods;
- To compare different AOTs for the removal of chemical and biologic pollutants from UWW effluents collected after secondary treatment in UWWTPs;
- To perform bacterial community analysis to assess the potential disturbance on the bacterial community composition after the application of AOTs;
- To prepare and characterize structured catalysts (semiconductors and/or carbon materials) having into account the specifications of each treatment process.

Chapter 4

Preliminary results

4.1. Abstract

The present work deals with the treatment of waters and UWW contaminated with antibiotic amoxicillin (AMX) and anti-inflammatory drug diclofenac (DFC) by using different AOTs, namely ozonation, photolysis, photolytic ozonation, photocatalysis and photocatalytic ozonation. The most efficient treatment was also tested for the removal of certain OMPs present at actual concentrations in an UWW effluent. Organic pollutants at concentrations higher than those typically found in conventional UWWTPs were used in these preliminary experiments to determine which process was more efficient in the removal of both organic pollutants and respective reaction by-products. Furthermore, ultrapure water was used to mitigate the matrix effect on the efficiency removal.

DFC was degraded quickly under direct photolysis by artificial light (medium-pressure vapor arc, λ_{exc} > 300 nm), while AMX remained very stable. In the presence of ozone, regardless of the type of process, complete degradation of both organic pollutants was observed in less than 20 min. Photolysis or ozonation on their own led to modest values of total organic carbon (TOC) removal (<6% or 41%, respectively in 180 min), while for photocatalysis (no ozone present) a significant fraction of nonoxidizable compounds remained in the treated water (~15% after 180 min). In the case of photolytic ozonation, the kinetics of TOC removal was slow. In contrast, relatively fast and complete mineralization of AMX and DFC (30 and 120 min, respectively) was achieved when applying the photocatalytic ozonation process. The absence of toxicity of the treated waters was confirmed by growth inhibition assays using two different microorganisms, *Escherichia coli* and *Staphylococcus aureus*. Photocatalytic ozonation was also applied to an UWW spiked with both AMX and DFC. The parent pollutants were easily oxidized, but the TOC removal was only as much as 68%, mainly due to the persistent presence of oxamic acid in the treated sample. The same treatment allowed the effective

degradation of a wide group of OMPs (pesticides, pharmaceuticals, hormones and an industrial compound) detected in nonspiked UWW.

4.2. Materials and methods

AMX (beta-lactam antibiotic used to treat infections caused by a wide range of Gram-positive and Gram-negative bacteria) and DFC (non-steroidal anti-inflammatory drug with strong resistance to biodegradation) were used as model pollutants with a 0.1 mM concentration of each in aqueous solution. In each experiment, the reactor was filled with 250 mL of the model pollutant solution and stirred at 400 rpm. Regarding the ozone experiments, a constant ozone flow rate (150 Ncm^3 min^{-1}) and a constant inlet ozone concentration (50 g Nm^{-3}) were used. In photolysis, the ozone containing stream was replaced by the same flow rate of oxygen, and a Heraeus TQ 150 medium-pressure mercury vapor lamp (UV/Vis >300 nm with dominant emission lines at 314, 366, 405, 436, 546 and 578 nm) was held in a DURAN 50® glass immersion tube with water recirculation. Photocatalytic experiments were performed adding the TiO_2-P25 photocatalyst (0.5 g L^{-1}) to the solution. High performance liquid chromatography (HPLC) was used to determine the concentration of AMX, DFC and reaction by-products (oxalic acid and oxamic acid) concentrations. TOC content was also measured to verify the degree of mineralization achieved by a particular treatment in a Shimadzu TOC-5000A analyser. Ion chromatography analyses were performed in a Metrohm 881 Compact IC Pro apparatus, for quantification of nitrates, nitrites, sulphates and chlorides.

In order to assess if some compounds more toxic than the parent ones were formed during photocatalytic ozonation, growth inhibition assays were carried out in 96-well microtiter plates using *E. coli* DSM 1103 and *S. aureus* DSM 1104 as test strains.

The removal of a set of OMPs found in urban wastewater samples (non-spiked) was evaluated by photocatalytic ozonation, under the experimental conditions described above and testing other two different inlet dosages of ozone (70 and 90 g Nm^{-3}), all in triplicate. Ozonation and photocatalysis were also tested using the previously described experimental conditions. 37 OMPs were analysed by a fully validated method of solid phase extraction followed by ultra-high performance liquid chromatography with tandem mass spectrometry (SPE – UHPLC - MS/MS), before and after the application of AOTs at lab-scale, specifically: DFC, ibuprofen, naproxen, tramadol, azithromycin, clarithromycin, erythromycin, sulfamethoxazole, trimethoprim, fluticasone propionate, montelukast, warfarin, clopidogrel, metoprolol, propranolol, hydrochlorothiazide, atorvastatin, bezafibrate, simvastatin, carbamazepine, citalopram, fluoxetine, norfluoxetine, venlafaxine, diphenhydramine, 17-a-ethinylestradiol, 17-b-estradiol, estrone, alachlor, atrazine, simazine, diuron, isoproturon, chlorfenvinphos, pentachlorophenol, clofibric acid and perfluorooctanesulfonic acid.

4.3. Results and discussion

DFC was rapidly removed under direct photolysis by artificial light, whereas AMX was very stable, what can be explained by the overlap in some extent between the absorption spectrum of DFC and the UV-Vis spectrum of the irradiation reaching the aqueous solution. Complete removal of both organic pollutants was achieved (in less than 20 min) with the processes where ozone was applied. The higher mineralization rates were found for photocatalytic ozonation, achieving a complete TOC removal after 30 and 120 min for AMX and DFC, respectively. Although single ozonation showed high efficiency removal of both parent compounds (AMX and DFC), the concentration of both oxalic and oxamic acids (typical reaction by-products) increased during the experiment, indicating that the degradation of the reaction by-products is difficult by this process. Regarding photolysis, the formation of both acids did not occur, in agreement with the low mineralization levels. For both photocatalysis and photolytic ozonation, the concentration of carboxylic acids increased in the first minutes of reaction and afterwards started to decrease. A similar behaviour was verified for photocatalytic ozonation; though, formed in lower concentrations and removed faster. Considering the above-mentioned results, photocatalytic ozonation was considered the best process for AMX and DFC degradation and their complete mineralization.

Afterwards, to test if AMX and DFC are toxic and if compounds more toxic than the parent pollutants were formed during photocatalytic ozonation, growth inhibition assays were performed using two different microorganisms, *E. coli* and *S. aureus*. Regarding AMX, 15 min of treatment was sufficient to eliminate the antimicrobial properties of the AMX solution, allowing *S. aureus* and *E. coli* to grow. Moreover, by-products of AMX photocatalytic ozonation did not inhibit the growth of these organisms.

In opposition to AMX, both tested organisms grew not only in the presence of the DFC by-products but also in the presence of the parent compound, which can be explained by the fact that DFC is not an antimicrobial compound.

To study the matrix effect on the efficiency of photocatalytic ozonation, experiments were carried out with spiked (AMX and DFC) UWW collected after the secondary treatment from an UWWTP located in Northern Portugal. Once again, a complete degradation of both compounds was achieved in less than 20 min; however, accumulation of oxamic acid occurred. In addition, only 68% decrease of the TOC content was verified, which might be attributed to the consumption of free radicals by inorganic ions and organic matter.

Finally, photocatalytic ozonation (50, 70 and 90 g Nm^{-3}), ozonation (50 g Nm^{-3}) and photocatalysis were applied to treat a non-spiked UWW. Despite the ozone dose employed and the reaction time, all OMPs found in the UWW were completely removed by photocatalytic ozonation. In the case of ozonation and photocatalysis, some OMPs were not completely removed. Moreover, the intermediate oxamic acid generated in these conditions is highly refractory to oxidation.

4.4. Conclusions

The photocatalytic ozonation process was an efficient solution for fast (5 min) breakdown of AMX and DFC into small low weight compounds and, more important, for their complete mineralization (in 30 and 120 min, respectively), which is of major relevance for developing effective water/wastewater treatment technologies. Ozonation, photolysis, photolytic ozonation and photocatalysis were not efficient due to the slow TOC removal and/or the formation of nonoxidizable organic compounds. Oxalic and oxamic acids were identified as the low molecular weight reaction intermediates of AMX and DFC degradation, but they were readily degraded (in less than 20 min) by photocatalytic ozonation. In photocatalytic ozonation of AMX solutions, no activity against *E. coli* and *S. aureus* was determined after 15 min. In addition, for both compounds (AMX and DFC) no toxic by-products were detected for these two microorganisms.

Experiments performed with an UWW from a UWWTP, non-spiked or spiked with AMX and DFC, showed that the intermediate oxamic acid, generated in these conditions is much more refractory to oxidation, in opposition with the results obtained with distilled water, probably due to the presence of oxoanions. Even so, all the identified parent micropollutants (pesticides, pharmaceuticals, hormones and an industrial compound) were removed by photocatalytic ozonation of the non-spiked UWW.

A copy of the full research article with the following reference and reproduced by permission of Elsevier is provided as Appendix A.

> **Moreira, N.F.F.**, Orge, C.A., Ribeiro, A.R., Faria, J.L., Nunes, O.C., Pereira, M.F.R., Silva, A.M.T. *Fast mineralization and detoxification of amoxicillin and diclofenac by photocatalytic ozonation and application to an urban wastewater, Water Research*, 87 (2015) 87-96.

The obtained results herein referred were the driving force and motivation for the following studies presented in chapters 5-7, towards the application of AOTs for the removal of OMPs, potentially harmful microorganisms (human pathogens and ARB&ARGs). These studies were designed as close as possible to realistic scenarios, *i.e.* considering the application of reaction systems operating in continuous mode, the catalyst development and immobilization in proper supports, and the application of more efficient light sources in terms of converting electricity into UV/Vis light, size, robustness, toxicity and lifespan, such as LEDs. Moreover, the impact of AOTs on the bacterial diversity of treated UWW was also studied.

PART II

Advanced wastewater treatment

CONTENT

Chapter 5

Photocatalytic ozonation of urban wastewater and surface water using immobilized TiO_2 with LEDs: Micropollutants, antibiotic resistance genes and estrogenic activity

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The work described in Chapter 5 was published in the following reference and reproduced by permission of Elsevier (the original version is provided as **Appendix B)**:

> **Moreira, N.F.F.**, Sousa, M.J., Macedo, G., Ribeiro, A.R., Barreiros, L., Pedrosa, M., Faria, J.L., Pereira, M.F.R., Castro-Silva, S., Segundo, M.A., Manaia, C.M., Nunes, O.C., Silva, A.M.T. *Photocatalytic ozonation of urban wastewater and surface water using immobilized* TiO_2 *with LEDs: micropollutants, antibiotic resistance genes and estrogenic activity, Water Research*, 94 (2016) 10-22.

Specific nomenclature in Chapter 5:

- TiO_2 corresponds to the commercial TiO_2-P25 material;
- Urban WWTPs corresponds to UWWTP.
- Organic micropollutants corresponds to OMPs.

5.1. Abstract

Photocatalytic ozonation was employed for the first time in continuous mode with TiO_2-coated glass Raschig rings and light emitting diodes (LEDs) to treat urban wastewater as well as surface water collected from the supply area of a drinking water treatment plant (DWTP). Different levels of contamination and types of contaminants were considered in this work, including chemical priority substances (PSs) and contaminants of emerging concern (CECs), as well as potential human opportunistic antibiotic resistant bacteria and their genes (ARB&ARG). Photocatalytic ozonation was more effective than single ozonation (or even than TiO_2 catalytic ozonation) in the degradation of typical reaction by-products (such as oxalic acid), and more effective than photocatalysis to remove the parent micropollutants determined in urban wastewater. In fact, only fluoxetine, clarithromycin, erythromycin and 17-alpha-ethinylestradiol (EE2) were detected after photocatalytic ozonation, by using solid-phase extraction (SPE) pre-concentration and LC-MS/MS analysis. In surface water, this treatment allowed the removal of all determined micropollutants to levels below the limit of detection (0.01–0.20 ng L^{-1}). The efficiency of this process was then assessed based on the capacity to remove different groups of cultivable microorganisms and housekeeping (16S rRNA) and antibiotic resistance or related genes (*intI*1, *bla*$_{TEM}$, *qnr*S, *sul*1). Photocatalytic ozonation was observed to efficiently remove microorganisms and ARGs. Although after storage total heterotrophic and ARB (to ciprofloxacin, gentamicin, meropenem), fungi, and the genes 16S rRNA and *intI*1, increased to values close to the pre-treatment levels, the ARGs (*bla*$_{TEM}$, *qnr*S and *sul*1) were reduced to levels below/close to the quantification limit even after 3-days storage of treated surface water or wastewater. Yeast estrogen screen (YES), thiazolyl blue tetrazolium reduction (MTT) and lactate dehydrogenase (LDH) assays were also performed before and after photocatalytic ozonation to evaluate the potential estrogenic activity, the cellular metabolic activity and the cell viability.

Compounds with estrogenic effects and significant differences concerning cell viability were not observed in any case. A slight cytotoxicity was only detected for Caco-2 and hCMEC/D3 cell lines after treatment of the urban wastewater, but not for L929 fibroblasts.

5.2. Introduction

The environmental occurrence of priority substances (PSs), contaminants of emerging concern (CECs), human pathogens and antibiotic resistance genes (ARGs) is a serious issue affecting ecosystem services (such as drinking water supplies) and urban wastewater treatment plants (WWTPs) are hot spots for such type of contamination [1, 2]. For instance, conventional WWTPs are not specifically designed to eliminate PSs and CECs occurring at trace concentrations, ending into the environment. Among these, specific pesticides, endocrine disrupting compounds, industrial compounds, flame retardants, polycyclic aromatic hydrocarbons, dioxins and dioxin-like compounds, have already been identified as PSs in Directive 2013/39/EU [3]. Furthermore, a watch list of 10 substances/groups of substances that should be monitored within the European Union was recently included in Decision 495/2015/EU [4], namely pharmaceuticals (diclofenac, 17-alpha-ethinylestradiol (EE2) and three macrolide antibiotics), two natural hormones (17-beta-estradiol (E2) and estrone (E1)), some pesticides, an UV filter and a food additive.

Besides the chemical contamination, the biological pollution is also a matter of concern in aquatic environments, in particular in human-impacted areas, calling for adequate responses from WWTPs [5-8]. Conventional WWTPs, although able to remove the bacterial loads up to 2 logarithmic cycles, do not contribute to significant reductions of antibiotic resistance prevalence and, in some cases, can even contribute to its increase [7]. It is estimated that urban conventional WWTPs using distinct types of biological treatments, and operating to achieve the recommended quality thresholds, release at least 10^9 antibiotic resistant coliforms per minute to the surrounding environment [9]. Therefore, the spread of PSs, CECs and antibiotic resistant bacteria and their genes (ARB&ARG) emitted from WWTPs contributes for the contamination of surface and groundwater, existing the possibility that some of these pollutants may reach the drinking water systems [9, 10]. This scenario makes also vulnerable the quality of treated wastewater and, thus, its reuse in activities such as irrigation in agriculture when this wastewater is not properly treated [11]. In this context, new treatment options should be capable of eliminating PSs and CECs and reducing ARGs to levels at which further dissemination will be improbable [1, 12].

Chemical oxidation technologies (COTs) and advanced oxidation processes (AOPs), such as Fenton-based processes, heterogeneous photocatalysis and ozonation-based processes, have been widely studied to eliminate PSs and CECs from different types of wastewater, but most studies are focused on single compounds, normally at concentrations higher than those found in urban wastewater [2]. In addition, photocatalysis (with or without H_2O_2) and the photo-Fenton process were already tested for the inactivation of an antibiotic resistant *Escherichia coli* strain [5, 6, 8]. However, more studies are still needed to better understand the effect of the oxidative approaches on the control of ARB&ARG. For instance, photocatalytic ozonation has been studied for the removal of pharmaceuticals in effluents from urban WWTPs [13-21], and a couple of reviews were published last year [22, 23], but not much is known about the potential of photocatalytic ozonation to remove ARB&ARGs from urban wastewater. Much less information can be found regarding surface water. In fact the literature is still scarce, less than 300 publications being found in Scopus for “photocatalytic ozonation”, against more than 35,500 for “photocatalysis” and 12,000 for “ozonation or ozonolysis”, in January 2016. The high efficiency of the photocatalytic ozonation treatment has been explained by a synergistic effect between ozonation and photocatalysis. The photogenerated electrons can react with ozone molecules generating ozonide radicals while decreasing the possible recombination of electron–hole pairs [22, 24].

Based on this background information, the present study aimed at performing a comprehensive evaluation of the capacity of photocatalytic ozonation to treat wastewater collected after the secondary treatment of an urban WWTP. These experiments were performed in continuous mode implementing an innovative approach with TiO_2-coated glass Raschig rings [25] and light emitting diodes (LEDs). The same process was tested for surface water treatment, aiming at producing water fulfilling the requirements of drinking water. Thus, different levels of contamination and different types of contaminants were considered in this work, including a set of chemical PSs and CECs, some selected from Directive, 2013/39/EU [3] and Decision 495/2015/EU [4], as well as potential human opportunistic pathogens and ARGs. Yeast estrogen screen (YES), thiazolyl blue tetrazolium reduction (MTT) and lactate dehydrogenase (LDH) assays were also performed before and after photocatalytic ozonation to evaluate biological effects, including the potential estrogenic activity, the cellular metabolic activity and the cell viability in fresh and treated waters. Because biological contaminants have the potential to regrow, when the stress conditions imposed by the water treatment processes are relieved, microbiological indicators were also monitored after storage of treated water.

5.3. Materials and methods

5.3.1. Chemicals and materials

TiO_2 (80% anatase and 20% rutile crystalline phases) was provided by Evonik Degussa GmbH (P25). Ultrapure water (resistivity > 18 MΩ cm) was supplied by a Milli-Q water system. Methanol and acetonitrile (MS grade) were acquired from VWR International (Fontenay-sous-Bois, France) whereas methanol and ethanol (HPLC grade) as well as orthophosphoric acid were purchased from Fisher Scientific UK Limited (Leicestershire, UK). Sulphuric acid was purchased from Merck (Darmstadt, Germany). Oxamic acid (>96%) was supplied by Fluka (Buchs, Switzerland).

Anhydrous sodium dihydrogen phosphate, sodium chloride and oxalic acid (>99%) were purchased from Sigma-Aldrich (Steinhein, Germany), as well as all reference standards for liquid chromatography (>98% purity): diclofenac sodium, ibuprofen sodium, naproxen, tramadol hydrochloride, azithromycin dihydrate, clarithromycin, erythromycin, sulfamethoxazole, trimethoprim, fluticasone propionate, montelukast sodium, warfarin, clopidogrel hydrogen sulphate, metoprolol tartrate, propranolol hydrochloride, hydrochlorothiazide, atorvastatin calcium salt trihydrate, bezafibrate, simvastatin, carbamazepine, citalopram hydrobromide, fluoxetine hydrochloride, norfluoxetine oxalate, venlafaxine hydrochloride, diphenhydramine, 17-alpha-ethinylestradiol (EE2), 17-beta-estradiol (E2), estrone (E1), alachlor, atrazine, simazine, diuron, isoproturon, chlorfenvinphos, pentachlorophenol, clofibric acid and perfluorooctanesulfonic acid. Stock solutions of each individual compound (approximately 1000 mg L^{-1}) were prepared in methanol, ethanol or acetonitrile, depending on their solubility, and a working solution (200 µg L^{-1}) was prepared by diluting these solutions in ethanol. The deuterated compounds used as internal standards were also purchased from Sigma-Aldrich (Steinhein, Germany), namely: ketoprofen-d3, ofloxacine-d3, propanolol-d7 solution, fluoxetine-d5 solution, E2-d5 solution and atrazine-d5. A working solution containing 10 mg L^{-1} of all internal standards was prepared by diluting individual stock solutions (1000 mg L^{-1}) in ethanol.

For cell culture experiments, dimethyl sulfoxide (≥99.9%), Dulbecco's phosphate buffered saline (PBS) pH 7.4, Triton™ X-100, thiazolyl blue tetrazolium (MTT) and trypan blue powder were purchased from Sigma-Aldrich. Dulbecco's modified eagle media (DMEM; ref: 31966-021), heat inactivated fetal bovine serum (FBS), penicillin-streptomycin (PenStrep) and trypsin-EDTA were purchased from Gibco® by Life Technologies™ (UK). EndoGRO™ - MV complete media kit was obtained from Merck

Millipore and LDH Cytotoxicity Detection Kit was acquired from Takara Bio Inc. (Shiga, Japan). Caco-2 cell line was purchased to the American Type Culture Collection (ATCC, Wesel, Germany) and used between passage number 35 and 42. hCMEC/D3 cell line was kindly supplied by the Institut National de la Santé et de la Recherche Médicale (INSERM, Paris, France) and used between the passage number 31 to 34. Murine fibroblasts L929 were also obtained from ATCC.

5.3.2. Experimental set-up and procedure

A bubble column reactor (Figure 5.1a; 2.2 I.D. × 60 cm height) equipped with a loop column (Figure 5.1b; 2.2 I.D. × 15 cm height) was used for all the experiments.

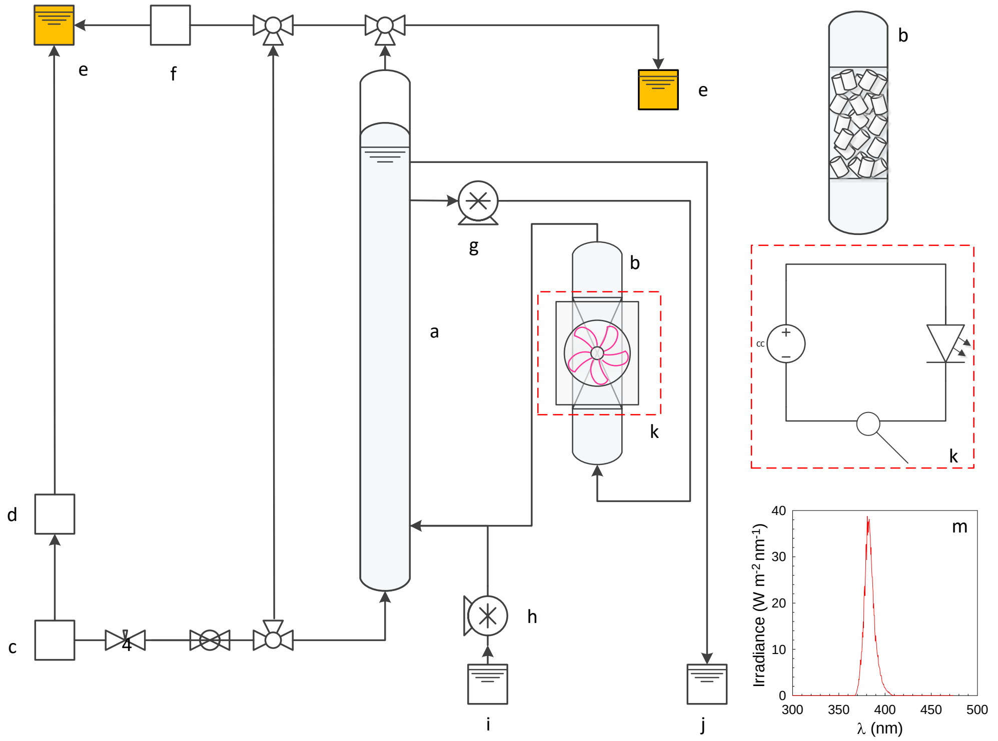

Figure 5.1. Experimental set-up of the homemade photocatalytic reactor able to work in continuous mode: (a) Column for gas-liquid contact; (b) Column for liquid-solid contact (Raschig rings); (c) Ozone generator; (d) Mass flow controller; (e) Ozone gas destroyer; (f) Ozone gas monitor; (g) Peristaltic pump for recirculation; (h) Peristaltic pump; (i) Inlet solution; (j) Outlet solution; (k) LEDs; (m) LEDs irradiance spectrum.

This reactor was designed for ozonation experiments operating in continuous mode [26] and equipped with LEDs in the present work. Two 10 W UV high intensity LEDs (15.5 mm × 23 mm), with dominant emission line at 382 nm and long service life (intensity remains above 70% after 10,000 h work) (Figure 5.1k and m), were placed perpendicularly to each other, irradiating the stream (distance of 5 cm) passing through the loop column, by setting a recirculation flow rate of 60 mL min^{-1} (Figure 5.1g), as performed in a previous work dealing with catalytic ozonation [27]. The wavelength around 382 nm was chosen considering a compromise between the cost of LEDs (increasing when the emission wavelength decreases) and the possibility to achieve an overlapping between the LEDs main emission wavelength and the TiO_2 absorption spectrum. LEDs emitting in UV-C wavelengths can be up to 10 times more expensive than those emitting in near visible wavelengths. In addition, operation at lower wavelengths demands more energy, the LEDs lifetime decreases significantly [28], and energy is wasted in the form of heat. For these reasons, the wavelength near 382 nm was seen as the best option to the system under study, in this way using the minimum energy to activate the TiO_2 photocatalyst.

The loop column was packed with seventy three TiO_2-coated glass Raschig rings (Figure 5.1b) that were prepared as described elsewhere [25], from a procedure adapted from literature [29]. Uncoated Raschig rings were used in non-catalytic assays. In photolysis and photocatalytic experiments, the LEDs were switched on at the beginning of the experiment, with a constant oxygen flow rate (15 $\text{Ncm}^3\ \text{min}^{-1}$). In ozonation and photocatalytic ozonation assays, a BMT 802X ozone generator (Figure 5.1c) was used to produce ozone (from pure oxygen) at a constant inlet concentration (50 g Nm^{-3}) and flow rate (15 $\text{Ncm}^3\ \text{min}^{-1}$). A BMT 964 ozone analyser (Figure 5.1f) was used to control the concentration of ozone in the gas phase. Gas washing bottles filled with potassium iodide solution were used to remove the ozone leaving the reactor in the gas phase (Figure 5.1e).

Regardless of the type of test performed, the reactor was always filled with ultrapure water and the experiment started (t = 0 min) when the water to be treated (wastewater or surface water) (Figure 5.1i) was pumped to the reactor (Figure 5.1h), *i.e.*, being diluted until achieving the steady state. Thus, since the reactor was fully filled with ultrapure water before starting the experiment with a realistic matrix, some dilution occurred up to 90 min in every run. This reactor is designed to operate in continuous mode, with a continuous entrance of realistic matrix at the bottom of the biggest column (liquid flow rate of 15 mL min^{-1}), a continuous outlet stream of treated surface water/wastewater at the top of the same column, and a continuous recirculation (60 mL min^{-1}) between the biggest column and the smaller column holding the photocatalyst, in this way simulating a perfectly mixed reactor. Thus, the removal efficiency was inferred when the steady state was achieved, *i.e.* when the outlet solution had always the same concentration and there was no more dilution effect. For this reason, preliminary studies on hydraulic retention time (HRT) were performed using NaCl as tracer (inlet concentration of 2000 mg L^{-1}) and uncoated Raschig glass rings were placed randomly inside the loop column, measuring the conductivity with a conductimeter Crison GLP 31 (Barcelona, Spain). The gas and liquid streams were maintained at 15 $\text{Ncm}^3\ \text{min}^{-1}$ and 15 mL min^{-1}, having into account preliminary ozonation experiments with different ozone flow rates (using oxalic acid as prove molecule) and that these conditions were optimized for catalytic ozonation in a previous work [27]. The HRT studies allowed to determine the time needed to achieve the steady state and the time spent by the fluid inside the reactor (retention time). Figure 5.2 shows the evolution of the normalized conductivity (σ/σ_t) where σ is the conductivity of the outlet solution and σ_t the conductivity of the tracer injected in the column. The time needed to achieve the steady state is ca. 90-100 min and the retention time (26 min) was determined from the area under the representation of $1-(\sigma/\sigma_t)$ vs. time. Other run was performed with vertically aligned uncoated glass rings, but the results were similar to those obtained with randomly distributed glass rings. Having into account these results, the next experiments were performed during 180 min with randomly distributed Raschig glass rings (retention time = 26 min).

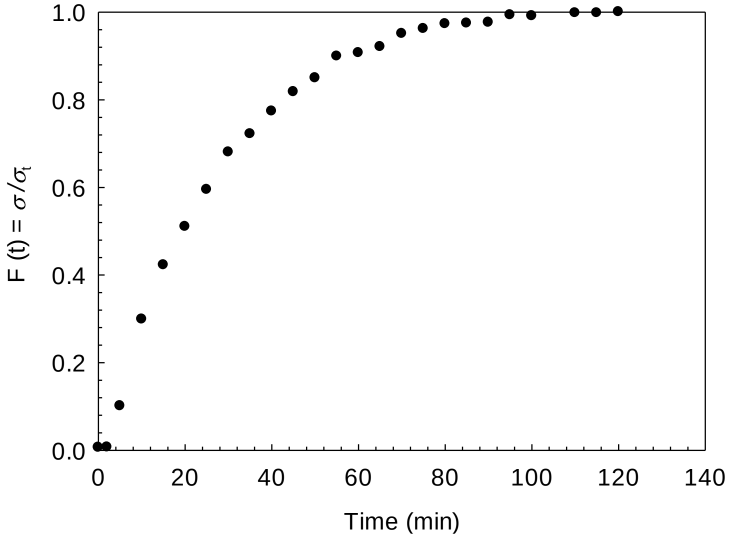

Figure 5.2. Normalized conductivity (σ/σ_t) of the outlet solution, introducing an inlet NaCl solution (2000 mg L^{-1}). σ is the conductivity of the outlet solution and σ_t the conductivity of the tracer injected in the column.

The first set of experiments with chemical pollutants was performed with ultrapure water spiked with diclofenac (32 mg L^{-1}; pH = 4.5) and the removal of this pharmaceutical was followed over time by analysing the treated effluent (Figure 5.1j). In a second set of experiments, wastewater samples collected after the activated sludge biological treatment of an urban WWTP located in Northern Portugal were treated by ozonation, photocatalysis and photocatalytic ozonation (n = 3, *i.e.* a total of three samples for each treatment, each sample collected in a different day). For the catalytic treatments, coated Raschig glass rings were replaced between experiments. The sampling (April-May 2015) was performed using pre-rinsed amber glass bottles (2.5 L). Surface water samples were collected (May-June 2015) in the supply area of a drinking water treatment plant (DWTP) and treated by photocatalytic ozonation (n = 3). All samples were transported at 4 ºC to the laboratory and processed immediately.

5.3.3. Materials characterization

TiO_2-coated glass rings were characterized before and after the photocatalytic ozonation treatment. Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS) were performed in a FEI Quanta 400 FEG ESEM/EDAX Genesis X4M instrument. The materials hydrophilicity was assessed by measuring the water contact angle in an Attension equipment (Theta model) which allows image acquisition and data analysis. The measurements were performed at room temperature using the sessile drop method in five different locations over glass rings. Thermogravimetric (TG) analysis was performed using a STA 490 PC/4/H Luxx Netzsch thermal analyser by heating the samples from room temperature until 800 ºC at 10 ºC min^{-1} under helium flow, holding this temperature for 7 min and finally changing to air atmosphere during 13 min.

5.3.4. Chemical analysis

High performance liquid chromatography with diode array detection (HPLC-DAD) was performed to analyse the removal of diclofenac, using a Hitachi Elite Lachrom apparatus equipped with a YMC Hydrosphere C18 column (250 mm × 4.6 mm i.d.), working at room temperature under isocratic elution. The mobile phase consisted in a mixture of phosphate buffer solution (pH 2.8) and methanol (30/70, v/v). The flow rate was 1 mL min^{-1} and the volume of injection was 15 μL. A pH meter pHenomenal® pH 1100L (VWR, Germany) was used for all pH adjustments. The oxalic and oxamic acid concentrations were determined by HPLC-UV, using an Altech AO-1000 column (300 mm × 6.5 mm i.d.) operating under isocratic elution with 5 mM H_2SO_4 at 0.5 mL min^{-1}. The total organic carbon (TOC) content was determined using a Shimadzu TOC-5000A analyser. The concentrations of the target micropollutants (listed in section 5.3.1) were determined in urban wastewater and surface water samples, before and after the treatment by the advanced oxidation processes, using a fully validated method of solid phase extraction (SPE) and ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) in a Shimadzu Corporation apparatus (Tokyo, Japan), as described elsewhere [30]. Wastewater (100 mL) and surface water (250 mL) samples were acidified to pH 3 with sulphuric acid, followed by extraction of the micropollutants using Oasis® HLB (Hydrophilic-Lipophilic-Balanced sorbent, 150 mg, 6 mL) cartridges (Waters, Milford, Massachusetts, USA). For internal calibration, isotopically labelled internal standards were added to the samples before SPE.

5.3.5. Microbiological characterization, DNA extraction and qPCR analysis

Wastewater and surface water samples were characterized for the abundance of different groups of cultivable microorganisms, quantification of selected antibiotic resistance and related genes (herein referred to ARG), before and immediately after photocatalytic ozonation, and after three days of water storage at room temperature. For each photocatalytic ozonation experiment, all assays were performed in triplicate. The membrane filtration method was used for the enumeration of cultivable microorganisms. Briefly, adequate serial dilutions of water samples were filtered through cellulose membrane filters (0.22 µm porosity; Whatman, UK) and incubated on culture media targeting different microbial groups: Plate Count Agar (PCA) (Merck, 30 ºC, 1-7 days) for total heterotrophs; membrane Fecal Coliforms (Difco, 37 ˚C, 1 day) for enterobacteria; m-Enterococcus agar (Difco, 37 ºC, 2 days) for enterococci; and Rose Bengal Chloramphenicol agar (VWR International, 30 ºC, 7 days) for fungi. Additionally, PCA supplemented with ciprofloxacin (4 mg L^{-1}), gentamicin (16 mg L^{-1}) or meropenem (4 mg L^{-1}) were used to assess resistance prevalence in surface water before and after treatment. These antibiotics were selected because resistance to carbapenems (e.g., meropenem) and aminoglycosides (e.g., gentamicin) is commonly found in drinking water bacteria or because selection of fluoroquinolone resistance (e.g., ciprofloxacin) is sometimes observed in aquatic environments [9]. For culture-independent assays, total DNA was extracted from water samples with volumes ranging from 100 mL to 1 L, after filtration through polycarbonate membranes (0.22 µm porosity; Whatman, UK), using the commercial kit PowerWater® DNA Isolation (MO BIO Laboratories, Inc., USA). DNA extracts were cryopreserved at −20 °C until the analyses of the abundance of ARG. Quantitative PCR (qPCR) was used according to the conditions indicated in Table 5.1, as described elsewhere [31].

Table 5.1. Conditions used in quantitative PCR assays.

Gene	Primers	Reference	Efficiency (%)	Conditions	Reference
16S rRNA	1114F (CGGCAACGAGCGCAACCC) 1275R (CCATTGTAGCACGTGTGTAGCC)	*Escherichia coli* ATCC 25922	100	95 ºC for 10 min (1 cycle) 95 ºC for 15 s, 55 ºC for 20 s and 72 ºC for 10 s (35 cycles) Other: 1a	[32]
bla$_{TEM}$	*bla*$_{\text{TEM-F}}$ (TTCCTGTTTTTGCTCACCCAG) *bla*$_{\text{TEM-R}}$ (CTCAAGGATCTTACCGCTGTTG)	*Escherichia coli* (A2FCC14)	96	95 ºC for 10 min (1 cycle) 95 ºC for 15 s, 60 ºC for 30 s and 72 ºC for 10 s (40 cycles) Other: 2a	[33]
*intI*1	*int*I1-F (CCTCCCGCACGATGATC) *int*I1-R (TCCACGCATCGTCAGGC)	*Escherichia coli* (A2FCC14)	94	95 ºC for 10 min (1 cycle) 95 ºC for 15 s, 55 ºC for 30 s and 72 ºC for 10 s (40 cycles) Other: 1a	[34]
*qnr*S	*qnr*SrtF11(GACGTGCTAACTTGCGTGAT) *qnr*SrtR11 (TGGCATTGTTGGAAACTTG)	*Enterobacter cloacae* (S1+)	95	95 ºC for 5 min (1 cycle) 95 ºC for 15 s – 60 ºC for 1 min (40 cycles) Other: 2c	[35]
*sul*1	*sul*1-FW (CGCACCGGAAACATCGCTGCAC) *sul*1-RV (TGAAGTTCCGCCGCAAGGCTCG)	*Achromobacter* sp.	94	95 ºC for 5 min (1 cycle) 95 ºC for 15 s – 60 ºC for 1 min (40 cycles) Other: 2b	[36]
*van*A	*van*A3FP (CTGTGAGGTCGGTTGTGCG) *van*A3RP (TTTGGTCCACCTCGCCA)	*Enterococcus faecalis* (H1EV23)	98	95 ºC for 5 min (1 cycle) 95 ºC for 15 s – 60 ºC for 1 min (40 cycles) Other: 2a	[37]

1) KAPA SYBR® FAST ABI Prism® qPCR Master Mix; 2) SYBR® Select Master Mix; a) 200 nM of primer; b) 400 nM of primer; c) 600 nM of primer.

5.3.6. Biological assays

5.3.6.1. Yeast estrogen screen (YES) assay

The YES assay was performed using the commercially available kit XenoScreen YES/YAS assay (Xenometrix, Hégenheim, France). Water samples (wastewater and surface water) collected before and after photocatalytic ozonation were filtered through 0.45 µm hydrophilic membranes and directly analyzed, without any pre-concentration. The assay and potential estrogenic activity calculations were carried out following the manufacturer's instructions. Briefly, samples were transferred to a 96-well microplate, mixed with assay medium and inoculated with the transformed yeast cells. Assay mixture consisted of growth medium containing the chromogenic substrate chlorophenol red-ß-d-galactopyranoside (CPRG). The natural estrogen E2, at concentrations between 10^{-6}-10^{-9} M, was used as positive control and standard. Ultrapure water was used as negative control. A solvent blank was also assayed. The plate was incubated for 48 h at 31 ºC with orbital shaking. After incubation, spectrophotometric measurements were performed in a microplate reader (Synergy HT, Bio-Tek Instruments, Winooski, USA) at 690 nm for growth and at 570 nm for expression of ß-galactosidase. The potential estrogenic activity was determined through the calculation of the parameters growth factor (G) and induction ratio (IR). The G parameter was calculated as the ratio of absorbance at 690 nm for the sample $(A_{690})_{sample}$ and the absorbance at 690 nm for the solvent $(A_{690})_{solvent}$. The IR parameter was calculated as $(1/G) \times ((A_{570} - A_{690})_{sample}/(A_{570} - A_{690})$solvent).

5.3.6.2. Thiazolyl blue tetrazolium reduction (MTT) and lactate dehydrogenase (LDH) assays

Thiazolyl blue tetrazolium reduction (MTT) assay evaluates cellular metabolic activity while lactate dehydrogenase (LDH) assay provides information about cell viability through evaluation of membrane integrity. Cell culture procedures are described in detail elsewhere [38]. Briefly, samples were diluted 1:2.5 in culture medium. Afterwards, 100 µL of diluted sample were added to cell layers or suspension, containing 100 µL of culture medium. After 24 h, the supernatant was removed for LDH assay, while the remaining content was used for MTT assay. For MTT assay, absence of cytotoxicity (100%) was evaluated by replacing test sample by culture medium. For LDH assay, absence of cell viability (100%) was evaluated by replacing test sample by 1% (v/v) Triton X-100 solution in PBS.

5.4. Results and discussion

5.4.1. Degradation of organic micropollutants

The first set of experiments with chemical pollutants was performed using ultrapure water spiked with diclofenac as model compound, due to the known high occurrence of this pharmaceutical in the environment, its recalcitrance, and inclusion in the watch list for European Union monitoring in the field of water policy [4]. The evolution of the normalized concentration of diclofenac is shown in Figure 5.3a, the time needed to achieve the steady state in all the experiments being around 90–100 min, as concluded from HRT studies (Figure 5.2). Figure 5.3a shows that complete removal of this compound was achieved in all the experiments where ozone was employed (ozonation, photolysis assisted ozonation, catalytic ozonation, and photocatalytic ozonation). For (non-catalytic) photolysis, the outlet and inlet diclofenac concentrations were practically the same in steady state. This result was expected since this model compound absorbs maximally radiation at wavelengths below 325 nm while the radiation peak of the used LEDs was centered at 382 nm, above 367 and below 410 nm (Figure 5.1m). In the case of TiO_2-coated glass rings photocatalysis, a modest diclofenac removal in steady state was observed (ca. 26%; *i.e.* $C/C_0 = 0.74$).

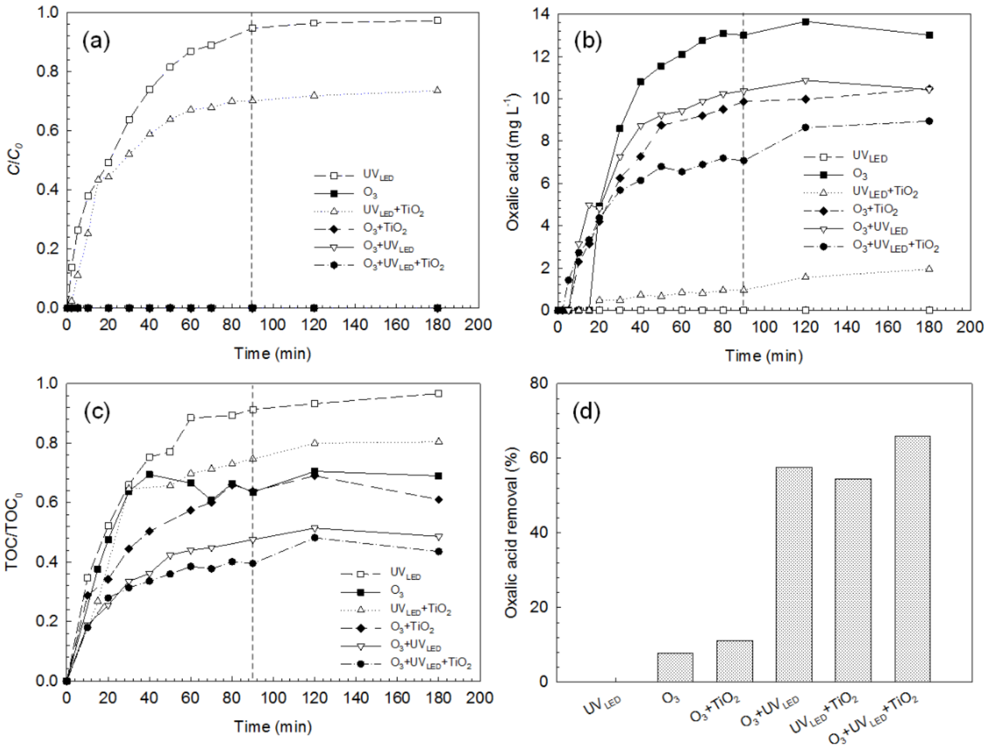

Figure 5.3. (a) Normalized concentration (C/C_0) of diclofenac in ultrapure water (C_0 = 32 mg L^{-1}), (b) evolution of its oxalic acid by-product concentration, and (c) respective normalized TOC content (TOC/TOC$_0$) under different advanced processes. (d) Removal of oxalic acid when used as feed (C_0 = 90 mg L^{-1}). Experiments started with the column filled with ultrapure water only (without pollutant at t = 0 min), the steady state was obtained at ca. 90 min and the retention time is 26 min.

Oxalic and oxamic acids are well known reaction products during ozonation of larger molecules [39], and thus they were also analysed, but only oxalic acid was detected above the limit of detection – LOD (Figure 5.3b). As expected, oxalic acid was not formed during photolysis, while photocatalysis originated a low amount of this acid in steady state (ca. 2.0 mg L^{-1}), which is probably related to the poor efficiency of this process for the degradation of diclofenac. In contrast, ozonation alone led to the largest amount of oxalic acid (ca. 13.0 mg L^{-1}), followed by photolysis assisted ozonation and catalytic ozonation (ca. 10.5 mg L^{-1}), and then by photocatalytic ozonation (ca. 9.0 mg L^{-1}).

TOC analysis was performed to account for the presence of other organic compounds that can be formed during the degradation of diclofenac (Figure 5.3c). Once again, photolysis and photocatalysis were the least effective treatments, with TOC removals of ca. 3 and 20% in steady state, respectively, while photolysis assisted ozonation and photocatalytic ozonation were the best performing options in terms of TOC removal (ca. 51% and 56%, respectively).

Since oxalic acid was the main by-product detected from diclofenac degradation, additional experiments were performed having oxalic acid only (90 mg L^{-1}) in ultrapure water as feed (Figure 5.3d). Oxalic acid was completely recalcitrant in the photolysis assay, while low removal values were obtained with ozonation (8%) and catalytic ozonation (11%). All the other processes (photocatalysis, photolysis assisted ozonation and photocatalytic ozonation) were quite effective in the removal of oxalic acid (55%, 58% and 66%, respectively). Even so, photocatalytic ozonation was more efficient than photolysis assisted ozonation for the degradation of oxalic acid, probably due to the overlap between the absorption spectrum of TiO_2 and the emission spectrum of the LEDs, enough to get the minimum energy needed to surpass the energy band gap of TiO_2. In batch conditions [18], it was already observed that oxalic acid (one of the main reaction by-products in ozonation-based processes) is oxidized efficiently by photocatalytic ozonation, while photolysis assisted ozonation was not able to remove the oxalic acid accumulated. In addition, it is known that UV at 382 nm (far from UV-C) is inefficient for disinfection in the absence of a photocatalyst [40]. Thus, considering the targeted simultaneous treatment of both chemical and biological contaminants, and since a photocatalyst can increase the process disinfection efficiency by the formation of highly reactive radicals, photocatalytic ozonation was applied to treat wastewater samples collected after the secondary biological treatment of an urban WWTP. Ozonation and photocatalysis experiments were also performed in order to assess the individual contribution of each process on their own.

The micropollutants (pharmaceuticals, a metabolite, hormones, pesticides and an industrial compound listed in section 5.3.1) were analysed (before and after the treatment) by UHPLC-MS/MS, after pre-concentration of the samples by SPE. Figure 5.4 shows the concentration range for the detected micropollutants in the effluent of the biological treatment used in the urban WWTP during the sampling campaign (April–May 2015). The highest concentrations were observed for the following compounds with a frequency of detection of 100% (9/9): the anti-inflammatory naproxen, the diuretic hydrochlorothiazide, the lipid regulator bezafibrate and the synthetic hormone EE2 included in the watch list of the Decision 495/2015/EU [4]. Diclofenac, azithromycin, clarithromycin and erythromycin, also included in the watch list, as well as ibuprofen, clopidogrel, propranolol, atorvastatin, carbamazepine, fluoxetine and norfluoxetine, were quantified in all samples. Citalopram and venlafaxine were also detected, but not always above the limit of quantification (LOQ), whereas sulfamethoxazole, trimethoprim, diphenhydramine, E2 and isoproturon were not detected in some samples.

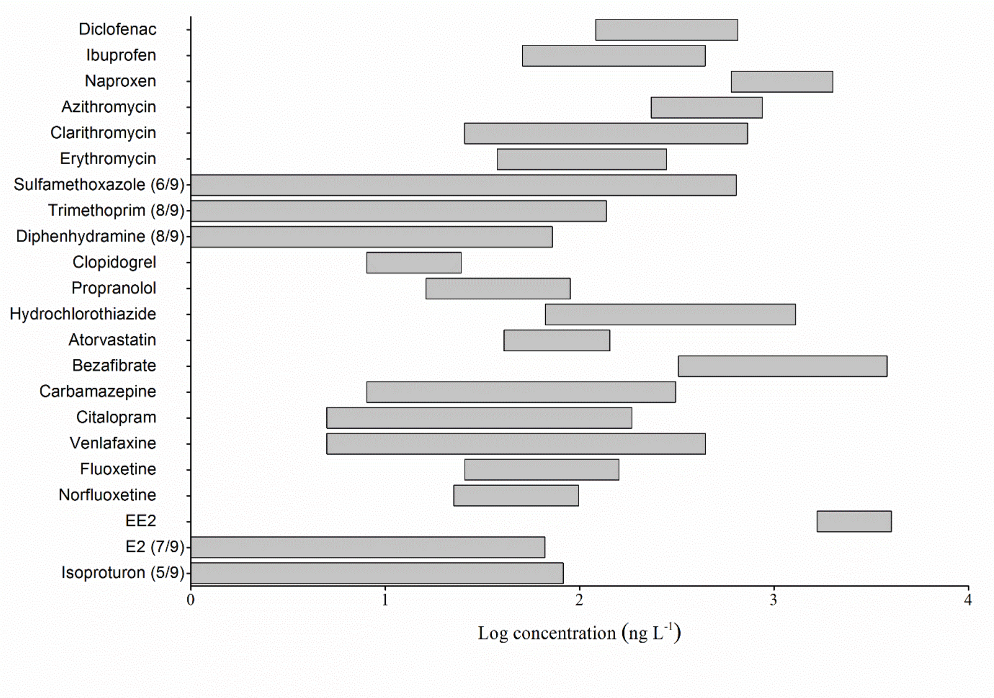

Figure 5.4. Logarithmic range of concentrations (ng L^{-1}) of the detected micropollutants in WWTP samples collected after the secondary biological treatment. The frequency of occurrence was 100 % (9/9) for all compounds, except when indicated.

Figure 5.5. shows that the higher efficiency to remove the detected micropollutants was found for ozonation and photocatalytic ozonation. Photocatalysis was comparatively less effective in the degradation of these micropollutants. Only fluoxetine, clarithromycin, erythromycin and EE2 were detected in the outlet solution after photocatalytic ozonation. While both antibiotics were only detected in the final effluent of one of the triplicate experiments, fluoxetine and EE2 were detected twice. Naproxen was only quantified in one replicate assay. It is also important to note that samples were collected daily for each experiment. Regarding the TOC removal, the initial TOC of ca. 25 mg L^{-1} decreased to 19 mg L^{-1} in steady state conditions of photocatalytic ozonation. The initial and final pH values were quite similar (7.4 and 7.2, respectively) while the low weight carboxylic acids and ions were below the LOQs. Thus, both single ozonation and photocatalytic ozonation treatments were quite effective to remove the chemical

organic micropollutants (Figure 5.5), but it should be also taken into account that ozonation is typically much less effective than photocatalytic ozonation in the mineralization (Figure 5.3c) and degradation of typical reaction by-products (Figure 5.3b). Among different treatments described elsewhere in batch conditions [18], photocatalytic ozonation was considered the best performing process: a fast mineralization of amoxicillin and diclofenac was observed in spiked urban wastewater; toxic intermediate products for *Escherichia coli* and *Staphylococcus aureus* were not detected; and the parent micropollutants determined were removed from an urban wastewater.

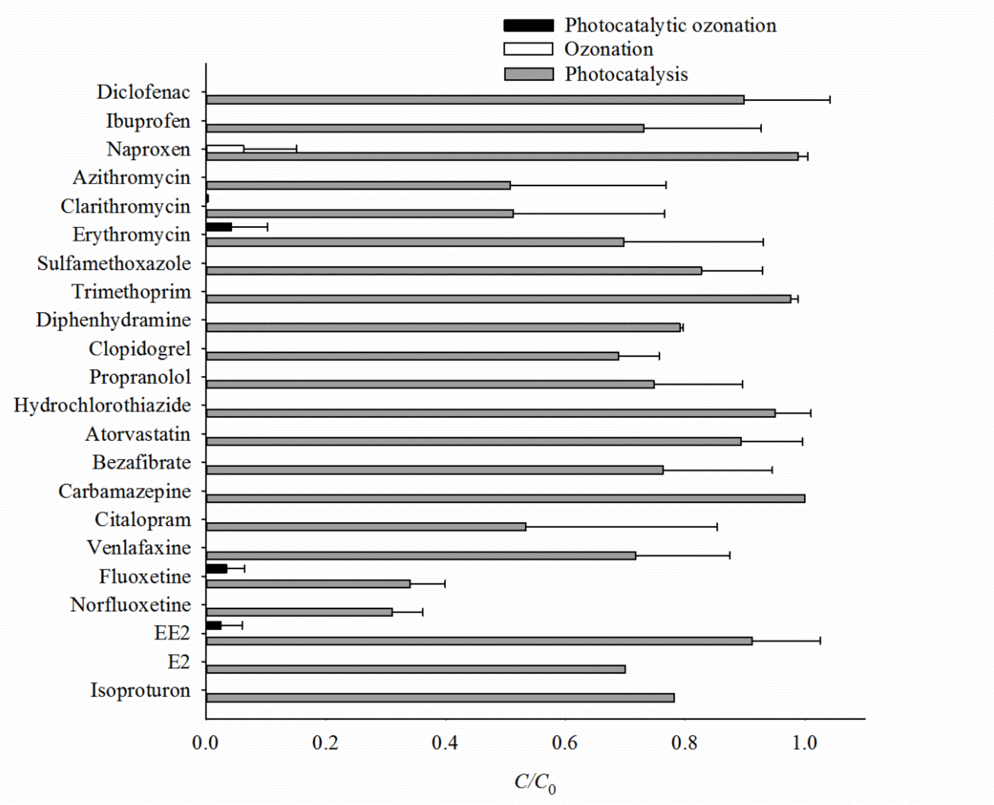

Figure 5.5. Normalized concentration of micropollutants (C/C_0) in the effluents from urban WWTPs treated by photocatalysis, ozonation and photocatalytic ozonation; C_0 refers to the concentration before treatment and C to that after treatment (retention time: 26 min); compounds with C_0 below the LOD before treatment are not shown.

Photocatalytic ozonation, the most efficient process for the degradation and mineralization of chemical pollutants in urban wastewater, was also tested to treat surface water samples collected near a DWTP. The micropollutants detected after pre-concentration of the samples by SPE were: clarithromycin, fluoxetine, norfluoxetine, carbamazepine, EE2, E2 and isoproturon (Table 5.2). The antibiotic clarithromycin, recently included in the watch list of substances for monitoring [4], was quantified at trace concentrations (up to 4.13 ng L^{-1}). Fluoxetine was also detected in all samples, but only quantified in one sample (at 5.72 ng L^{-1}), while its human metabolite norfluoxetine was detected once in the same sample, with a higher concentration (7.66 ng L^{-1}) than the parent compound. The highest levels were obtained for the antiepileptic carbamazepine (up to 56.2 ng L^{-1}) and for the pesticide isoproturon (up to 24.3 ng L^{-1}), the last considered as PS [3]. All these micropollutants were completely removed by photocatalytic ozonation. Although not quantifiable, EE2 and E2 were detected before photocatalytic ozonation and completely removed. Therefore, photocatalytic ozonation was observed as an efficient treatment for the degradation of organic micropollutants in both urban wastewater and surface water.

Table 5.2. Concentration of the micropollutants detected in surface water collected during three sampling campaigns, between May and June 2015, and normalized concentration after treatment (C/C_0).

	C_0 (ng L^{-1})	C/C_0	C_0 (ng L^{-1})	C/C_0	C_0 (ng L^{-1})	C/C_0
Clarithromycin	4.13	0	1.36	0	2.11	0
Fluoxetine	5.72	0	<LOQ	0	<LOQ	0
Norfluoxetine	7.66	0	<LOD	n.a.	<LOD	n.a.
Carbamazepine	<LOQ	0	35.1	0	56.2	0
E2	<LOQ	0	<LOQ	0	<LOQ	0
EE2	<LOD	n.a.	<LOQ	0	<LOQ	0
Isoproturon	<LOQ	0	24.3	0	21.3	0

LOD: limit of detection; LOQ: limit of quantification; n.a.: not applicable.

5.4.2. TiO_2-coated glass rings characterization

The TiO_2-coated glass rings (shown in Figure 5.6a) were characterized by different techniques before and after photocatalytic ozonation of the urban wastewater samples.

A negligible weight loss (WL) was obtained by TG analysis of the fresh TiO_2-coated glass rings (not shown), regardless of the atmosphere employed (helium or air). In the case of the rings recovered after photocatalytic ozonation, desorption of water occurred below 100 °C under helium atmosphere while 4% of the weight decreased when the gas phase was shifted from helium to air at 800 ºC, most probably due to the oxidation of some organic matter remaining in the surface of these rings after photocatalytic ozonation. SEM micrographs, EDS spectra and contact angles of water droplets over the glass rings are shown in Figure 5.6b and c, respectively before and after the photocatalytic ozonation treatment. TiO_2 presence is evident in both samples, with Figure 5.6d showing a higher SEM magnification of the TiO_2 particles. It is also possible to conclude that TiO_2-coated glass rings are highly hydrophilic since very low contact angles were obtained (<15º). Overall, no significant differences were observed between the fresh and used samples, even considering the high matrix complexity of effluents from urban WWTPs.

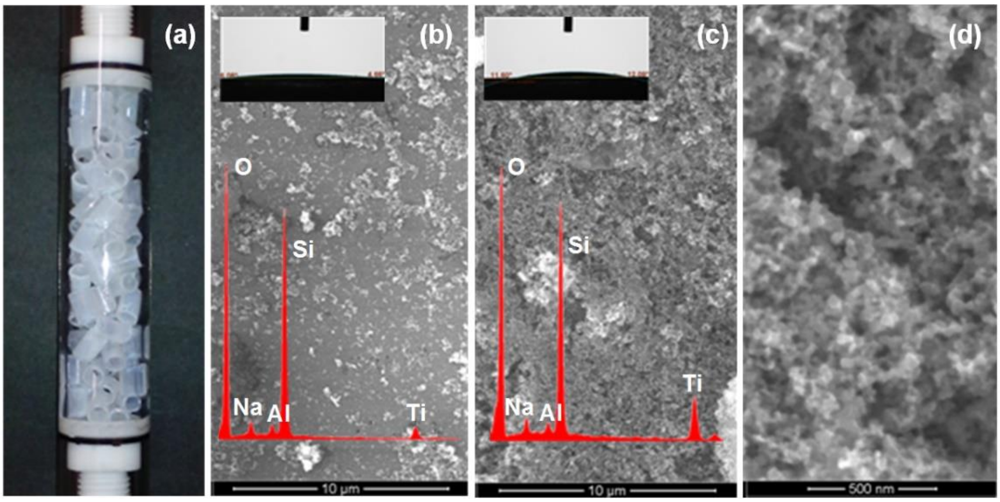

Figure 5.6. (a) TiO_2-coated glass Raschig rings and respective (b, c, d) SEM micrographs, EDS and contact angles (b) before and (c, d) after photocatalytic ozonation.

5.4.3. Microbial inactivation and reactivation

The photocatalytic ozonation of secondarily treated wastewater showed the reduction of enterococci, enterobacteria, and fungi from ~10^5 – 10^6 colony forming units (CFU) per 100 mL to values around or below ~10^1 CFU/100 mL. In contrast, total heterotrophs that may comprise bacteria with different levels of endurance, such as endospore producers, presented lower reductions, with about ~10^2 CFU/100 mL being detected after the advanced treatment (Figure 5.7a). These results were confirmed in the assays testing the regrowth capacity, with significantly higher counts of total heterotrophic populations and fungi than of the other microbial groups analysed. Regrowth may have resulted from the combination of treatment survival and capacity to use any available carbon and energy sources. Indeed, the occurrence of microbial regrowth after water treatment has been attributed to the presence of assimilable organic carbon, generated during the oxidation of recalcitrant organic matter [41, 42]. The comparison of the regrowth capacity in the presence of light or in the dark, suggested that light may have facilitated the recovery of cell injuries, mainly in bacterial heterotrophs and fungi (Figure 5.7a), as previously described [43].

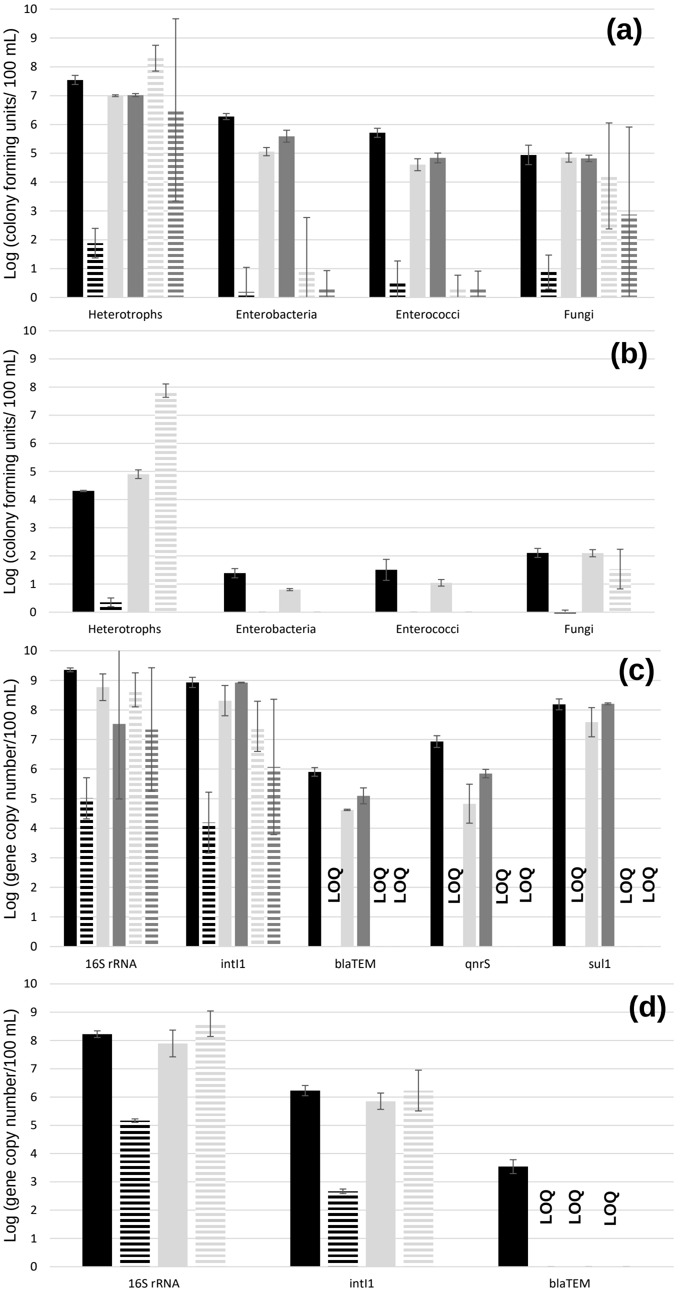

Figure 5.7. Microbial inactivation by photocatalytic ozonation in continuous reaction performed on wastewater effluents (a, c) and surface water (b, d) was assessed based on the quantification of selected microbial groups using culture-dependent methods (a, b) and qPCR of selected genes (c, d), in non-treated control samples (filled bars) and after a treatment retention time of 26 min (striped bars), at the initial time (black) and after 3-days storage at room temperature with light (grey) or in the dark (dark grey). LOQ, below or close to the limit of quantification (at least more than half of the quantification reactions were below the quantification limit and none was more than 3 times above that value).

Since most of the microorganisms in water are not cultivable, and advanced water treatment may enhance the fraction of non-cultivable populations, the use of culture-independent methods was necessary in this study. In spite of their stress status, some bacteria maintain viability, being able to regrow when the disinfection stress is relieved. From some of these bacterial cells, DNA extraction may be not possible immediately after surface water/wastewater treatment, therefore the measurement of the same target genes after three days of incubation was a way to assess the abundance of bacteria fitted to survive after disinfection. Of the analysed genes, 16S rRNA, *intI*1, *bla*$_{\text{TEM}}$, *qnr*S and *sul*1 were above the LOQ in the secondarily treated wastewater samples analyzed. The gene *van*A was below the LOD (Figure 5.7c). Photocatalytic ozonation led to a significant reduction of total prokaryotic cells (assessed based on the abundance of the 16S rRNA gene) and of the gene *intI*1, associated with mobile gene cassettes, also with widespread distribution in different bacterial groups. In addition, also the antibiotic resistance genes *bla*$_{\text{TEM}}$, *qnr*S and *sul*1 were significantly reduced after the photocatalytic ozonation. These genes, although highly prevalent in human impacted environments were originally at lower abundance than 16S rRNA and *intI*1 genes, and were reduced to levels below or close the LOQ (~10 gene copies per mL) after photocatalytic ozonation. For the genes 16S rRNA and *intI*1, the reduction observed after treatment was apparently a transient effect. After storage in the dark or presence of light, the gene copy numbers per 100 mL of 16S rRNA and *intI*1 genes were about 1 logarithmic cycle below the initial value (Figure 5.7c). The other analysed genes did not yield a significant increase in their relative abundance during storage (Figure 5.7c). These results were also useful to measure the effectiveness of the treatment process, with the regrowth capacity representing a good indication of the fraction of live cells remaining immediately after treatment.

Regarding surface water, the photocatalytic ozonation treatment produced similar results to those obtained for the secondarily treated wastewater. Except for total heterotrophs, the photocatalytic ozonation treatment led to the removal of the analysed microbial groups (Figure 5.7b) to values close to or below the LOD (10^{-2} CFU/100 mL). However, regrowth was observed for fungi and was particularly enhanced for total heterotrophs. In addition, in this case, the analysis of specific potential antibiotic resistant populations suggested that bacteria with acquired antibiotic resistance traits may have the capacity to regrow in photocatalytic ozonated water up to values found in non-treated surface water (Figure 5.8). Similar conclusions were retrieved in a study where the effect of chlorination and H_2O_2/sunlight processes on the regrowth of a multidrug *Escherichia coli* strain was investigated [44].

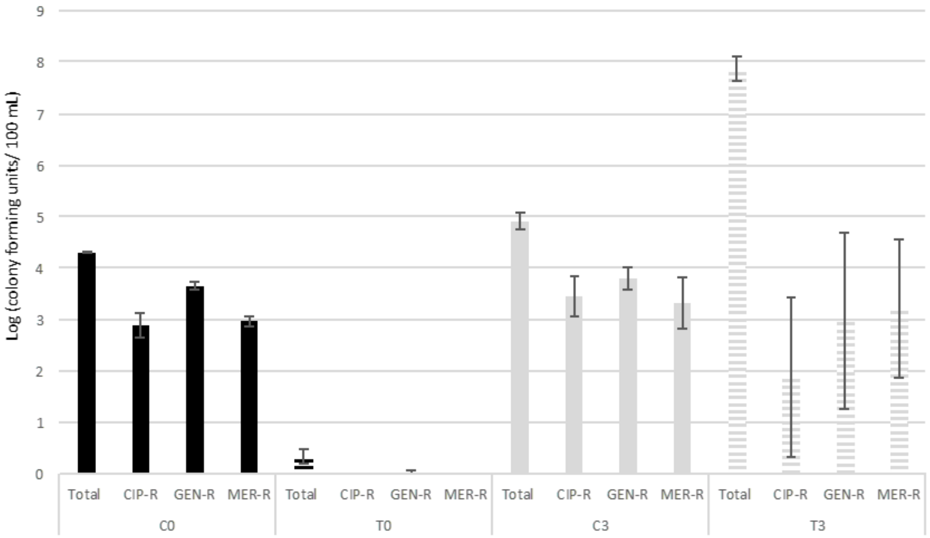

Figure 5.8. Total, ciprofloxacin resistant (CIP-R), gentamicin resistant (GEN-R) and meropenem resistant (MER-R) heterotrophic counts before (C_0), after photocatalytic ozonation treatment (T_0) and after 3-day incubation of untreated surface water (C_3) and treated surface water (T_3) at dark conditions for microbial reactivation assessment.

In surface water the genes *qnr*S, *van*A and *sul*1 were below the LOQ, while *bla*$_{TEM}$ presented 10^4 gene copy number per 100 mL (Figure 5.7d). Photocatalytic ozonation treatment led to reductions of around 3 logarithmic cycles of the 16S rRNA and *intI*1 genes, and the gene *bla*$_{TEM}$ was reduced to levels close to the LOQ. As for wastewater, for 16S rRNA and *intI*1 genes, the inactivation effect was apparently transient (Figure 5.7d), corroborating the cultivable-dependent data. Although these findings may suggest a potential increase of microbiological risks, it would be necessary to study the diversity of the populations and/or the prevalence of resistance genes among them to have a better estimation of potential risks.

5.4.4. Biological effects

YES assay results revealed that no agonistic estrogenic activity was found for samples collected before and after photocatalytic ozonation treatment of the WWTP effluent, along with no growth inhibition. The IR values determined for both samples were between 0.804 and 1.074, which is below IR10 (1.487 for E2). These observations indicate that the proposed treatment strategy did not originate compounds with estrogenic effects. Furthermore, cytotoxicity and cellular viability assays using cell models for digestive epithelium (Caco-2), blood-brain barrier (hCMEC/D3), and skin (L929) showed no significant difference concerning cell viability (Table 5.3, LDH assay). MTT assay is a suitable tool for assessment of cytotoxic effects from wastewater, surface water and drinking water samples as reported before [45, 46]. Slight cytotoxicity was observed for Caco-2 and hCMEC/D3 cell lines after treatment, but not for L929 fibroblasts (Table 5.3, MTT assay). The first two cell lines are human-derived, while L929 fibroblasts were originated in mouse, accounting for their different susceptibility. Furthermore, as reported before [45], the exposure to water testing sample mixed with culture media can originate different results, depending upon the final growth media composition.

Table 5.3. Results (percentage) from MTT[a] and LDH[b] assays before and after treatment of urban wastewater sample.

Cell line	MTT assay		LDH assay[c]	
	Before treatment	After treatment	Before treatment	After treatment
Caco-2	92 ± 20	59 ± 11	55.3 ± 3.1 (51.5 ± 4.6)	49.0 ± 3.3 (49.5 ± 4.0)
hCMEC/D3	106 ± 10	58 ± 21	61.1 ± 3.7 (66.6 ± 7.0)	60.3 ± 4.2 (67.4 ± 5.5)
L929	120 ± 19	91 ± 10	50.7 ± 2.5 (51.6 ± 3.0)	55.7 ± 3.2 (56.7 ± 3.3)

[a] Values for culture media were 100 % (RSD < 20 %) and between 1.3 and 10 % for Triton X-100 (total disruption of cells);
[b] Values for Triton X-100 (total disruption of cells) were 100 % (RSD < 8 %);
[c] Values in parentheses correspond to blank values obtained in culture media only (intact cells).

Regarding the surface water, no agonistic estrogenic activity was found for these samples when using the YES assay, along with no growth inhibition. The IR values determined for both samples were between 0.681 and 0.962, which is also below IR10 (1.487 for E2). These observations corroborate that the proposed treatment strategy did not originate compounds with estrogenic effects. Similar results were obtained when applying recombinant yeasts expressing estrogen receptor alpha to evaluate the estrogenic activity of wastewater, bottled waters and tap water in Finnish samples [47]. For LDH assay, cellular viability was maintained after treatment for both Caco-2 and hCMEC/D3 cells, providing values similar to those obtained for culture media.

5.5. Conclusions

Among different oxidative treatments tested in continuous mode, photocatalytic ozonation using TiO_2-coated glass Raschig rings with LEDs irradiation was the most efficient for the removal of PSs and CECs from urban wastewater. In these experiments, fluoxetine, clarithromycin, erythromycin and EE2 were more refractory to oxidation than the other compounds. The same process completely removed all the micropollutants detected in surface water (carbamazepine, isoproturon, clarithromycin, norfluoxetine, fluoxetine, E2 and EE2). In addition, the efficiency to remove different groups of cultivable microorganisms and housekeeping (16S rRNA) and antibiotic resistance or related genes (*intI*1, *bla*$_{\mathrm{TEM}}$, *qnr*S, *sul*1) was studied, with photocatalytic ozonation showing high efficiency on the removal of microbial loads, including ARG, from both urban wastewater and surface water. The tested ARGs were successfully removed to levels below/close to the threshold of quantification. Moreover, after the 3-days storage of treated surface water/wastewater at room temperature, there was no evidence of regrowth of the host, as inferred from the absence of an increase in the relative abundance of the respective ARG. However, both culture-dependent and -independent methods demonstrated that part of the surface water/wastewater microbiota was viable, able to regrow, and the potential risk of this regrowth is still to be assessed. From YES, MTT and LDH assays it follows that compounds with estrogenic effects were not formed and that differences concerning cell viability were not statistically significant for both urban wastewater and surface water. Therefore, photocatalytic ozonation proved to be a potential solution for simultaneous removal of organic micropollutants, different microbial groups of potential human pathogens, ARB and ARG present in wastewater or surface water. However, more studies are still required as well as the evaluation of the process associated costs.

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Chapter 6

Solar treatment (H_2O_2, TiO_2-P25 and GO-TiO_2 photocatalysis, photo-Fenton) of organic micropollutants, human pathogen indicators, antibiotic resistant bacteria and related genes in urban wastewater

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The work described in Chapter 6 was published in the following reference and reproduced by permission of Elsevier (the original version is provided as **Appendix C)**:

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Specific nomenclature in Chapter 6:

- P25 corresponds to the commercial TiO_2-P25;
- TiO_2 corresponds to bare-TiO_2;
- Organic micropollutants corresponds to OMPs.

6.1. Abstract

Solar-driven advanced oxidation processes were studied in a pilot-scale photoreactor, as tertiary treatments of effluents from an urban wastewater treatment plant. Solar-H_2O_2, heterogeneous photocatalysis (with and/or without the addition of H_2O_2 and employing three different photocatalysts) and the photo-Fenton process were investigated. Chemical (sulfamethoxazole, carbamazepine, and diclofenac) and biological contaminants (faecal contamination indicators, their antibiotic resistant counterparts, 16S rRNA and antibiotic resistance genes), as well as the whole bacterial community, were characterized.

Heterogeneous photocatalysis using TiO_2-P25 and assisted with H_2O_2 (P25/H_2O_2) was the most efficient process on the degradation of the chemical organic micropollutants, attaining levels below the limits of quantification in less than 4 h of treatment (corresponding to $Q_{UV} < 40\,kJ\,L^{-1}$). This performance was followed by the same process without H_2O_2, using TiO_2-P25 or a composite material based on graphene oxide and TiO_2. Regarding the biological indicators, total faecal coliforms and enterococci and their antibiotic resistant (tetracycline and ciprofloxacin) counterparts were reduced to values close, or beneath, the detection limit (1 CFU 100 mL^{-1}) for all treatments employing H_2O_2, even upon storage of the treated wastewater for 3-days. Moreover, P25/H_2O_2 and solar-H_2O_2 were the most efficient processes in the reduction of the abundance (gene copy number per volume of wastewater) of the analysed genes. However, this reduction was transient for 16S rRNA, *intI*1 and *sul*1 genes, since after 3-days storage of the treated wastewater their abundance increased to values close to pre-treatment levels. Similar behaviour was observed for the genes *qnr*S (using TiO_2-P25), *bla*$_{CTX\text{-}M}$ and *bla*$_{TEM}$ (using TiO_2-P25 and TiO_2-P25/H_2O_2). Interestingly, higher proportions of sequence reads affiliated to the phylum *Proteobacteria* (*Beta-* and *Gammaproteobacteria*) were found after 3-days storage of treated wastewater than before its treatment. Members of the

genera *Pseudomonas*, *Rheinheimera* and *Methylotenera* were among those with overgrowth.

6.2. Introduction

Conventional urban wastewater treatment plants (UWWTPs) are not specifically designed for the removal of organic micropollutants, and in many cases neither for effective disinfection (removals of bacterial loads up to 2 log cycles) which considers the inactivation of bacteria that can contribute to the spread of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARG) into the environment. These contaminants can reach the natural waters, such as surface and ground waters that are serving as drinking water sources [1, 2]. Moreover, the continuous disposal of antibiotics and related products into the environment can lead to the development and proliferation of ARB, decreasing the efficiency of these antibiotics when supplied to human and animals [3, 4]. Since high bacterial density can be found in effluents of UWWTPs (*i.e.*, bacterial cells are close to each other), horizontal gene transfer and selection of ARB can be considered important mechanisms for ARG enrichment [5]. The dissemination of these contaminants urges the development of new technologies able to improve the simultaneous removal of organic micropollutants and microorganisms of concern.

Advanced oxidation processes (AOPs) are conceptually based on the generation of the highly reactive hydroxyl radicals ($HO^\bullet$), but other reactive species can also be formed. AOPs, such as UVC/H_2O_2 and ozonation, have been widely studied for the removal of hazardous organic chemical compounds and disinfection, and they have been applied as viable solutions to enhance the quality of secondary effluents before discharged or reused [6]. However, several drawbacks have been associated to the aforementioned processes. For instance, ozonation-based treatments are known to produce bromate (from bromide), which is classified as a “possible human” carcinogen [7], whereas the processes dependent on high/medium vapour pressure lamps (such as UV_{254} nm) have higher operating costs and they are less environmentally friendly than those implementing solar radiation. Furthermore, in a recent study [8], we have shown that oxidation processes (such as UV_{254} nm, ozonation and photocatalytic ozonation) might have the potential to act selectively over some bacterial groups. In that bench-scale study, irrespective of the type of treatment used, it was observed a significant modification of the residual bacterial community after 3-days storage, which was characterized by higher proportions of *Proteobacteria* (*Gamma-* and *Betaproteobacteria*) than those observed in non-treated wastewater. This is an example of bacterial community disturbance induced by a disinfection treatment, which eventually may affect negatively the biological quality of the final stream if the reduction of the bacterial diversity and disequilibrium of the microbial ecosystem favour the possible selection of ubiquitous bacteria associated with acquirement and spread of virulence or ARGs [8].

Among the different AOPs, those driven by natural sunlight have raised a great interest during last decades (*i.e.* natural solar radiation as source of photons), reducing the need to costly lamps or maintenance of systems. Photocatalysis with irradiated titanium dioxide (TiO_2) and the photo-Fenton process have demonstrated to be powerful wastewater treatment options. A number of experimental studies are available in literature recognizing the high potential of these solar techniques for water decontamination and disinfection of a wide range of water sources [9-14]. Especially interest for water disinfection has attracted the solar photo-assisted treatment with H_2O_2, which has achieved fast inactivation of several types of microorganisms in water [15-17]. In addition, the development of solar Compound Parabolic Collector (CPC) reactors has resulted in the development of technological solutions with increased efficiency for water treatment [9, 18]. However, the performance of these solar-based processes has not been investigated in terms of changes of the bacterial community composition and simultaneous removal of organic contaminants and ARB&ARG.

Thus, the present study aims at comparing the efficiency of different solar-driven AOPs on the simultaneous removal of micropollutants and disinfection of a secondary effluent of an UWWTP. Solar-H_2O_2, heterogeneous photocatalysis (with and/or without the addition of H_2O_2) and the photo-Fenton process were tested using a pilot-plant CPC solar photoreactor. For the first time, the performance of each process was assessed based on the removal efficiency of organic contaminants and ARB&ARG, as well as on the changes of the bacterial community composition. Microbial characterization was performed before treatment, after 5 h of treatment and after 3-days storage of treated wastewater at room temperature. Thus, the simultaneous removal of chemical and biological contaminants by using solar-driven AOPs at Pilot scale and considering possible changes in the bacterial community, which can affect the water quality, is the main novelty of the present work.

6.3. Materials and methods

6.3.1. Chemicals and materials

Degussa (Aeroxide) TiO_2-P25 catalyst from Evonik Corporation (hereafter referred to as P25) and a composite consisting of TiO_2 and 4.0 wt% of graphene oxide (GO-TiO_2) – respective preparation method and characterization described elsewhere [19] – were used to conduct heterogeneous photocatalytic experiments. For comparative purposes, bare TiO_2 was also prepared following the same method used for GO-TiO_2, but without the addition of GO (hereafter referred to as TiO_2). The H_2O_2 30% (w/v) solution, analytical grade sulphuric acid (H_2SO_4, 98%), bovine liver catalase and ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) were obtained from Riedel-de Haën (Germany), Merk (Germany), Sigma-Aldrich (USA) and PANREAC (Spain), respectively. Carbamazepine

(CBZ), sulfamethoxazole (SMX) and diclofenac (DFC) were all high-purity grade (>99%), and purchased from Sigma-Aldrich (Spain). Stock solutions were prepared by dissolving the individual compounds (2.5 g L^{-1}) in methanol due to water solubility limitations. Required volumes of the stock solutions were directly added to the urban wastewater samples into the CPC pilot reactor to obtain the initial concentration of 100 µg L^{-1} of each organic micropollutant. Methanol (J.T.Baker) and acetonitrile (Sigma-Aldrich) were HPLC-grade. Ultrapure water was supplied by a Milli-Q water system.

6.3.2. Municipal wastewater treatment plant samples

All solar-driven treatments were carried out using urban wastewater samples collected (every day in batches of 60 L) after the secondary treatment from the UWWTP of El Bobar (Almería, Spain), and stored at 4 ºC no more than 2 h before solar experiments. The UWWTP uses conventional activated sludge plus decantation as secondary treatment. In 2015 (year of the sampling campaign), it produced an average of secondary effluent daily flow of ca. 33,000 m^3, with a capacity of 100,000 inhabitant-equivalent. The main physicochemical characteristics of the effluent, including turbidity, pH, conductivity, dissolved inorganic carbon (DIC), dissolved organic carbon (DOC) and inorganic ions concentration, are listed in Table C-S6.1 (Supplementary Information).

6.3.3. Pilot scale CPC photoreactor and solar experiments

All the solar-driven oxidation processes were performed in a pilot-scale CPC photoreactor, at Plataforma Solar de Almeria (PSA), Spain (37°84′N and 2°34′W), on sunny days between June and August 2015, with a duration of 5 h. The configuration of the CPC photoreactor is described elsewhere [20]. The CPC photoreactor tube module, tilted at an angle of 37° relative to the horizontal plane, is connected to a recirculation tank and a centrifugal pump. The water flow rate was set at 10 L min^{-1}. The total volume of the photoreactor was 20 L, while the illuminated volume and the irradiated collector surface area were 15 L and 1 m^2, respectively. Target chemical and biological contaminants were simultaneously monitored in the same experiment.

In heterogeneous photocatalysis (P25, TiO_2 and GO-TiO_2) a catalyst load of 200 mg L^{-1} was used. In photo-Fenton (Fe^{2+}/H_2O_2), $FeSO_4 \cdot 7H_2O$ was used as source of 10 mg L^{-1} of Fe^{2+}. In H_2O_2 assisted processes (H_2O_2, Fe^{2+}/H_2O_2, P25/H_2O_2 and GO-TiO_2/H_2O_2), the initial concentration of H_2O_2 was 20 mg L^{-1} reached by adding the H_2O_2 30% (w/v) solution. Different amounts of H_2O_2 added to the system may have different effect on the removal efficiencies [12, 21]. The catalyst and H_2O_2 concentrations were selected considering the optimization done in our previous studies with solar-driven oxidation processes [12, 22]. Immediately after the collection of a sample, residual H_2O_2 was eliminated by adding a freshly prepared solution of bovine liver catalase (0.1 g L^{-1}) in a ratio 0.1/5.0 (v/v). Photolysis (Blank) assays were performed to study the effect of solar radiation without the addition of any catalyst or reactant.

Besides the experimental time, the accumulated solar UVA energy received in the solar reactor per unit of treated water volume (Q_{UV}, kJ L^{-1}) was considered for comparison of the treatment efficiencies [9] and calculated using Eq. (1):

$$Q_{UV,n} = Q_{UV,n-1} + \frac{\Delta t_n \overline{UV}_{G,n} A_r}{V_t}; \Delta t_n = t_n - t_{n-1} \tag{1}$$

where $UV_{G,n}$ is the global UV irradiance (W m^{-2}) averaged during exposure time; t_n the exposure time (s); V_t the total reactor volume (L); and A_r the illuminated reactor surface (m^2).

A global UVA (300-400 nm, Model CUV4, Kipp & Zonen, Netherlands) pyranometer tilted 37° was used to measure the solar radiant UVA rate incident (W m^{-2}) as described elsewhere [20]. The average value of solar UVA irradiance was 40 W m^{-2}.

Before treatment, the CPC photoreactor was covered by an opaque sheet while the wastewater and reagents were added to the reactor and recirculated during 15 min to guarantee homogenisation. Samples were collected each hour over the treatment. During this period, temperature (Checktemp, Hanna instruments, Spain) and pH (multi 720, WTW, Germany) were measured. The temperature of the wastewater varied from 16.6 ± 1.5 °C to 43.2 ± 4.2 °C.

6.3.4. Chemical analysis

High performance liquid chromatography (HPLC) was used to analyse the evolution of the target organic micropollutants using an apparatus from Agilent Technologies (series 1260, Palo Alto, CA, USA), equipped with a diode array detector (UV-DAD) and a C-18 column. The mobile phase consisted of 90% formic acid aqueous solution at 25 mM and 10% acetonitrile. A linear gradient was used from 10% to 85% of acetonitrile during 13 min at a flow rate of 1 mL min^{-1}. The injection volume was set at 100 μL. Before injection, samples were filtered through a 0.2 μm syringe-driven filter, and afterwards the filter was washed with 1 mL of acetonitrile to remove the adsorbed compounds. CBZ, SMX and DFC were selected as model organic micropollutants since they have been frequently found in aquatic environments, including wastewater, surface and groundwater and even in drinking water [23]. The detection wavelength values were 267 nm for both CBZ and SMX, and 273 nm for DFC. The limits of quantification (LOQ) were 4.7, 6.2 and 4.1 μg L^{-1} for CBZ, SMX and DFC, respectively.

DX-600 and DX-120 (Dionex Corporation, Sunnyvale, CA) equipments were used for quantification of anions and cations, respectively. DOC and DIC measurements were performed using a 5050 A TOC (Shimadzu Corporation, Kyoto, Japan) analyzer, after sample filtration using 0.2 μm syringe filters. Turbidity was determined with a turbidimeter (Model 2100N, Hach, USA). The H_2O_2 concentration was determined by a spectrometric method, as described elsewhere [24], and the Fe^{2+} concentration by the ISO 6332 method. Natural iron was not detected in any of the urban wastewater samples by using this method, *i.e.* a spectrophotometric technique with phenanthroline/acetic acid (UV-Vis measurements, limit of detection 0.05 mg L^{-1}).

6.3.5. Microbiological cultivation, DNA extraction, qPCR and bacterial community analysis

To assess the disinfection efficiency, cultivable faecal indicator bacteria, targeted ARB and selected ARGs were quantified before and immediately after the 5 h-treatment, and after 3-days of storage of treated wastewater at room temperature. The abundance of faecal coliforms, enterococci and their tetracycline and ciprofloxacin resistant counterparts was assessed by using the membrane filtration method. Faecal coliforms and enterococci are used worldwide as indicators of faecal contamination, in particular to assess the microbiological water quality [25, 26]. In addition, the ubiquity of these bacteria in human impacted environments and their high persistence in the environment, as well as genome plasticity, make of faecal coliforms and enterococci important tracers to assess the antibiotic resistance status of environmental samples. Furthermore, in these groups high resistance prevalence has been observed for antibiotics such as tetracycline and ciprofloxacin in aquatic environments, including wastewater [27]. After serial 10-fold dilutions in sterile saline solution (0.85% NaCl), 100 mL of each dilution was filtered through cellulose membrane filters (0.22 µm porosity; Whatman, UK). The filtering membranes were incubated on selective media for each target bacterial groups - membrane Faecal Coliforms agar (m-FC) (Difco, 30 °C, 24 h) for faecal coliforms, and Slanetz & Bartley agar (S&B) (Difco, 30 °C, 48 h) for enterococci. In addition, the prevalence of ARB was assessed in the same media supplemented with tetracycline (Fluka, 16 mg L^{-1}) and ciprofloxacin (Applichem, 1 mg L^{-1}). The antibiotic stock solutions were sterilized by filtration (0.2 µm syringe driven-filters).

The use of adequate indicators of antibiotic resistance in both culturable and non-culturable bacteria has been recommended [28]. For culture-independent assays, a volume of 250 mL of each sample (before and after the 5 h treatment, and after 3-days of storage) was filtered through polycarbonate membranes (0.22 µm porosity; Whatman, UK). DNA was extracted using the commercial kit PowerWater® DNA Isolation (MO BIO Laboratories, Inc., USA) and stored at −20 °C. These extracts were used for the quantification of the house keeping gene 16S rRNA, *int*I1 (related with ARG mobilization) and a set of ARGs encoding resistance to different classes of antibiotics frequently detected in wastewater [29] by quantitative PCR (qPCR) of samples collected before treatment, immediately after treatment, and after 3-days of storage. In addition, the same extracts were also used for the bacterial community analysis, in this case except for the samples collected immediately after treatment, due to DNA scarcity. The qPCR (StepOne™ Real-Time PCR System; Life Technologies, Carlsbad, CA) assays were performed according to the conditions shown in Table C-S6.2 - Supplementary Information [30-36], and as described elsewhere [37].

The bacterial community composition was analysed based on the hypervariable V3/V4 region (forward primer Bakt_341F 5′-CCTACGGGNGGCWGCAG-3′ and reverse Bakt_805R 5′-GACTACHVGGGTATCTAATCC-3’) of 16S rRNA gene Illumina sequencing (Genoinseq, Cantanhede, Portugal). Nucleotide sequencing data were processed and analysed using the QIIME pipeline [38]. Briefly, sequences shorter than 250 bp and with average quality scores lower than 25 were eliminated, and bases with average quality lower than 25 in a window of 5 bases, were trimmed using the software PRINSEQ [39]. Chimeric sequences were identified and removed using USEARCH v6.1 [40]. Free-chimeric sequences were further grouped into operational taxonomic units (OTUs) using USEARCH v6.1 [40] with a phylotype threshold of ≥97% sequence similarity and were taxonomically assigned using

QIIME default values. The sequences comprising each OTU were aligned using PyNAST [38] and were taxonomically classified using Greengenes Database version 13_8 (updated: August 2013) [41]. As a variable number of sequences was obtained between samples, the alpha diversity indices Shannon, phylogenetic diversity (PD) whole tree, and Simpson were calculated after rarefying to 54,771 sequences per sample (value of the smallest dataset) [42-44]. An alpha diversity index represents the diversity within a particular community and it is affected by its size, in particular by the number of different species and the abundance of each one [45]. The cumulative sum scaling (CSS) normalization procedure was applied to the sequence data to assess the beta diversity patterns [46]. The weighted UniFrac metric distances [47] were calculated in the QIIME pipeline and the results shown as Principal Coordinates Analysis (PCoA) biplots that include the position of the ten most prevalent bacterial classes. Correlations between the relative abundance of populations at different taxonomic levels were analysed using the statistics software STAMP v2.1.3 [48].

6.4. Results and discussion

6.4.1. Degradation of organic micropollutants

The evolution of the concentrations of targeted organic micropollutants (*i.e.*, CBZ, SMX and DFC) spiked in urban wastewater under solar-driven oxidation treatments are shown in Figure 6.1 Control experiments were performed under the same conditions but without the addition of any catalyst or H_2O_2 (*i.e.*, Blank = photolysis). CBZ and SMX were very resistant upon irradiation in the absence of a catalyst or H_2O_2 (only 20 ± 6% and 17 ± 4% of removal, respectively). In contrast, DFC was efficiently removed by photolysis after 4 h. This result was expected since the DFC absorption spectrum (not shown) has a tail entering well above the 300 nm [49].

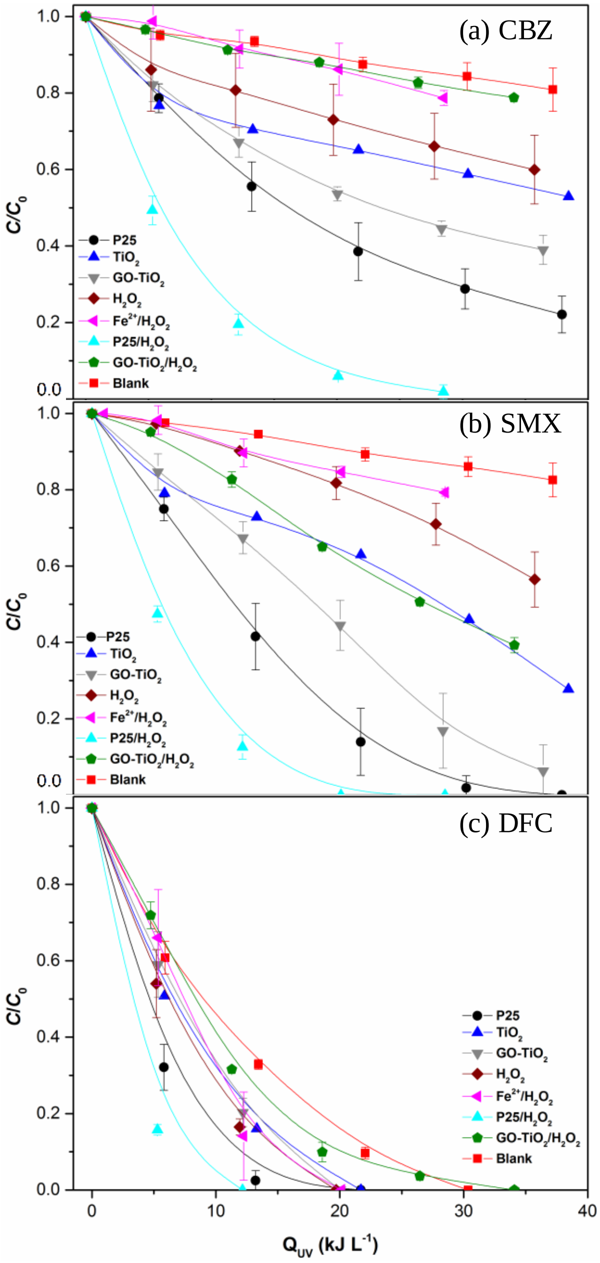

Figure 6.1. Normalized concentration (C/C_0) of (a) CBZ, (b) SMX and (c) DFC spiked in urban wastewater as function of accumulated energy (Q_{UV}) using different solar-driven treatments. Except for TiO_2, values are the average of four (P25), three ($GO-TiO_2$, H_2O_2, Fe^{2+}/H_2O_2) and two ($P25/H_2O_2$, $GO-TiO_2/H_2O_2$, Blank) independent assays. Error bars represent standard deviations.

The heterogeneous photocatalysts (P25, TiO_2 and $GO-TiO_2$), without the addition of H_2O_2, converted the targeted organic micropollutants in the following order of decreasing efficiency: P25 > GO-TiO_2 > TiO_2 (Figure 6.1). The high photocatalytic efficiency of GO-TiO_2 on the degradation of different types of organic micropollutants in synthetic matrices has been already demonstrated in our previous studies, namely for: diuron, alachlor, isoproturon and atrazine – pesticides classified by the EU as priority pollutants [50]; microcystin-LA – cyanotoxin [51]; bisphenol A – xenoestrogen [52]; diphenhydramine – antihistamine pharmaceutical, and methyl orange – azo dye [19]. The GO-TiO_2 photocatalyst has been much more active than P25 under Vis-light illumination, but under near UV-Vis radiation the activity of this type of photocatalysts depends on the target pollutant. The better performance of GO-TiO_2 in comparison to bare TiO_2 has been attributed to the good assembly and interfacial coupling between TiO_2 and GO sheets as well as the respective quenching of photoluminescence (inhibiting charge recombination) [19]. In the present study, P25 was the most efficient photocatalyst for the degradation of the organic micropollutants, most probably, because of the better performance of P25 under UV radiation.

The addition of H_2O_2 to the P25 photocatalytic system, increased the degradation efficiency of the targeted micropollutants. H_2O_2 captures and reacts with the photoinduced surface electrons (supressing the electron/hole recombination) and it also reacts with the superoxide radical anions, both pathways leading to the formation of additional hydroxyl radicals [11, 53]. There is markedly a competing process, which may be mediated by the active surface of the photocatalyst. Interestingly, the removal efficiency decreased when H_2O_2 was added to the photocatalytic system containing GO-TiO_2. Degradation of GO-TiO_2 may eventually occur, for instance by the H_2O_2 attack to the underlying C-C bonds in the superficial defect sites of GO [54].

Solar-H_2O_2 and photo-Fenton processes (Fe^{2+}/H_2O_2) also led to very modest removals of CBZ and SMX (Figure 6.1a and b, respectively). One of the downsides of photo-Fenton is the formation of iron sludge due to the precipitation of iron hydroxide at neutral pH. In this work, the pH was adjusted to a circumneutral value (5.5) by using H_2SO_4, which could explain the low photo-Fenton efficiency that is known to be maximized at pH values around 3[15, 55, 56]. In a previous work [57], solar photo-Fenton (also operated at mild pH conditions) was effective for the degradation of organic micropollutants, but the total inorganic carbon was removed by sample acidification since it competes with the organic contaminants for hydroxyl radicals [58].

Overall, P25/H_2O_2 followed by the P25 and GO-TiO_2 photocatalytic processes were the best performing treatments for removal of the targeted organic micropollutants. Regarding the mineralization of the organic matter present in the urban wastewater, P25/H_2O_2, solar-H_2O_2, and the photo-Fenton process were the most efficient treatments (DOC removals always around 23 ± 3%), other processes removing no more than ca. 10% of the initial DOC. However, due to the chemical complexity of this matrix, and the differences between the DOC (in the range of mg L^{-1}) and the concentration of the studied organic pollutants (in the range of μg L^{-1}), it is not possible to correlate the DOC and micropollutants removals.

6.4.2. Bacteria inactivation and reactivation

The performance of different solar-driven processes was assessed before and over the treatment, in terms of removal of total faecal coliforms (Figure 6.2a), enterococci (Figure 6.2c) and respective fraction of resistant populations (Figure 6.2b and d). The reduction of the bacterial indicators loads was observed in all the treatments, with the highest inactivation rates leading to values bellow or close the LOD (1 CFU 100 mL^{-1}), for the processes where H_2O_2 was used (H_2O_2, Fe^{2+}/H_2O_2, P25/H_2O_2, GO-TiO_2/H_2O_2). Among these, and despite the iron precipitation, the photo-Fenton process was the most efficient treatment on the reduction of resistant and non-resistant faecal coliforms and enterococci (Figure 6.2a-d, Fe^{2+}/H_2O_2). However, photo-Fenton showed similar disinfection profiles to solar-H_2O_2 for faecal coliforms (Figure 6.2a and b) and to

$P25/H_2O_2$ for enterococci (Figure 6.2c and d). These three processes removed those bacteria for $Q_{UV} < 30\,kJ\,L^{-1}$, suggesting that H_2O_2 plays a major role on disinfection, including in the case of solar-H_2O_2 and photo-Fenton that were not effective for the removal of all the organic micropollutants. In contrast, moderate inactivation rates were observed for photolysis (Figure 6.2a, b and c, Blank), except for antibiotic resistant enterococci that were reduced to values below the LOD (Figure 6.2d, Blank). Interestingly, $P25/H_2O_2$ showed high efficiency on the removal of organic micropollutants and resistant and non-resistant enterococci, whereas the efficiency for inactivation of resistant and non-resistant faecal coliforms was not so high in comparison with solar-H_2O_2.

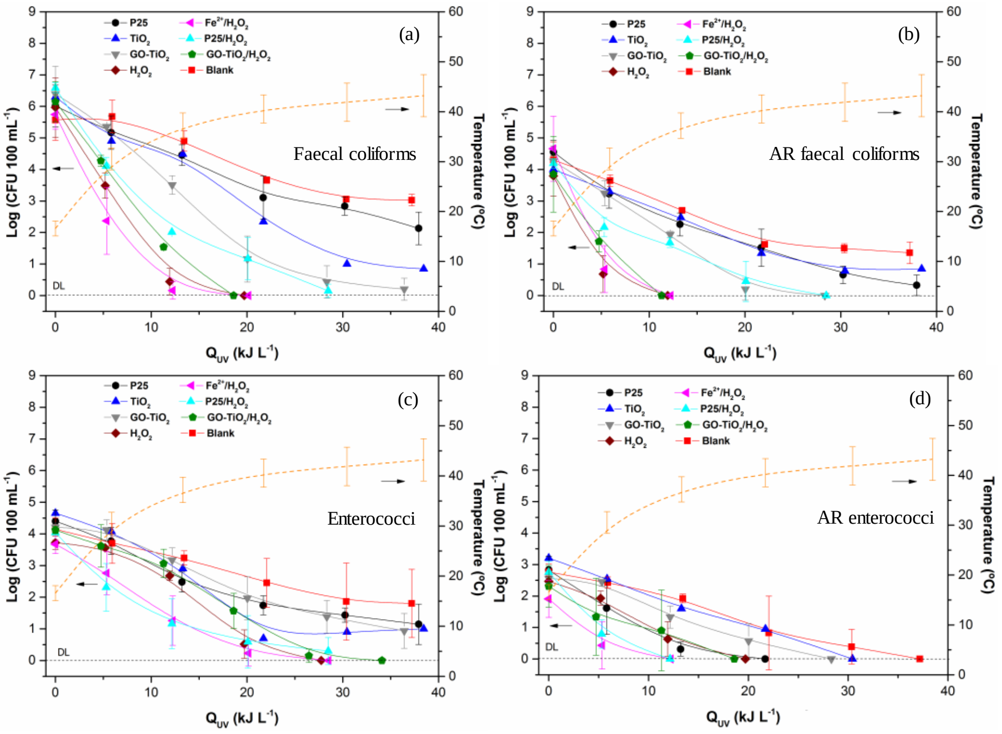

Figure 6.2. Faecal coliforms (a) and enterococci (c) and their antibiotic resistant counterparts (b, d) inactivation in urban wastewater as function of accumulated energy (Q_{UV}) using different solar-driven treatments. Except for TiO_2, values are the average of four (P25), three ($GO\text{-}TiO_2$, H_2O_2, Fe^{2+}/H_2O_2) and two ($P25/H_2O_2$, $GO\text{-}TiO_2/H_2O_2$, Blank) independent assays. Error bars represent standard deviations. DL, detection limit.

The accepted hypotheses and mechanism that explain the inactivation of microorganisms by exposure to solar-H_2O_2 is based on the accumulated damages inside cells by internal cellular injures occurring under sunlight and accelerated in the presence of H_2O_2. It is well accepted that solar radiation produces internal damages affecting different intracellular vital components leading to bacterial death or lack of viability [59]. A recent study attributed bacterial inactivation during solar photolysis to the combined effect of intracellular production of reactive oxygen species by UV photons absorption and water temperature increase [60]. When H_2O_2 is added, it may diffuse inside bacteria cells promoting additional internal photo-reactions with naturally present iron and other metals via Fenton and Fenton–like reactions, activating, thus, a photo-Fenton cycle under sunlight at intracellular level also [59]. Both photo-effects act jointly producing an accelerated disinfection that has been also reported to be very efficient for other types of bacteria, viral indicators, and fungi including, *Escherichia coli* and *Enterococcus faecalis* [20], *Legionella jordanis* [61], F-specific RNA bacteriophage [55], *Fusarium* sp. [12, 17], *Phytophthora capsici* [62], *Curvularia* sp. [59], and several antimicrobial resistant bacteria [63].

Although H_2O_2 assisted processes performed better that non-assisted ones, heterogeneous photocatalysis without H_2O_2 also performed quite well on the inactivation of total or resistant populations of faecal coliforms and enterococci. Among these processes, the GO-TiO_2 composite was the most efficient catalyst for the removal of total and resistant populations of faecal coliforms (Figure 6.2a and b, GO-TiO_2). The good efficiency of photocatalytic disinfection using this type of composites (but prepared by other methods) and under visible radiation only was already shown in previous studies [64, 65]. Its high photocatalytic activity has been attributed to the improvement in charge separation since GO may promote the electron transfer with TiO_2 particles, acting as an electron bridge, and to the decrease of the bandgap energy of the composite catalysts as well as to an enhancement of the adsorptive properties. These authors concluded that hydrogen peroxide, hydroxyl radicals, and singlet oxygen were the main species involved in the disinfection process under UV–Vis irradiation and only singlet oxygen under visible illumination [64]. Moreover, very recently [66], the photocatalytic efficiency of P25 (in the absence of H_2O_2) was compared to that of a TiO_2-reduced GO composite on the removal of three organic micropollutants (including SMX) and *E. coli* resistant and non-resistant to antibiotics in urban wastewater. P25 performed better than TiO_2-reduced GO for the photocatalytic degradation of SMX, but higher bacteria permanent inactivation was observed in the presence of the composite, in accordance with our results. However, in the same study, TiO_2-reduced GO performed better than P25 for the removal of erythromycin (*i.e.* the opposite of that observed for SMX), the photocatalytic efficiency thus depending on the target organic molecule. In our particular case, P25 was better than the GO-TiO_2 composite for the removal of all the studied organic micropollutants (CBZ, SMX and DFC).

Since microbial inactivation, monitored via culture-based methods, can be transient [10, 67, 68], further assays testing the regrowth capacity after 3-days storage of the treated wastewater at room temperature were performed. Bacterial reactivation is influenced by factors such as the storage conditions, temperature, availability of nutrients and the UV dose, among others [69, 70]. The bacterial loads before, after the treatment and after 3-days storage at room temperature are shown in Figure 6.3a and b for faecal coliforms and Figure 6.3c and d for enterococci. No regrowth was observed in stored wastewater treated by the H_2O_2-assisted processes, and total faecal coliforms and enterococci as well as their ARB counterparts were kept below the detection limit. In stored wastewater treated by heterogeneous photocatalysis without H_2O_2 (P25, TiO_2 and GO-TiO_2), the bacterial loads of these groups were 2–3 log values lower than before the treatment. Similar observations were registered for the control (Blank = photolysis). These results indicate the inability of these bacterial groups to recover to values close to those found in raw wastewater after the tested solar-driven processes. This can be also explained by the mode of action attributed to solar-H_2O_2 disinfection, where oxidative damages alter the bacteria viability at intracellular level, as proven in the literature [61]. In such report, an EMA-qPCR method for the detection of membrane integrity damages of *L. jordanis* cells under two photo-oxidative processes was used, *i.e.* solar-H_2O_2 and P25 with solar radiation. It was confirmed the well-accepted mechanism of heterogeneous (P25) photocatalysis via oxidative attacks of the external cell membrane, whereas the mechanism for solar-H_2O_2 was based on internal photochemical reactions. This may explain the results reported in Figure 6.3 on the non-recover capability of ARB when H_2O_2 was used in these treatment processes, while they can regrow when H_2O_2 is not in the media [61]. In general, the selected solar-driven oxidation processes were effective in reducing faecal indicators, including those resistant to tetracycline and ciprofloxacin, from wastewater samples.

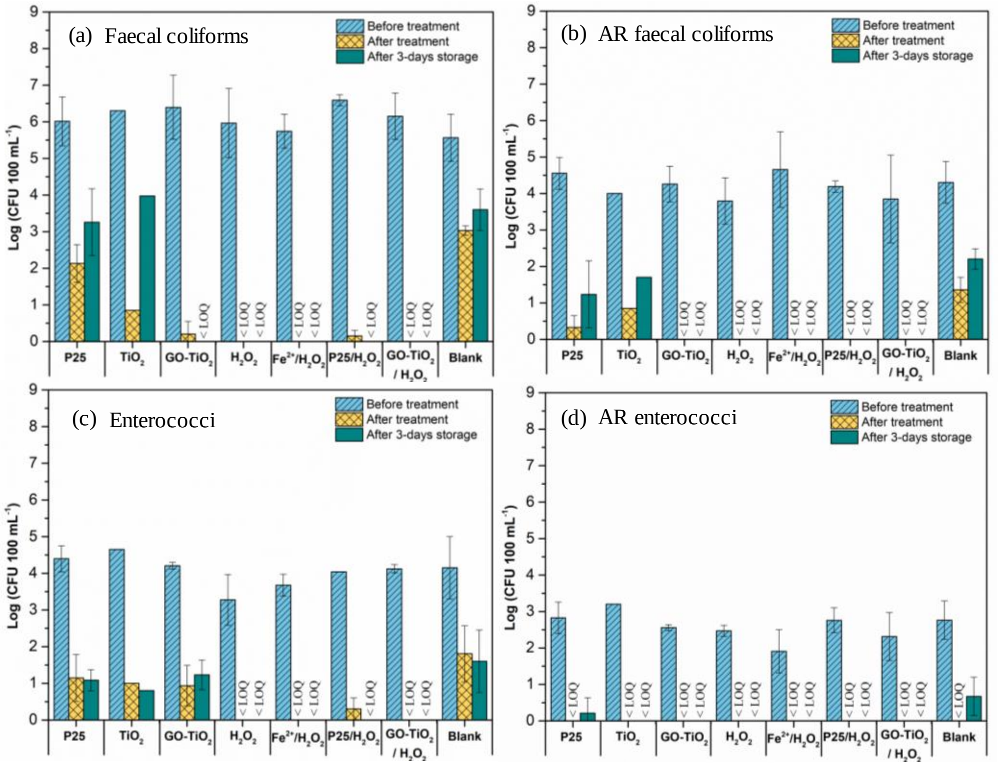

Figure 6.3. (a) Faecal coliforms and (c) enterococci and their (b, d) antibiotic resistant counterparts counts before, after treatment and after 3-days storage using different solar-driven treatments. Except for TiO_2, values are the average of four (P25), three ($GO\text{-}TiO_2$, H_2O_2, Fe^{2+}/H_2O_2) and two ($P25/H_2O_2$, $GO\text{-}TiO_2/H_2O_2$, Blank) independent assays. Error bars represent standard deviations.

6.4.3. Effect of disinfection on ARGs and bacterial community composition

Considering that the bacterial community is much more diverse and complex than that assessed based on the cultivation methods used, and that some bacteria may be injured and hence, unable to grow, culture-independent methods were carried out to give additional insights on the disinfection effectiveness of the studied solar-driven processes. Since the main purpose of this work was the simultaneous removal of chemical and biological contaminants, the processes showing better performance on the degradation of organic micropollutants (*i.e.*, $P25/H_2O_2$, P25 and $GO\text{-}TiO_2$ photocatalytic processes), disinfection ($P25/H_2O_2$ and solar-H_2O_2), and also the reference process (photolysis), were selected for further investigation based on culture-independent methods - Fe^{2+}/H_2O_2 was not chosen due to its bad performance for degradation of the organic micropollutants (Figure 6.1).

Among the analysed genes (*i.e.*, 16S rRNA, *intI*1, *qnr*S, *bla*$_{CTX-M}$, *sul*1, *bla*$_{TEM}$ and *van*A), only *van*A was below the LOD before treatment (not shown). For the other genes, $P25/H_2O_2$ photocatalysis and solar-H_2O_2 were the most efficient processes (*i.e.*, lower abundance after treatment), both leading to log average reduction of 1 value, except for *bla*$_{CTX-M}$ (3 log reduction) (Figure 6.4). However, after 3-days storage, regardless the treatment used, the abundance of 16S rRNA gene, a house keeping gene of prokaryotes, was close or even higher (up to 1 log for $P25/H_2O_2$) than those found before treatment, suggesting the ability of some bacteria to recover after the treatment. Similar results were observed for *intI*1 and *sul*1 genes, encoding integrase and conferring resistance to sulphonamides, respectively. Among the studied processes, only solar-H_2O_2 and GO-TiO_2 prevented the reactivation of *bla*$_{CTX-M}$ and *bla*$_{TEM}$ genes (encoding resistance to beta-lactams) above the pre-treatment levels. For the *qnr*S gene (a plasmid-mediated quinolone resistance gene), besides these two processes, also $P25/H_2O_2$ prevented its reactivation.

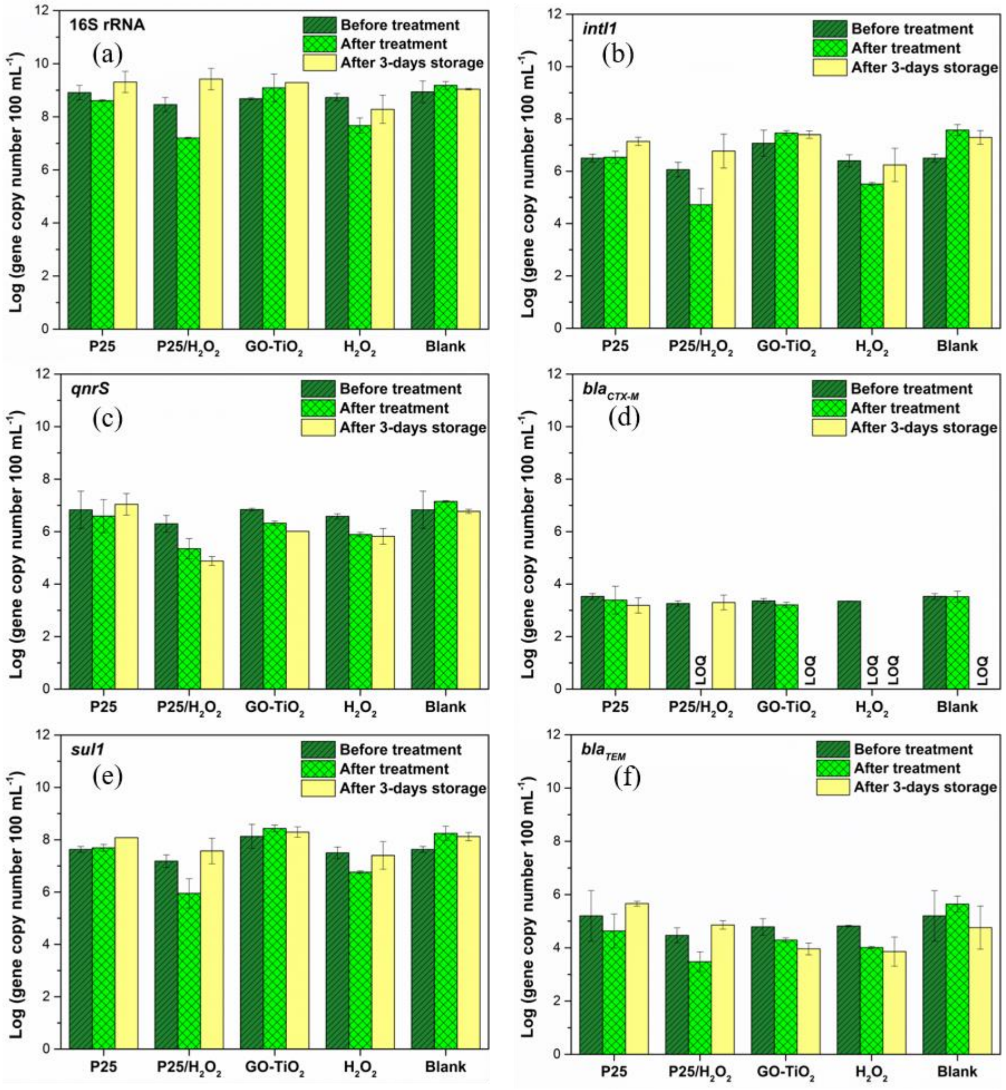

Figure 6.4. Abundance of target genes before and after treatment, and after 3-days storage at room temperature using different solar-driven treatments: (a) 16S rRNA, (b) *intI*1, (c) *qnr*S, (d) *bla*$_{CTX-M}$, (e) *sul*1 and (f) *bla*$_{TEM}$. Values are the average of two independent assays. Error bars represent standard deviations.

Other authors [3, 71] investigated the potential of UV/H_2O_2 process to control the potential spread of ARB into the environment. Despite of a successful inactivation of total coliforms, *E. coli* and antibiotic resistant counterparts, the genes *bla*$_{TEM}$, *qnr*S and *tet*W were still present in wastewater after 240 min of treatment, in accordance with the results of the present study.

Overall, the studied processes led to a slight reduction in the abundance of targeted genes immediately after treatment. Only *bla*$_{\text{CTX-M}}$, with a relatively low initial abundance, was reduced after H_2O_2 associated treatments to values below the limit of quantification. Although these types of treatment were observed to cause higher reductions in the other genes analysed, none of these ARGs were reduced to levels below LOQ. A further investigation would be necessary to assess whether disinfection driven reductions of ARGs were due the loss of the ARG (for instance plasmid loss or ARG excision), with maintenance of the host cell viability or if such reductions were due to cell inactivation. However, the observation of reactivation after 3-days storage in most of the cases, suggest that at least part of the ARG removal was due to cell inactivation and not to ARG loss. These results suggest that at least some of the bacterial cells survived these treatments, being able to reproduce during storage. However, further research on this subject is needed to withdraw safe conclusions. Regarding the apparent inconsistency between data obtained based on qPCR analyses and cultivation methods, in fact it could be expected given the low fitness of the indicators of faecal contamination to grow in water. Moreover, it suggests that regrowth is due to other bacterial populations, as it can be confirmed below with the data based on the bacterial community analysis.

The effect of the different treatment processes on the bacterial communities was another aim of this study. Out of the 49 phyla found in freshly collected wastewater (before treatment), *Proteobacteria* (62 ± 7%) and *Bacteroidetes* (10 ± 2%) were, in average ($n = 8$), the most abundant (Figure 6.5a, t_0). *Proteobacteria* comprised mainly members of the classes *Beta-* (28 ± 2%), *Gamma-* (20 ± 10%), *Alpha-* (7 ± 2%) and *Deltaproteobacteria* (5 ± 1%). Beside these groups, several other bacterial classes were detected in the freshly collected wastewater in relative abundances ranging from 2 ± 1% to 4 ± 2%; examples are "*Saprospirae*", *Flavobacteriia* and *Bacteroidia* (Bacteroidetes), *Clostridia* (Firmicutes), *Planctomycetia* (Planctomycetes), *ZB2* (OD1) and *TM7-1* (TM7). The other identified classes had abundances <1.3% (Figure 6.5a, t_0).

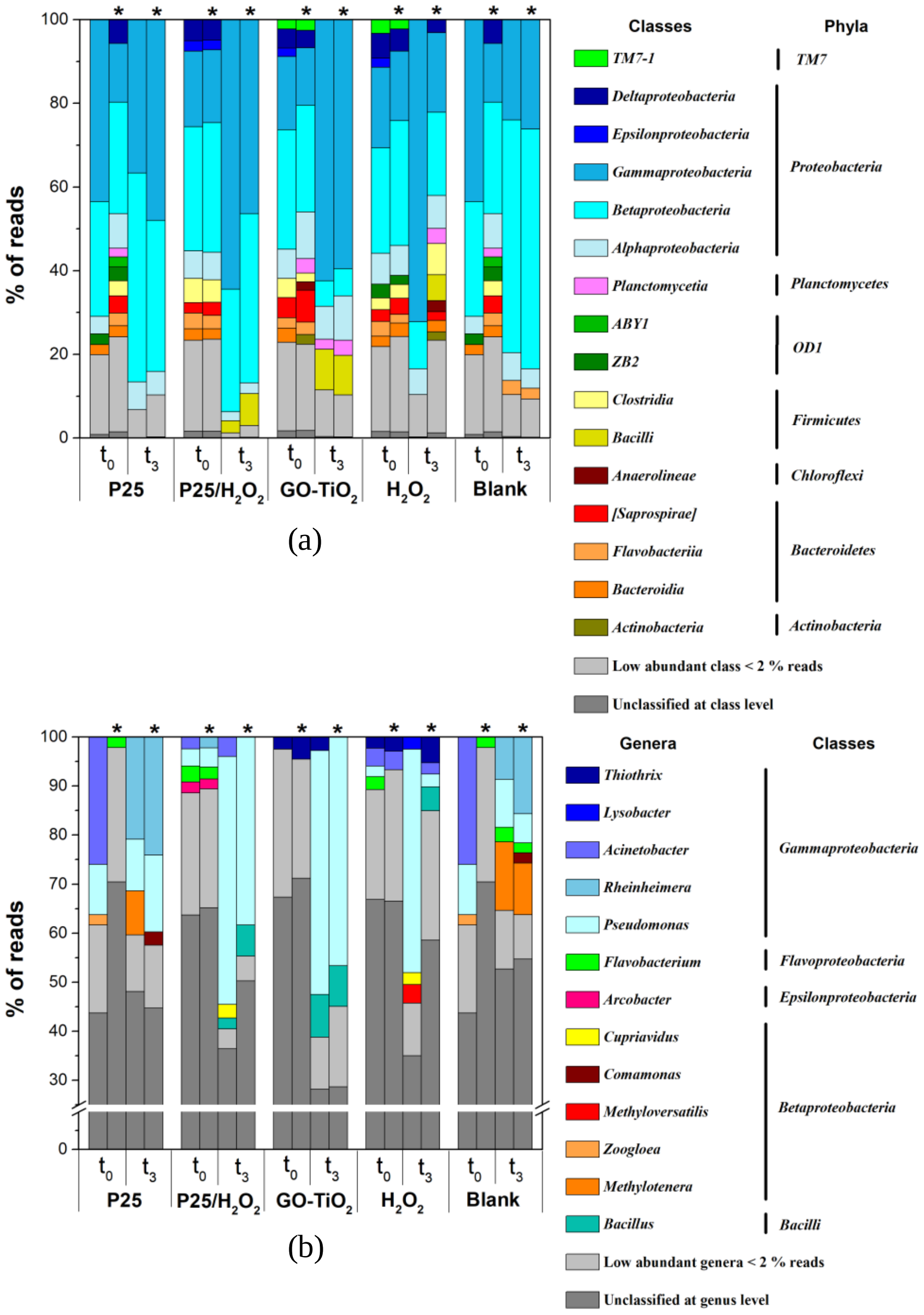

Figure 6.5. Relative abundance of (a) classes and (b) genera before (t_0) and 3-days after treatment (t_3). *, indicates a second independent assay.

Regardless of the solar driven process, treatment followed by storage at room conditions led to important bacterial community rearrangements that, in general, had the same pattern. The relative abundance of the members of *Proteobacteria* was higher ($p < 0.01$) in the stored treated wastewater than in the freshly collected wastewater samples, whereas it was lower ($p < 0.01$) for the majority of members of the other phyla (Figure 6.5a, t_3). The lower values of the alpha-diversity indices of the stored treated wastewater samples when compared with those of freshly collected wastewater samples (Table 6.1) corroborate this loss of diversity and equitability. These rearrangements are well depicted in the PCoA biplot, where the bacterial communities of the freshly collected wastewater samples were separated from those treated and stored over axis PC1 (31.0%) (Figure 6.6, squares and stars, respectively).

Table 6.1. Alpha diversity indices of the wastewater samples before (t_0) and after 3-days storage after treatment (t_3) calculated based on the average of 10 rarefaction OTU tables.

Experiment	Time	OTUs No.	Shannon	Simpson	PD whole tree
P25	t_0	3023	7.0	0.95	125.6
P25 ^{a}	t_0	3181	8.3	0.99	158.7
P25	**t_3**	3267	6.6	0.92	101.8
P25 ^{a}	**t_3**	4010	7.3	0.96	123.8
P25 / H_2O_2	t_0	3353	7.8	0.98	156.7
P25 / H_2O_2 ^{a}	t_0	3586	8.1	0.98	164.8
P25 / H_2O_2	**t_3**	2462	6.1	0.93	59.4
P25 / H_2O_2 ^{a}	**t_3**	2047	4.9	0.84	58.7
GO-TiO_2	t_0	3425	8.1	0.98	161.2
GO-TiO_2 *	t_0	2687	7.6	0.97	136.3
GO-TiO_2	**t_3**	2419	5.6	0.83	95.5
GO-TiO_2 ^{a}	**t_3**	2628	6.5	0.93	92.7
H_2O_2	t_0	3161	7.8	0.98	155.7
H_2O_2 *	t_0	3567	8.1	0.98	166.3
H_2O_2	**t_3**	2179	5.2	0.84	79.4
H_2O_2 ^{a}	**t_3**	2595	6.3	0.91	136.2
Blank	t_0	3023	7.0	0.95	125.6

Blank [a]	t_0	3181	8.3	0.99	158.7
Blank	**t_3**	3487	6.7	0.94	112.4
Blank [a]	**t_3**	3511	6.3	0.91	111.0

[a], indicates a second independent assay.

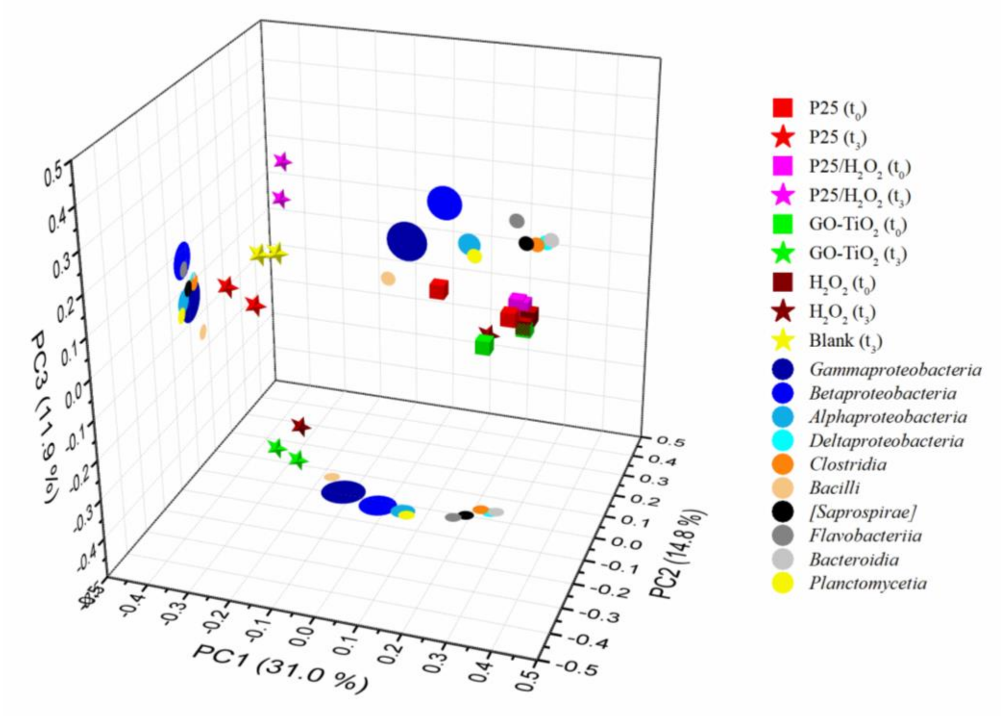

Figure 6.6. Biplot of principal coordinates analysis (PCoA) based on weighted Unifrac distances of samples before (t_0 - squares) and 3-days storage after treatment (t_3 – stars).

Differences on the structure and composition of the bacterial community of the stored treated wastewater samples were mainly based on the relative abundance of *Beta-* and *Gammaproteobacteria* and *Bacilli* (Figure 6.5a, t_3 and Figure 6.6., stars). The structure and composition of the bacterial communities of treated stored water was similar. Nevertheless, the relative abundance of *Betaproteobacteria* was higher for photolysis > P25 > P25/H_2O_2 > solar-H_2O_2 > GO-TiO_2, varying between 57% and 7% (Figure 6.5a). Consequently, the relative abundance of *Gammaproteobacteria* followed a kind of inverse order (*i.e.*, GO-TiO_2 > P25/H_2O_2 > P25 > solar-H_2O_2 > photolysis), varying between 61% and 25% (Figure 6.5a). Despite the lower values when compared with these proteobacterial classes, the relative abundance of *Bacilli* followed a similar order (8% and 0%, Figure 6.5a). The ability of some members of class *Bacilli* to survive under harsh stressful conditions through the production of resistance forms (endospores) as well as their ability to withstand oxidative stress [72, 73] may explain their survival upon these treatments.

Altogether, the results obtained suggest that the solar-driven AOPs inactivated less efficiently *Beta*- and/or *Gammaproteobacteria* or that at least some bacteria belonging to these classes have higher capacity to regrow. Curiously, at the genus level, it is possible to observe that members of the ubiquitous genus *Pseudomonas* were, in general, the group with the sharpest increase during storage, followed by *Rheinheimera* and *Methilotenera* (Figure 6.5b). Alterations in the composition and structure of the bacterial communities leading to higher proportions of *Proteobacteria* in stored water than before the treatment were reported in the literature for photolysis, ozonation and photocatalytic ozonation [8], coupled biological and photocatalysis treatments [74] and ozonation coupled to a sequencing batch biofilm reactor [75].

6.5. Conclusions

Among the tested solar-driven oxidation processes, photo-Fenton at circumneutral pH was the worst performing one (quite similar to photolysis), whereas the combination of P25 and H_2O_2 was the most efficient approach for the removal of organic micropollutants in the urban wastewater sampled.

Regarding the biological indicators, a decrease in the abundance of total faecal coliforms and enterococci and their antibiotic resistant counterparts was found for all the processes employing H_2O_2, which was permanent after 3-days storage of the treated wastewater. P25/H_2O_2 and solar-H_2O_2 were also able to reduce the total bacterial load, assessed based on the abundance of the 16S rRNA gene. Nevertheless, the abundance of total bacterial load increased after 3-days storage to values close or higher than those verified before treatment. Similar observations were found for the genes *intI*1 and *sul*1. Hence, none of the studied processes was able to prevent bacterial reactivation, including antibiotic resistant populations.

Thus, among all the studied processes, P25/H_2O_2 seemed to be that achieving the best compromise for the removal of both organic micropollutants and biological contaminants, although not able to prevent bacterial reactivation.

Interestingly, regardless of the oxidation process studied, higher relative abundance of the phylum *Proteobacteria* (*Beta*- and *Gammaproteobacteria*), namely of genera *Pseudomonas*, *Rheinheimera* and *Methylotenera*, was observed in treated wastewater after 3-days storage. Since within the phylum *Proteobacteria*, in particular of the classes *Beta*- and *Gammaproteobacteria*, it is possible to find diverse multidrug-resistant bacteria, the increase of this group of organisms in stored treated water may deserve further investigation. Moreover, the potential disturbance of the water bacterial communities may have relevant ecology implication and should be considered in the design of advanced oxidation technologies.

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Chapter 7

Metal-free g-C_3N_4 photocatalysis of organic micropollutants in urban wastewater under visible light

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The work described in Chapter 7 was published in the following reference and reproduced by permission of Elsevier (the original version is provided as **Appendix D)**:

> **Moreira, N.F.F.**, Sampaio, M.J., Ribeiro, A.R., Silva, C.G., Faria, J.L., Silva, A.M.T. *Metal-free g-C_3N_4 photocatalysis of organic micropollutants in urban wastewater under visible light, Applied Catalysis B: Environmental*, 248 (2019) 184-192.

Specific nomenclature in Chapter 7:
- MPs corresponds to OMPs;
- TiO_2 corresponds to the commercial TiO2-P25 material.

7.1. Abstract

Heterogeneous photocatalysis under visible light was employed for the degradation of organic micropollutants (MPs) found in the biologically treated effluents of an urban wastewater treatment plant (UWWTP). The irradiation source consisted in 4 light emitting diodes (LEDs) with a nominal power of 400–500 W m^{-2} at 417 nm. Metal-free exfoliated graphitic carbon nitride (gCN_T), which was synthetized through a simple thermal treatment using dicyandiamide as precursor, was employed as photocatalyst. The gCN_T material was characterized using different techniques: N_2 adsorption isotherms at 77 K, electron microscopy (scanning and transmission) and diffuse reflectance UV–Vis. The photocatalytic performance of the material was compared to the commercial TiO_2-P25, confirming that gCN_T led to a remarkable higher removal efficiency of the target MPs. Most of these MPs were removed in less than 10 min to levels below the limit of quantification (carbamazepine > isoproturon > clopidogrel > diclofenac > atenolol > bezafibrate > tramadol > venlafaxine > fluoxetine). Indirect photolysis, resulting from the secondary reactive oxidants generated during the irradiation of the aqueous matrix components, was found to partially contribute for the elimination of the occurring MPs. In addition, the gCN_T photocatalyst was immobilized on glass rings for use under continuous mode operation, a minimum residence time of 25 min being required to attain significant removal efficiencies. Phytotoxicity experiments showed that heterogeneous photocatalysis did not enhance the toxicity of the wastewaters.

7.2. Introduction

Organic micropollutants (MPs) are frequently found in the aquatic environment at concentrations levels between ng L^{-1} and μg L^{-1}. The difficult elimination of MPs by physicochemical and biological treatments conventionally applied in the urban wastewater treatment plants (UWWTPs) triggers the need of alternative approaches to remove them. Although discharge limits are not yet regulated for MPs, some recommendations have been launched by European Union (EU) in order to monitor harmful substances, namely the Directive 2013/39/EU [1], which indicates a group of 45 priority substances/groups of substances and recommends new treatment strategies, and the Decision 2015/495/EU [2], setting a Watch List of 17 contaminants of emerging concern (CECs) for EU-monitoring, which was very recently repealed by a new Watch List of 15 CECs [3].

Novel treatment tools have been sought such as advanced oxidation processes (AOPs), which have been largely studied to reach high degradation rates of MPs from UWWTP effluents [4]. However, only UV (mainly for disinfection) and ozonation have been implemented so far at full-scale [5-7], both approaches presenting some limitations but affordable costs when compared with most AOPs. Solar applications of heterogeneous photocatalysis are an interesting alternative to degrade a broad range of MPs [8]. Wide-bandgap optical semiconductors able to act as visible light photocatalysts require absorption edges above the UV limit since this only accounts for less than 5% of the solar spectrum reaching Earth's surface. Visible-light photocatalysts with a narrower bandgap, such as graphitic carbon nitride (g-C_3N_4 with a bandgap of 2.7 eV, corresponding to an absorption edge at 459 nm), have been receiving an increasing interest for a wide variety of photocatalytic applications (e.g., CO_2 and NOx reduction, degradation of organic pollutants, organic selective synthesis, hydrogen production and water splitting) [9-18]. The high photocatalytic activity of g-C_3N_4 materials on the degradation of different types of organic contaminants in water has been already demonstrated in previous studies, namely for methylene blue [19-22], rhodamine B [22-25], methyl orange [26-28], basic fuchsin, malachite green and crystal violet [29], acid orange 7 [30], oxalic acid [31], tetracycline [32, 33], phenol [34-36], atrazine [35, 37], carbamazepine and sulfamethoxazole [35, 38] and humic acids [39]. However, in the majority of these reports, the experiments are often carried out using ultrapure water, concentrations higher than those usually found in the environment, slurry (batch) systems, metal loaded g-C_3N_4 materials, and spiked matrices [40]. To the best of our knowledge, none of these studies report the treatment of an actual urban wastewater (*i.e.*, not spiked) employing g-C_3N_4 as metal-free photocatalyst.

g-C_3N_4 has been synthesized by thermal polymerization of several nitrogen-rich precursors, namely urea, thiourea, cyanamide, dicyandiamide or melamine [17, 41]. Since its photocatalytic activity is struggled by the low surface area and by the fast recombination of electrons and holes, several approaches have been used to overcome these drawbacks, namely the chemical, mechanical and thermal exfoliation treatments of g-C_3N_4 [17, 42]. Other procedures include the design of g-C_3N_4 based nanoarchitectures using templating approaches, doping with metals, and modification with other semiconductors, well discussed in the literature [41].

The chemical and thermal stability of the low-cost, metal-free g-C_3N_4 turns this visible light photocatalyst an outstanding option for solar-driven photocatalysis [9, 43]. In addition, energy-efficient and low-cost visible light emitting diodes (LEDs) with a narrow emission band can be employed as light sources, as replacement of xenon or mercury lamps, and matching the absorption peak of the material for the economy of the process [43].

The novelty of the present study relies on the application of a highly-efficient photocatalytic process based on a metal-free g-C_3N_4 photocatalyst with enhanced activity towards degradation of a wide range of MPs in realistic urban wastewaters, by using a low-cost and energy-efficient visible light source (LED). The photocatalytic treatment was also performed in continuous mode, by employing an innovative approach with g-C_3N_4-coated glass rings. The phytotoxicity of the wastewater was evaluated, before and after photocatalytic treatment, to infer about the potential formation of reaction by-products toxic for plants, which could compromise water reuse. To the best of our knowledge, this is the first study on heterogeneous photocatalysis using g-C_3N_4 as a semiconductor for the removal of MPs in actual UWWTP effluents.

7.3. Materials and methods

7.3.1. Chemicals and materials

Dicyandiamide (99%) supplied from Sigma-Aldrich was used as precursor in the preparation of the bulk g-C_3N_4. Aeroxide® TiO_2-P25 (~80% anatase:20% rutile) powder was supplied by Evonik. Polyvinyl alcohol (PVA), reference standards for liquid chromatography (> 98%) and surrogate standards were purchased from Sigma-Aldrich, which include atenolol, bezafibrate, carbamazepine, clopidogrel, diclofenac, fluoxetine, isoproturon, tramadol venlafaxine, azithromycin-d3, atrazine-d5, diclofenac-d4 and fluoxetine-d5. Ethanol (99.5%) and 2-propanol (100%) were obtained from Fisher Scientific UK Limited. Acetonitrile (MS grade) was supplied by Panreac AppliChem. Formic (99.5%) and sulfuric (98%) acids were acquired from Merck (Darmstadt, Germany). Ultrapure water was produced in a Milli-Q water system (resistivity of 18.2 MΩ cm, at 25 °C). The Oasis® HLB (Hydrophilic-Lipophilic-Balanced) cartridges (150 mg, 6 mL) used for sample preparation, were purchased from Waters (Milford, MA, USA).

7.3.2. Catalyst preparation and characterization

The bulk g-C_3N_4 material was prepared by thermal decomposition of dicyandiamide, as previously described [17]. Briefly, a certain amount of dicyandiamide precursor was placed in a covered crucible and then heated to 550 °C in air atmosphere for 4 h. In a second step, thermal oxidative exfoliation of the bulk material was conducted at 500 °C.

The resulting material was labelled as gCN_T. TiO_2 was used as received, for comparison purposes.

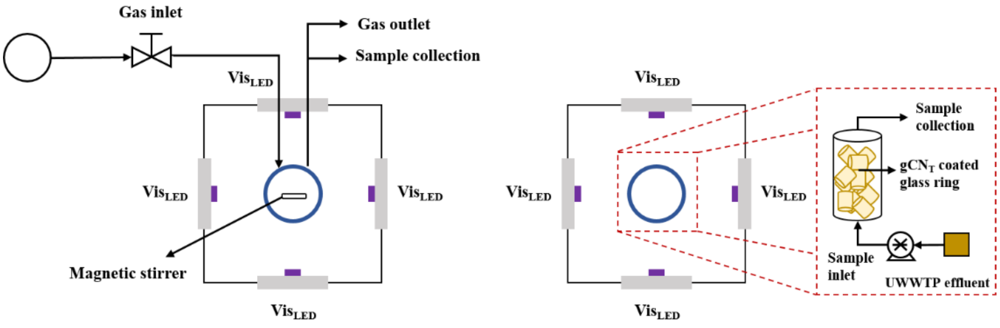
The specific surface area (S_{BET}) of the mentioned photocatalysts was calculated by using the Brunauer-Emmett-Teller (BET) method and N_2 adsorption isotherms obtained at −196 °C in a Quantachrome NOVA 4200e apparatus. The optical absorption was determined in the 220–800 nm range by diffuse reflectance UV–Vis (DRUV-Vis) in a JASCO V-560 spectrophotometer equipped with an integrating sphere. The results were recorded to equivalent absorption Kubelka–Munk units and used to obtain the optical bandgap.

Uncoated glass rings (diameter = 3 mm, length = 3 mm) were cleaned with an anionic detergent in water, under sonication. The washed rings were then immersed in 2-propanol and sonicated for 15 min. Before coating with gCN_T, the glass rings were immersed in a 2% (w/V) aqueous solution of PVA by dip-coating. This procedure was immediately followed by a second dip-coating immersion of the glass rings in a 5% (w/V) gCN_T:ethanol suspension, previously sonicated for 30 min.

The surface morphology of bulk $g\text{-}C_3N_4$, gCN_T powders and gCN_T immobilized on glass rings was observed by scanning electron microscopy (SEM), using a FEI Quanta 400FEG ESEM/EDAX Genesis X4 M instrument. In the case of the immobilized gCN_T, the SEM apparatus was equipped with a sample holder, in which the broken glass rings were positioned to observe and measure the cross-section of the coated material, by using the respective SEM instrument software. Transmission electron microscopy (TEM) was performed on a JEM 220FS microscope (Jeol, Japan) with a LaB6 electron gun operating at 200 kV.

7.3.3. Experimental set-up

Wastewater samples from the secondary biological treatment of a UWWTP located in the North region of Portugal, were collected and separated in aliquots which were stored at – 4 °C. The photocatalytic performance of the gCN_T powder was studied in batch mode, using a borosilicate reactor containing 60 mL of UWWTP effluent, equipped with four high-power visible LEDs ($\lambda_{max} = 417$ nm, Full-Width Half-Maximum (FWHM) = 18.2 nm) perpendicularly placed to each other and equidistant (4.0 cm) from the reactor (Scheme 7.1a). The nominal irradiation of each LED varied between 400–500 W m^{-2}, as determined by UV-Vis spectroradiometry (USB2000+, OceanOptics, USA). The catalyst load was fixed at 1.0 g L^{-1} (selected in preliminary studies as optimal gCN_T load for the degradation of model organic pollutants). A dark period of 30 min was performed to establish the adsorption-desorption equilibrium under oxygenated conditions (air). Under these conditions, the removals of the 9 studied MPs never exceeded 6% after 30 min of adsorption period with either TiO_2 or gCN_T. Then, the suspension containing the UWWTP effluent and the photocatalyst was irradiated for 60 min, and samples were withdrawn regularly from the reactor.



a) Batch mode system b) Continuous mode system

Scheme 7.1. Photocatalytic experimental set-up under batch (a) and continuous (b) operation modes.

Photolysis experiments were performed in UWWTP effluent and also in ultrapure water spiked with the MPs found in the UWWTP effluent and at similar concentrations, in this case under air and deoxygenated (argon) conditions. For that, the required volumes of each standard solution were added to a volumetric flask and the organic solvent was evaporated before filling it with the ultrapure water, avoiding the presence of the organic solvent and its possible scavenging effect. Control experiments using ultrapure water as matrix were also run in the dark, under oxygenated and deoxygenated conditions. All the experiments were carried out in triplicate.

Continuous flow experiments were performed using the gCN_T material immobilized on glass rings (Scheme 7.1b). A borosilicate cylindrical reactor (internal diameter = 2.7 cm, length = 7.0 cm) was packed with 115 gCN_T-coated glass rings. The filling volume after packing was approximately 31.5 mL. The reactor was continuously filled with UWWTP effluent using a peristaltic pump at constant flow rate (Q), and LEDs were then turned on after establishment of the adsorption-desorption equilibrium. Samples were regularly withdrawn after reaching the steady state. Different residence times (2, 5, 12, 25, 55 min) were tested, by changing the liquid flow rate (0.57, 1.26, 2.6, 6.3, 15.7 mL min^{-1}). Uncoated glass rings were used for photolysis experiments (residence time of 55 min) to maintain the hydrodynamic behaviour and light diffusion conditions, allowing the comparison with the photocatalytic experiments.

7.3.4. Analytical methods

The concentration of each organic MP in the UWWTP effluent was determined by ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) in a Shimadzu Corporation apparatus (Tokyo, Japan), consisting of a UHPLC equipment (Nexera) with two pumps (LC-30AD), an autosampler (SIL-30AC), an oven (CTO-20AC), a degasser (DGU-20 A 5R) and a system controller (CBM-20 A) with proper software (LC Solution Version 5.41SP1), coupled to a triple quadrupole mass spectrometer detector (Ultra-Fast Mass Spectrometry series LCMS-8040). Before UHPLC-MS analysis, a pre-concentration and clean up procedure was performed by solid phase extraction (SPE), adding isotopically labelled internal standards to the samples before SPE as described elsewhere [44]. A Cortecs™ C18+ column (100 × 2.1 mm i.d.; 1.6 μm particle diameter), supplied by Waters (Milford, MA, USA), was used with a mobile phase composed by ultrapure water and acetonitrile (20:80, v/v), both acidified with 0.1% formic acid. The chromatographic analytical method was performed at isocratic mode, using a flow rate of 0.3 mL min^{-1} and the column oven temperature set at 30 °C. The autosampler temperature was set at 4 °C and the injection volume was 5 μL. For quantification purposes, the selected reaction monitoring (SRM) transition between the precursor ion and the most abundant fragment ion was determined; whereas the second most intense fragment ion was used for confirmation of the identity of each target MP (SRM1/SRM2 ratio).

The dissolved organic carbon (DOC) was determined using a Shimadzu TOC-L apparatus (Shimadzu Scientific Instruments, Japan).

7.3.5. Phytotoxicity evaluation

PHYTOTOXKIT microbiotests (MicroBioTests Inc.) were used to assess the phytotoxicity, by comparing the germination of the seeds of 3 given plant species, as well as their root and shoot lengths, after exposure during 72 h to distilled water, non-treated and treated UWWTP effluents (after 10 and 60 min using TiO_2 and gCN_T-photocatalysts). The PHYTOTOXKIT microbiotest strictly adheres to ISO standard 18763. The plants were monocotyl *Sorghum saccharatum* (Sorgho), dicotyls *Lepidium sativum* (garden cress) and *Sinapis alba* (mustard). The measurements of root and shoot lengths were performed by image analysis using ImageJ® software (NIH, USA).

7.4. Results and discussion

7.4.1. Photocatalyst characterization

The characterization of the bulk g-C_3N_4 and gCN_T materials was presented in our previous work [17] (labeled as bulk and T500, respectively), studying its use for the photocatalytic synthesis of benzaldehyde. In the present work, the most relevant characterization for interpretation of the results was selected, and additional data on the gCN_T immobilized on glass rings before and after the photocatalytic reactions, was included. Briefly, the S_{BET} of the bulk g-C_3N_4 material prepared by direct pyrolysis of dicyandiamide is typically low around ~10 $m^2\,g^{-1}$, as experimentally determined. However, after thermal post-treatment at 500 °C, the S_{BET} of the resulting material, gCN_T, increased by ca. 12 times to 117 $m^2\,g^{-1}$. As already described in the literature, oxidation treatment under air atmosphere may lead to unstable structures, due to the low resilience of the hydrogen bonding between strands of polymeric melon by NH or NH_2 groups [41]. The procedure adopted in this work may promote the gradual exfoliation of the bulk material, due to layer-by-layer thermal oxidation and splitting, resulting in a higher S_{BET} [45, 46]. For comparison purposes, the S_{BET} determined for TiO_2 was 54 $m^2\,g^{-1}$.

The DRUV-Vis spectrum of gCN_T, shows an unresolved broad band fading out between 450 and 500 nm (Figure 7.1). It is significantly shifted to the red region leading to a higher light absorption range through the visible spectrum, in comparison to TiO_2. The commercial TiO_2 photocatalyst shows the characteristic absorption band sharp edge rising at 400 nm. The bandgap energies (E_g) of both photocatalysts were estimated by the Tauc plot (inset Figure 7.1), using the equivalent absorption Kubelka-Munk (*KM*) units of the absorption edges, $(KM.h.v)^{1/2}$ versus E_g, where *h* is Plank' constant and v is the light frequency. The E_g determined for TiO_2 and gCN_T were 3.14 and 2.76 eV, respectively.

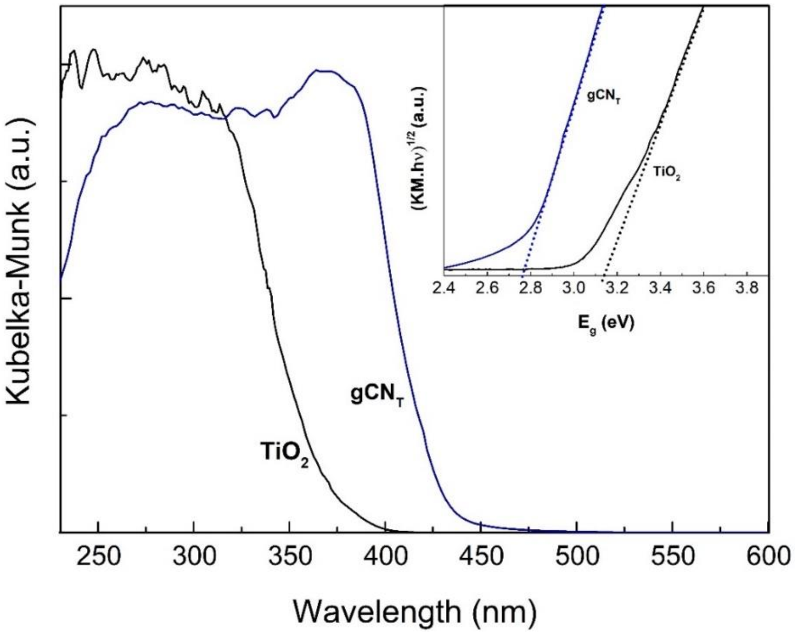


Figure 7.1. DRUV-Vis spectra of TiO_2 and gCN_T photocatalysts; inset: plot of Kubelka-Munk units as a function of the light energy.

The morphology of bulk $g\text{-}C_3N_4$ and gCN_T materials was assessed by SEM and TEM micrographs (Figure 7.2.), revealing differences in their morphology.

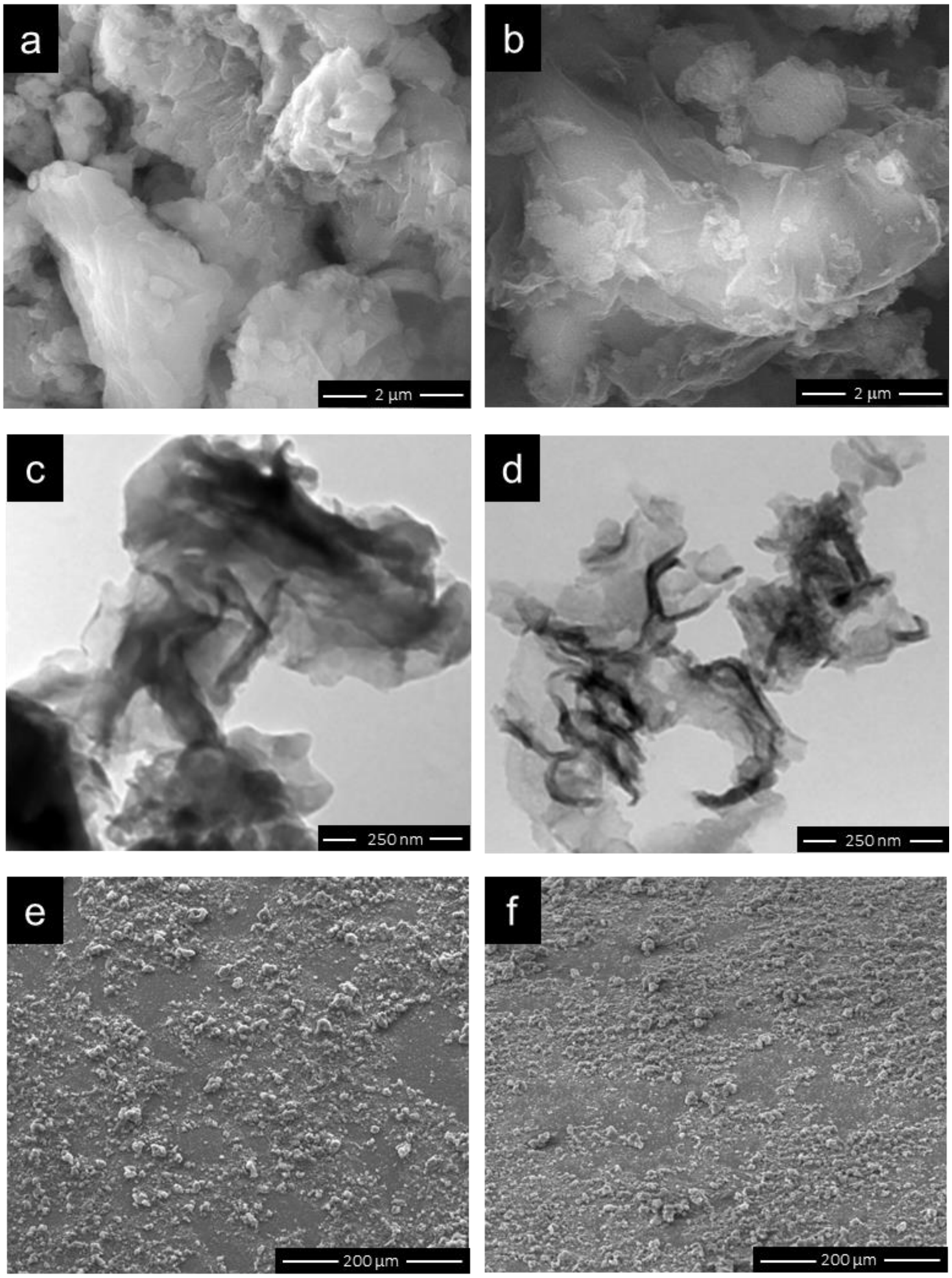


Figure 7.2. SEM (a, b) and TEM (c, d) images of bulk g-C_3N_4 (a, c) and gCN_T (b, d) powder materials. SEM of gCN_T immobilized on glass rings before (e) and after (f) usage in the photocatalytic reactions.

The bulk material is constituted by g-C_3N_4 sheets assembled in dense aggregates (SEM, Figure 7.2a), resulting from the stacking of the layers. In the case of gCN_T (SEM, Figure

7.2b), thinner plates surrounded by small aggregates of non-exfoliated g-C_3N_4 material were observed, resulting from the progressive oxidation of the hydrogen bonds between the layers, *i.e.* the exfoliation originated by the thermal oxidation treatment, in agreement with the results obtained from S_{BET} measurements. TEM analysis of the materials was also performed (Figure 7.2c and 7.2d) and the results corroborate with those given before, less dense layers being observed in the case of the gCN_T material. A representative SEM micrograph of the fresh gCN_T coated glass rings reveals its regular distribution over the support (Figure 7.2e). These gCN_T coated glass rings were analysed after being employed in the photocatalytic treatment of the UWWTP effluent, under continuous mode and during several hours, the morphology remaining uniform and, thus, confirming their stability under the tested conditions (Figure 7.2f).

7.4.2. Degradation of MPs in batch mode using powder photocatalysts

The photochemical degradation of a set of 9 organic MPs quantified in the UWWTP effluent was carried out in the absence of catalyst by irradiating at 417 nm using LEDs as light source (Figure 7.3). Under these conditions, a surprisingly significant abatement of the MPs (clopidogrel > fluoxetine > diclofenac > isoproturon ≈ tramadol ≈ venlafaxine > carbamazepine ≈ atenolol > bezafibrate) was observed (Figure 7.3a). This occurrence could not arise from direct single photon absorption since none of the target compounds will absorb on the emission band of the LED (Figure D-S7.1). However, indirect photolysis may take place by irradiation of the nitrates naturally present in UWWTP effluents (~0.40 mg L^{-1} in this particular case, Table D-S7.1): nitrate photochemistry gives raise to reactive oxygen species (ROS), namely singlet oxygen ($^1O_2$), hydroxyl radicals ($HO^•$) or alkyl peroxyl radicals ($ROO^•$) and hydrated electrons from the ionization of water solvent molecules [47, 48]. In addition, photosensitization phenomena may occur by light absorbing species, such as coloured organic matter in its triplet state with the ability to break the organic MPs, leading to their indirect photochemical decomposition [47-49]. Less common will be the occurrence of non-linear photon absorption phenomena, such as the simultaneous absorption of two photons [50].

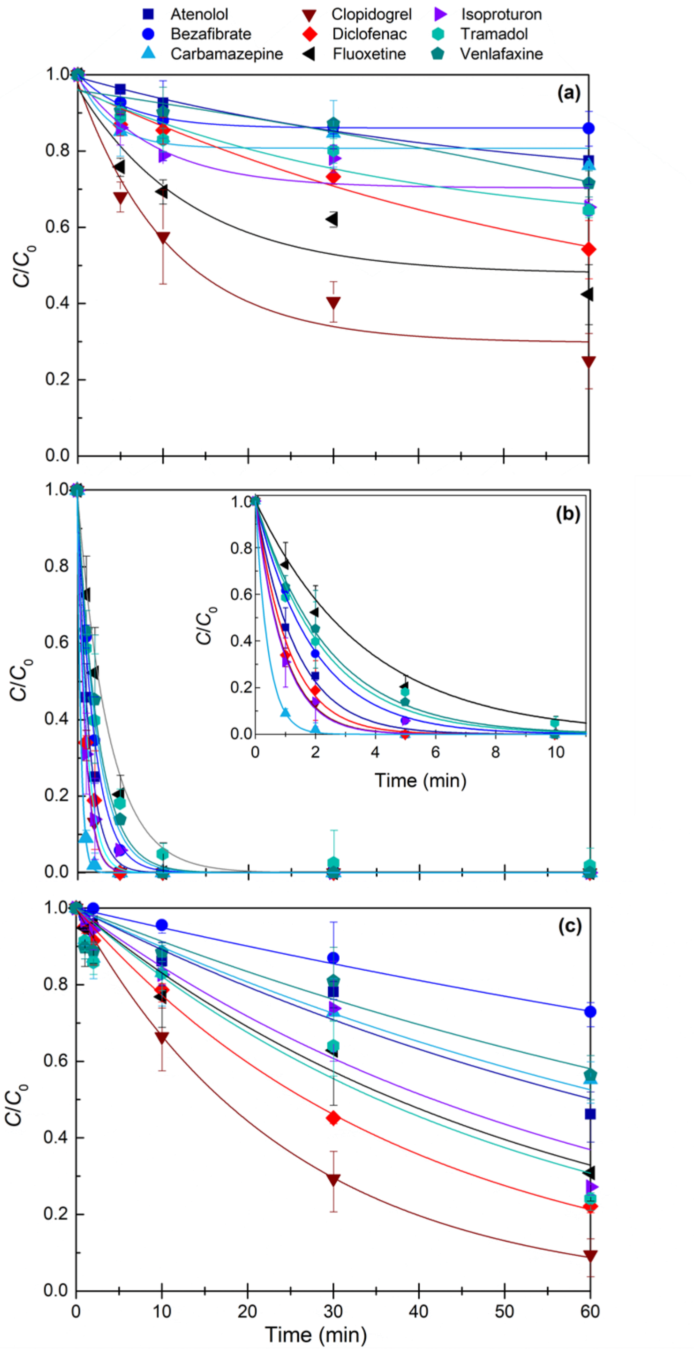


Figure 7.3. Normalized concentration of the MPs (C/C_0) identified in the UWWTP effluents, after treatment by photolysis (a), and by heterogeneous photocatalysis using gCN$_T$ (b) and TiO$_2$ (c). Experiments were performed in batch mode (60 mL) and with four LEDs, using a catalyst load of 1.0 g L^{-1}. LEDs were switched on after a dark period of 30 min to establish the adsorption-desorption equilibrium.

Considering that the complexity of the matrix may affect the degradation kinetics of the MPs [51-53], photolysis experiments were performed using ultrapure water spiked with a solution containing the 9 organic MPs with similar concentrations to those found in the UWWTP effluents, under oxygenated and deoxygenated conditions, and with/without light irradiation. Markedly lower removals were obtained when using ultrapure water as matrix (Figure D-S7.2) instead of the real wastewater (Figure 7.3a), and quite similar results were obtained when the solutions were saturated with argon or oxygen, under both dark and light conditions. Thus, photosensitization, indirect photolysis, or other mechanisms involving other matrix species, could take place under visible light driving the degradation of the MPs in the UWWTP effluent.

The photocatalytic degradation of the MPs in the UWWTP effluents followed a pseudo-first order rate law (Figure 7.3b and c). The apparent first order reaction rate constants (k_{app}) summarized in Table 7.1 were determined by exponential curve fitting to the experimental data.

Table 7.1. Initial concentration (ng L^{-1}) and apparent first order reaction rate constant (k_{app}, min^{-1}) for each MP using different treatment processes.

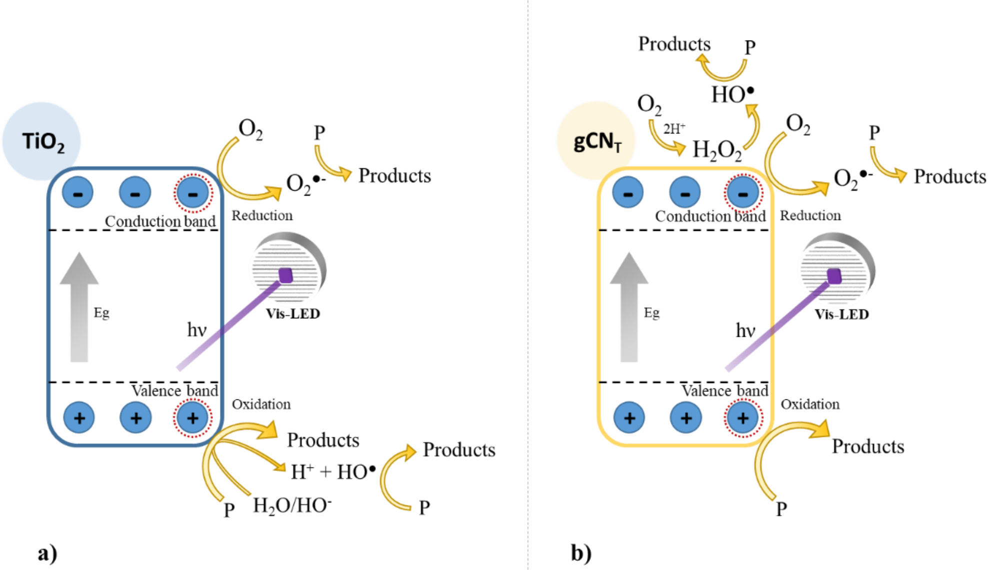
		k_{app} ($\times 10^{-2}$ min^{-1})	
Compound	Initial concentration (ng L^{-1})	VIS_{LED}/ gCN_T	VIS_{LED}/ TiO_2
Atenolol	12.5 ± 1.2	74.3 ± 2.3	1.2 ± 0.1
Bezafibrate	38.7 ± 6.0	51.9 ± 1.2	0.5 ± 0.0
Carbamazepine	763 ± 18	238 ± 1	1.1 ± 0.2
Clopidogrel	93.2 ± 10.7	108 ± 2	4.1 ± 0.0
Diclofenac	1102 ± 31	90.6 ± 2.6	2.6 ± 0.1
Fluoxetine	21.7 ± 4.5	27.4 ± 4.5	1.9 ± 0.2
Isoproturon	84.6 ± 7.4	110 ± 8	1.7 ± 0.3
Tramadol	3930 ± 244	44.6 ± 3.6	2.0 ± 0.3
Venlafaxine	349 ± 47	41.3 ± 1.2	0.9 ± 0.2

The photocatalytic experiments using the powdered gCN_T led to an almost complete removal of all MPs (carbamazepine > isoproturon > clopidogrel > diclofenac > atenolol > bezafibrate > tramadol > venlafaxine > fluoxetine) after 10 min of reaction (*i.e.*, after turning on the LEDs) (Figure 7.3b). It is important to refer that the k_{app} obtained for each MP hold one to three orders of magnitude compared with the TiO_2, proving the high efficiency of this catalyst under the reaction conditions used.

Regarding the standard TiO_2 photocatalyst (Figure 7.3c), it is observed that the conversion is only slightly better that the photolytic degradation of the MPs. In addition, a different degradation order of the MPs is observed (clopidogrel > diclofenac > tramadol > fluoxetine > isoproturon > atenolol > carbamazepine > venlafaxine > bezafibrate), when comparing with the gCN_T material. The distinct behaviour observed for both catalysts can be expected, especially by using a real matrix containing naturally occurring interferences that may act as promoters or inhibitors (such as organic matter consuming $HO^•$ radicals and other oxidizing species; anions acting as $HO^•$ scavengers; organic and inorganics species competing for the active reaction sites, etc.). Moreover, the photocatalytic activity of the TiO_2 under the light source used ($\lambda_{max} = 417$ nm) can also be explained in terms of the residual overlap between the absorption spectrum of TiO_2 and the emission of the LED (Figure D-S7.3). Obviously, it is not expected that a wide-bandgap optical semiconductor such as TiO_2 (E_g~3.14 eV) would be efficient under 417 nm excitation, since UV-light is required to effectively activate it. Nevertheless, the comparison between the TiO_2 (E_g~3.14 eV) and the gCN_T (E_g~2.76 eV) under visible light was performed based on previous studies using LEDs emitting either in the UV or visible ranges. In those experiments, the performance of both TiO_2 and gCN_T was evaluated on the degradation of diclofenac as model compound (Figure D-S7.4). The results demonstrated that even under UV light, the gCN_T material exhibited better performance compared with TiO_2, showing the high efficiency of gCN_T in both UV and visible ranges.

The efficiency of $g\text{-}C_3N_4$ based photocatalysts to eliminate organic contaminants (mostly dyes) is already known from the literature [19, 20, 23, 24, 26, 27, 29, 34, 37, 39]. In the unique study reported to a spiked effluent from a UWWTP [35], the photocatalytic degradation of four model compounds (phenol, atrazine, sulfamethoxazole and carbamazepine at 100 μM), under visible illumination (xenon lamp; λ > 400 nm), was attributed to reactive oxygen species and/or contaminant-photocatalyst interfacial interactions, depending on the g-C_3N_4 sample tested. Furthermore, the photocatalytic activity was similar regardless of the realistic matrix tested (water or wastewater), demonstrating the potential of g-C_3N_4 for the treatment of real case effluents.

Scheme 7.2 shows the possible pathway for gCN_T catalyst compared with the well-known TiO_2 [54], generally based from mechanism studies of model pollutants in aqueous solutions, *i.e.* considering that the others constituents present in the UWWTP effluent (organic matter, nitrates and other inorganics, etc.) do not interfere on the photocatalytic mechanism. However, it is important to remark that the photocatalytic experiments investigating the efficiency of the gCN_T were performed using UWWTP effluent as matrix, which turns the understanding of the mechanisms occurring and governing the degradation pathway at the catalyst surface much more challenging to predict.



Scheme 7.2. Schematic representation of a possible photocatalytic mechanism for the degradation of MPs using TiO_2 (a) or gCN_T (b).

Concerning the organic matter, no significant changes on the DOC content of the effluent from the UWWTP were observed before and after the treatment with both photocatalysts. This is not surprisingly due to the complexity of the matrix: there are several orders of magnitude of difference between the concentration of the studied organic MPs (in the range of ng L^{-1} to µg L^{-1}) and the DOC content (in the range of mg L^{-1}).

7.4.3. Degradation of MPs in continuous mode using gCN_T-coated glass rings

Slurry reactors have as typical disadvantage the need to separate the catalyst from the treated water, in many cases highly energy-consuming processes being required. The immobilization of the photocatalyst on a proper support is seen as a possibility to overcome this drawback in heterogeneous photocatalysis. However, there is a price to pay with immobilization since a decrease in the photocatalytic efficiency must arise from the fact that the reaction mostly occurs at the catalyst-solution interface and a catalyst dispersed in the matrix maximises the contact area [55-57]. The application of continuous mode operating systems, in some cases using immobilized catalysts, was already studied by various authors [56, 58-61].

In order to evaluate the feasibility of gCN_T photocatalysis as an option for application in a real case UWWTP, the removal of the target organic MPs of the effluent samples was further studied using the gCN_T immobilized on glass rings, under continuous mode operation. Different residence times (2, 5, 12, 25, 55 min) were tested, as shown in Figure 7.4. Control experiments were done conducting the photochemical degradation of the target organic MPs with uncoated glass rings during 55 min of residence time. More significant degrees of removal were found for clopidogrel, fluoxetine and diclofenac, as also observed for photolysis experiments in slurry (*i.e.* batch) mode, reinforcing the idea that other mechanisms besides direct photolysis affected the removal of MPs, namely indirect photolysis, photosensitization and/or photo(hydrolysis).

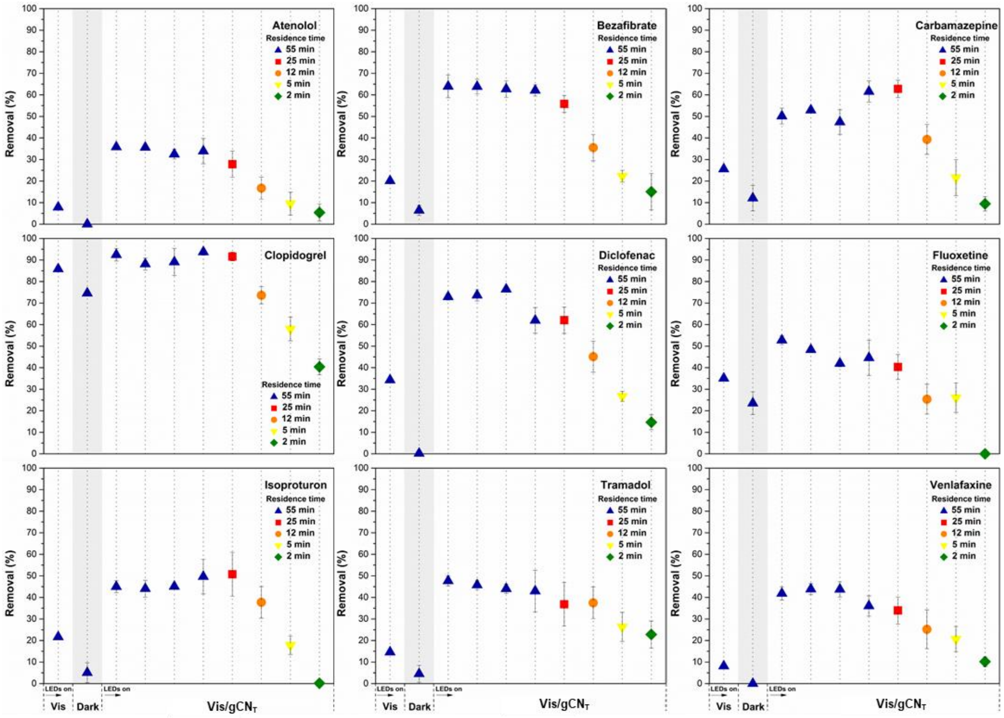


Figure 7.4. Removal (%) of the MPs found in UWWTP effluents, after treatment by photolysis, adsorption and photocatalysis using gCN$_T$ immobilized in glass rings under continuous mode operation for different treatment residence times. Experiments were performed in continuous mode (useful volume of 31.5 mL) with four LEDs, packing the reactor with 115 gCN$_T$-coated glass rings. LEDs were switched on after the adsorption-desorption equilibrium was established (UWWTP effluent was passed through the reactor during ca. 9 h for the lower flow rate (residence time of 55 min)). The samples were withdrawn after the steady state was achieved.

When the reactor was loaded with gCN$_T$ immobilized on glass rings and, before switching on the LEDs, the UWWTP effluent was passed through the reactor to establish the adsorption-desorption equilibrium, during ca. 9 h for the lower flow rate (residence time of 55 min).

Under the photocatalytic process, using gCN$_T$ coated glass rings after approximately 27 h at the highest residence time (55 min), the photocatalyst maintained its activity leading to identical removal efficiencies. Similar results were obtained for the residence times of 25 and 55 min, suggesting that residences times higher than 25 min will not significantly enhance the removal efficiencies of these MPs, probably due to limitations with the effectively irradiated active sites. For lower residence times, these efficiencies decreased, as expected due to the shorter contact between the organic compounds and the semiconductor.

Quite lower removals were obtained in continuous mode operation in comparison with the slurry (batch) system. As an example, nearly complete removal of the MPs was obtained in batch mode after 10 min (Figure 7.3b), whereas the following removal efficiencies were obtained in continuous mode for a residence time of 5 min (equivalent treatment time of 10 min in batch mode): atenolol (≈ 10%), bezafibrate (≈ 20%), carbamazepine (≈ 20%), clopidogrel (≈ 60%), diclofenac (≈ 25%), fluoxetine (≈ 20%), isoproturon (≈ 20%), tramadol (≈ 20%) and venlafaxine (≈ 20%). This finding was expected since the catalyst-solution interface decreases when using gCN_T coated glass rings. Again, in what concerns to the amount of organic matter, no significant changes on the DOC content were verified for all the tested residence times, by the same reasons given previously for batch mode. The application of coated-materials requires more treatment time for the effective degradation of pollutants due to the lower area of catalyst per unit of volume, when compared to the use of powder suspensions. However, some practical problems arising from the application of powder photocatalysts are avoided, such as the catalyst separation from the treated water.

The photocatalytic treatment under continuous mode using gCN_T coated glass rings to remove MPs from the effluents of UWWTP is nevertheless possible, but still a challenging technological solution. Further research is needed regarding the optimization of several parameters, such as the length of the column, the amount of catalyst immobilized on the support, the size and number of gCN_T-coated glass rings per unit volume, and the light distribution in the reactor, aiming to increase the process efficiency in continuous mode operation.

7.4.4. Phytotoxicity measurements

The phytotoxicity was assessed using the PHYTOTOXKIT microbiotest (MicroBioTests Inc.), according to the standard operating protocols for three plant species (*i.e. Sorghum saccharatum*, *Lepidium sativum* and *Sinapis alba*). The samples from the UWWTP before and after treatment by photocatalysis (using both TiO_2 and gCN_T), did not inhibited the germination of seeds (results not shown).

7.5. Conclusions

The heterogeneous photocatalysis of MPs in real case effluents of UWWTP was successfully achieved under visible light activation. Among the target MPs found in the UWWTP effluent, the gCN_T photocatalyst showed significantly higher removal rates than the benchmark TiO_2, which can be explained by the suitable E_g and good redox ability of the photogenerated carriers.

Complete removals (to values bellow the limits of quantification) were found in ca. 10 min of slurry photocatalytic treatment using gCN_T as catalyst. Moreover, only 5 min were enough to eliminate atenolol, carbamazepine, clopidogrel and diclofenac. As expected, lower efficiencies were observed for the removal of these MPs when the photocatalytic treatment was performed under continuous mode employing gCN_T immobilized on glass rings. Nevertheless, this strategy is important, considering the cost of separation processes and energy requirements for the recovery of the photocatalyst, prior to water reuse or discharge.

The present work showed the potential of gCN_T used in conjunction with visible LEDs to remove several organic MPs present in actual effluents from UWWTP. This can be relevant to the reduction of MPs in UWWTPs, or in other end-of-pipe solutions. It is however clear that parallel to the development of treatment solutions to minimize the impact of MPs on the environment, further optimization is needed.

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PART III

Final remarks

CONTENT

The continuous disposal of OMPs and potentially harmful microorganisms (including human pathogens, ARBs and ARGs) into water matrices affects directly the environmental and human health. The constant challenge for the development of effective water treatment processes led to a vast increase in the number of studies regarding new advanced technologies for the removal of both chemical and biological pollutants from different water matrices. However, the majority of the studies is still performed under unrealistic conditions, and some solutions are far away from being feasible to be applied in real case scenarios. Frequently, ultrapure or distilled water is spiked with the target contaminants at concentrations much higher than those actually reported in environmental compartments and, this way, excluding undesirable interactions between the oxidizing species and the water matrix components. Still, this methodology is important since it allows to study the degradation pathways of specific compounds and evaluate the performance of the chosen treatment towards that contaminant. Moreover, it enables the application of simple and widely used analytical techniques. Hence, future research on treatment technologies should consider the complex interactions between matrix constituents and the oxidative species formed, aiming to maximize the performance of AOTs, since lower efficiencies and higher costs could be expected in a real case scenario. Furthermore, it is essential to study the simultaneous removal of both chemical and biological pollutants, since their continuous proliferation is linked and can only be solved by interdisciplinary studies which will determine the delicate equilibrium between the treatment efficiency towards both pollutants.

Considering the above-mentioned statements, the work hereby presented followed the logic of performing preliminary studies under unrealistic conditions to better understand the processes feasibility and associated drawbacks with further studies being performed as close as possible to real case scenarios to assure their real efficiency, as well as the positive and the negative effects on the environment and in the human health.

The first set of experiments performed in batch mode, hereby considered as preliminary results (Chapter 4), consisted in the application of a wide set of AOTs (photolysis, ozonation, photolytic ozonation, heterogeneous photocatalysis and photocatalytic ozonation) for the removal of spiked and non-spiked organic pollutants in UWW. Through the results obtained for the different AOTs in spiked experiments, it was possible to conclude that photocatalytic ozonation was the best performing treatment for both removals of target parent compounds and their mineralization. Nonetheless, the other tested AOTs (excluding photolysis) were also able to remove both parent compounds in ultrapure water, though with lower mineralization rates. Therefore, it is possible to predict a synergistic effect when the photocatalytic and ozonation treatments are carried out simultaneously. Afterwards, photocatalytic ozonation, ozonation and photocatalysis were applied for the treatment of actual UWW effluents (not spiked), with high removal for both ozone-based treatments. However, the matrix effect led to lower mineralization rates. These results gave us new insights regarding the application of consolidated and perspective AOTs towards the removal of a wide set of OMPs actually present in UWW effluents and were the driving force for future studies.

From this point on, two different water treatment strategies were studied in conditions as near as possible to realistic scenarios, namely, ozone-based (Chapter 5) and light-driven oxidation technologies (Chapter 6 and 7).

The findings reported in Chapters 5 and 6, confirmed that both technologies (ozone- or light-driven based) could be feasible and efficient on the removal of chemical and biological pollutants considering the site-specific limitations and advantages. Nevertheless, despite of the AOT applied, it was found that even with the high inactivation rates of potentially harmful microorganisms (human pathogens, ARB&ARGs), part of the treated UWW microbiota was viable and able to regrow after 3-days storage of treated UWW, followed by the selection of some bacterial groups (namely, *Beta*- and

Gammaproteobacteria), which may have implications on the microbiological quality of the disinfected water.

Chapter 7 was an exception since no disinfection studies were performed. However, the main objective was the application of a highly efficient photocatalytic process based on a metal-free g-C_3N_4 photocatalyst (gCN$_T$) with enhanced activity towards the degradation of a wide range of OMPs in UWW, performed in continuous mode and applying visible light LEDs as the irradiation source. This study is highly important considering that solar radiation has its own drawbacks, such as, not being a continuous light source and that suffer discrepancies around the globe. The findings of this study demonstrated the potential of gCN$_T$ used in conjunction with visible light emitting LEDs to remove several OMPs present in actual effluents from UWWTP. As expected, lower efficiencies were observed for the removal of these OMPs when the photocatalytic treatment was performed under continuous mode using gCN$_T$ immobilized on glass rings.

In parallel, considering the results obtained in Chapter 5, additional studies were focused on the optimization of the reactor configuration and the respective operating conditions. Afterwards, a new reactor configuration was designed and assembled. The homemade reactor (Figure III.1) was designed to perform photocatalytic ozonation experiments in a single column also operating in continuous mode. The glass bubble column (3 I.D. × 70 cm height) was equipped with eight LEDs (15.5 mm × 23 mm) placed perpendicularly to each other, increasing the light distribution along the column. The distance between the LEDs and the reactor was set as 5 cm.

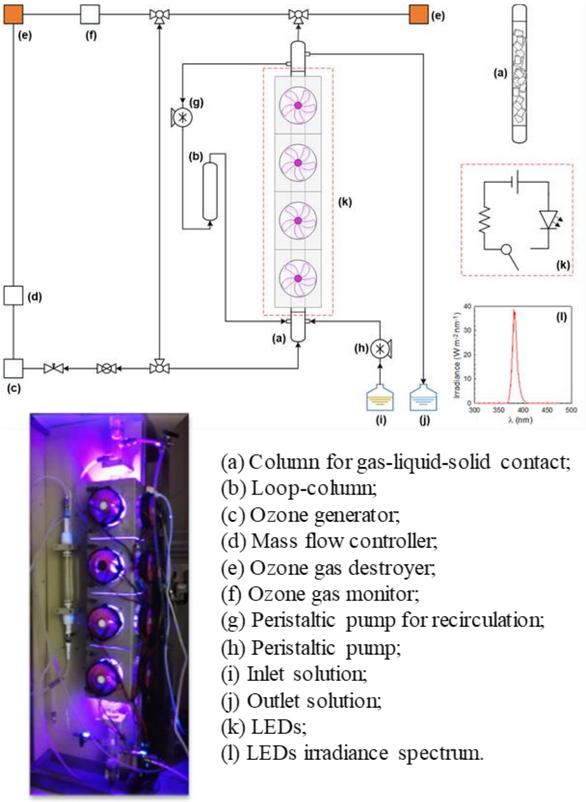

Figure III.1. Experimental set-up of the homemade photocatalytic reactor able to work in continuous mode: (a) Column for gas–liquid-solid contact (Raschig rings); (b) Loop-column; (c) Ozone generator; (d) Mass flow controller; (e) Ozone gas destroyer; (f) Ozone gas monitor; (g) Peristaltic pump for recirculation; (h) Peristaltic pump; (i) Inlet solution; (j) Outlet solution; (k) LEDs; (l) LEDs irradiance spectrum.

The new reactor configuration was tested during two collaboration studies which resulted in two publications:

1. Chávez, A.M, Ribeiro, A.R., Moreira, N.F.F., Silva, A.M.T., Rey, A., Álvarez, P.M., Beltrán, F.J. *"Removal of organic micropollutants from a municipal wastewater secondary effluent by UVA-LED photocatalytic ozonation"*, *Catalysts*, 9 (2019) 472.

The main objectives of this work were to assess the efficiencies of semi-batch and continuous processes and to determine the importance of direct ozonation and $HO^{\bullet}$ mediated reactions in the degradation of the OMPs. According to the obtained results, the presence of TiO_2-P25 and LEDs in an ozone process only added a small advantage to remove some OMPs. However, contributions of simultaneous ozone, radiation, and TiO_2-P25 application is deduced from the higher elimination of DOC as already reported in previous works. Still, in these experiments, the DOC values were mainly due to the water matrix components because the DOC contribution of OMPs actually present in UWW effluents was negligible. Thus, the improvement on the mineralization rates of targeted OMPs by the addition of TiO_2-P25 coated glass rings and LEDs was not possible. Additionally, photocatalytic ozonation could be advantageous regarding the economy of the process, since lower ozone doses might be needed to remove a given amount of organic carbon compared to ozonation.

It was possible to conclude that the new reactor configuration was efficient on the removal of a wide set of OMPs detected in actual UWW effluents by the tested ozone-based processes. Nevertheless, only few differences were observed between these processes. Yet, continuous experiments in deionized water revealed that a synergic effect takes place for ozone decomposition with UVA-LED radiation and TiO_2-P25 -coated glass rings, giving rise to a slight enhancement of contaminant removal in the treatment of UWW. Also, from kinetic studies, it was revealed that direct ozone reaction is the main pathway responsible for the removal of OMPs. Nevertheless, indirect and direct ozone reaction rates ratios indicate that the $HO^{\bullet}$ reaction pathway could prevail over direct reaction in the case of the most refractory compounds for the photocatalytic ozonation system (*i.e.*, isoproturon). Consequently, the use of TiO_2-P25-coated rings and energy-efficient LEDs for photocatalytic ozonation could be an attractive way for wastewater treatment in continuous operation. Different parameters such as retention time, liquid and gas stream flow rates, amount of oxidant agent supplied, among others, should be optimized in future works.

2. Iakovides, I.C., Michael-Kordatou, I, Moreira, N.F.F., Ribeiro, A.R., Fernandes, T., Pereira, M.F.R., Nunes, O.C., Manaia, C.M., Silva, A.M.T., Fatta-Kassinos, D. *"Continuous ozonation of urban wastewater: Removal of antibiotics, antibiotic-resistant Escherichia coli and antibiotic resistance genes and phytotoxicity"*, *Water Research*, 159 (2019) 333-347.

Considering the previous results, the present work was focused on the optimization of the ozonation process operated in continuous mode at different hydraulic retention times (*i.e.* 10, 20, 40 and 60 min) and specific ozone doses (*i.e.* 0.125, 0.25, 0.50 and 0.75 gO_3 $gDOC^{-1}$) for the simultaneous removal of both chemical and biological pollutants, namely eight antibiotics belonging to different classes, autochthonous total and antibiotic-resistant *E. coli*, the bacterial biomarker 16S rRNA gene, the class 1 integron integrase *intI*1 gene and ARGs (*i.e.*, *qac*ΔE1, *sul*1, *aad*A1 and *dfr*A1) from secondary treated UWW effluents. Additionally, since the bacterial inactivation could be a transient effect as observed in previous works, further studies evaluating their regrowth potential after treatment were assessed. Considering the application of UWW for reuse in crop irrigation, phytotoxicity studies were performed. Also, this was one of the very first attempts made to investigate if the variable region of class1 integrons might undergo excision after exposure to ozone (herein indicated by a reduction in *aad*A1 and *dfr*A1).

As expected, the efficiency of ozonation was highly ozone dose- and contact time-dependent. The inactivation of total cultivable as well as trimethoprim- or sulfamethoxazole-resistant *E. coli* was achieved under the experimental conditions of HRT 40 min and 0.25 gO_3 $gDOC^{-1}$, at which all antibiotic compounds were already degraded. The regrowth examinations revealed that higher ozone concentrations were required for the permanent inactivation of *E. coli* below the limit of quantification (LOQ). The abundance of the examined ARGs and related genes (*intI*1, *aad*A1, *dfr*A1, *qac*EΔ1 and *sul*1) was found to decrease with increasing HRT and ozone dose. However, regrowth could not be avoided after ozonation, as it was demonstrated that cultivable cells might still exist in the treated samples even after prolonged treatment.

Despite the fact that the mildest operating parameters (HRT of 10 min and 0.125 gO_3 $gDOC^{-1}$) were able to eliminate the parent compounds of the tested antibiotics in UWW effluents, it was clearly demonstrated in this study that higher ozone doses were required in order to confer permanent damage and/or death and prevent potential post-treatment re-growth of both total bacteria and ARB, and to reduce the abundance of ARGs.

In summary, the findings of this study clearly underline the importance of properly optimisation of the ozonation process (*e.g.* specific ozone dose and contact time) taking into consideration both the bacterial species and associated ARGs, as well as the UWW physicochemical properties (*e.g.* DOC), in order to mitigate the spread of ARB&ARGs and reduce the potential phytotoxicity.

Chapter 8

Conclusions and future work

The present study provides essential clues regarding the efficiency of a wide set of ozone-based and light-driven AOTs on the removal of OMPs and potentially harmful microorganism (human pathogens and ARB&ARGs) present in UWW effluents. Preliminary studies (Chapter 4) gave us insights concerning the application of various AOTs for the removal of organic pollutants either spiked in ultrapure water and UWW, or actual OMPs detected in UWW effluents. From those results, it was concluded that photocatalytic ozonation was the most promising process towards the removal of parent target OMPs, with significant levels of mineralization. However, the effect of water matrix on the treatment efficiency led to lower levels of mineralization in comparison with experiments performed with ultrapure water. Nevertheless, it should also be considered that ozonation and heterogeneous photocatalysis performed quite well on the removal of the parent compounds. From that point on, ozone-based and light-driven oxidation technologies, which could be implemented in different scenarios considering the site-specific advantages and limitations, were studied as close as possible to realistic scenarios, for the simultaneous removal of chemical and biological pollutants (Chapters 5-7).

In Chapter 5, a wide set of AOTs operated in continuous mode with TiO_2-P25-coated glass Raschig rings and LEDs were employed for the first time to treat UWW (as well as SW). Different types of contaminants were considered, including OMPs and ARB&ARGs. The main results presented in this work support the following conclusions:

- Among different tested AOTs, photocatalytic ozonation was the most efficient process for the removal of OMPs from UWW and SW;
- Fluoxetine, clarithromycin, erythromycin and EE2 were the most refractory compounds to oxidation;

- Photocatalytic ozonation demonstrated high efficiency on the removal of microbial loads and housekeeping (16S rRNA) and antibiotic resistance or related genes (*intI*1, *bla*$_{\text{TEM}}$, *qnr*S, *sul*1) from both water matrices;
- ARGs below/close to the quantification limit were found even after 3-days storage of treated water;
- However, both culture-dependent and -independent methods demonstrated that part of the treated SW/UWW microbiota was viable and able to regrow;
- Additionally, in SW samples, the analysis of specific potential antibiotic resistant populations suggested that bacteria with acquired antibiotic resistance traits may have the capacity to regrow in photocatalytic ozonated water up to values found in non-treated SW;
- From YES, MTT and LDH assays, it follows that compounds with estrogenic effects were not formed and that differences concerning cell viability were not statistically significant for both matrices;
- Photocatalytic ozonation proved to be a potential solution for simultaneous removal of OMPs, different microbial groups of potential human pathogens, ARB and ARGs present in UWW or SW. Nevertheless, due to the incapacity of ozonation to eliminate the whole water microbiota at the tested conditions, regrowth occurred giving rise to reduction of the bacterial community diversity driven by the selective pressure posed by the treatment. The survival of potential ARG carriers might have implications on the microbiological quality of the treated water.

Light-driven AOTs, that can take advantage from geographical areas with average annual solar radiation reaching high levels, were tested during a short-term mission for advanced training at *Plataforma Solar de Almeria*, Spain (Chapter 6). Different chemical and biological parameters were characterised from UWW samples obtained after applying a set of solar-driven AOTs performed in a pilot-plant CPC. The main conclusions of this work can be summarized as follows:

- H_2O_2-assisted heterogeneous photocatalysis with TiO_2-P25 showed the best compromise to remove both chemical and biological pollutants;
- A decrease in the abundance of antibiotic resistant and non-resistant bacteria was observed for all treatments, though reactivation of some microbial groups occurred for the processes without H_2O_2;
- Higher removal rates of the genes 16S rRNA, *intI*1, *qnr*S, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$ and *sul*1 were found in the experiments where H_2O_2 was used. Among the studied processes, only solar-H_2O_2 and GO-TiO_2 prevented the reactivation of *qnr*S, *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ genes in the stored water;
- Nevertheless, the abundance of total bacterial load increased after 3-days storage to values close to or higher than those verified before treatment. Similar observations were found for the genes *intI*1 and *sul*1. Hence, none of the studied processes was able to prevent bacterial reactivation, including antibiotic resistant populations;
- Regardless of the oxidative treatment, higher relative abundance of the phylum *Proteobacteria* (*Beta-* and *Gammaproteobacteria*), namely of genera *Pseudomonas*, *Rheinheimera*, *Methylotenera* and *Bacillus*, was observed in treated wastewater after 3-days storage.

Having in consideration the drawback of the fact that solar radiation is not an incessant irradiation source and it is able to suffer discrepancies around the globe and over the year, further studies were conducted with visible light LEDs (λ_{max} = 417 nm) as irradiation source and metal-free exfoliated carbon nitride (g-C_3N_4 referred to gCN_T) as photocatalyst for the removal of actual OMPs present in UWW (Chapter 7). The main results presented in this work support the conclusions:

- OMPs removals to values bellow the LOQ were found in ca. 10 min of slurry photocatalytic treatment using gCN_T as catalyst;
- The heterogeneous photocatalysis of OMPs in UWWTP effluents was successfully achieved under visible light activation, using LEDs;
- As expected, lower efficiencies were observed for the removal of these MPs when the photocatalytic treatment was performed under continuous mode employing gCN_T immobilized on glass rings. Still, this strategy is important, considering the cost of separation processes and energy requirements for the recovery of the photocatalyst, prior to water reuse or discharge;
- The samples from the UWWTP before and after treatment by photocatalysis (using both TiO_2 and gCN_T), did not inhibit the germination of seeds in phytotoxicity tests.

The present PhD studies provided new knowledge on the application of AOTs for the simultaneous removal of chemical and biological pollutants. However, some other questions that should be addressed in further studies have emerged in parallel with the conclusions.

Most of the studied AOTs led to very high degradation rates of targeted parent organic pollutants in various water matrices. Nevertheless, in most of the cases, and in particular, when an actual matrix such as UWW was used, reduced mineralization rates were found. The efficiency of each process results from the complex interplay between inhibitory and promoting effects originated by the water matrix constituents, the operating parameters, the abatement mechanisms occurring during the application of each treatment technology and site-specific limitations

The available information regarding the water matrix effects on the removal efficiency of OMPs in UWWTPs effluents is still limited and further studies are needed to identify the mechanisms and the impact of the main components of the water matrix. Future research on treatment technologies should consider the complex interactions between matrix constituents and the oxidative species formed, aiming to maximize the performance of AOTs, since lower efficiencies and higher costs could be expected in real case scenarios. Besides interfering with the process performance, the water matrix might also have an impact on the production and on the nature of the transformation by-products. The degradation of OMPs is usually followed by the formation of plentiful transformation by-products through unaccountable pathways. Thus, more studies addressing the toxicity and biodegradability of the effluents generated should be attempted in order to determine the deleterious effects on the ecosystems and human health. Complete mineralization of the organic matter is challenging and in general not needed if the by-products formed are biodegradable and non-toxic. Sometimes, a post-treatment with sand filters or a biologically activated carbon filter could be applied to minimize problems associated with the formation of by-products, though increasing the treatment costs. Moreover, standard biodegradability and toxicity tests may be used to establish the levels of treatment needed to achieve a safe effluent.

If we take a closer look to the state of the art, it is possible to conclude that some technologies, such as solar-driven AOTs (that appear competitive with other AOTs in locations with higher average annual solar irradiation levels) are currently developed to a lower Technology Readiness Level, which makes further comparison difficult with consolidated AOTs, such as ozonation (already implemented cost-efficiently at full-scale in several countries). Moreover, many innovative processes and novel combinations of existing processes have been studied only at small-scale or under unrealistic conditions.

Additionally, since multiple factors can affect the efficiency of the treatments, experiments should be performed as close as possible to the real conditions, considering the study of real water matrices and the actual chemical and biological pollutants detected at their natural concentrations. Besides, the available information regarding the simultaneous removal of both chemical and biological pollutants is still limited and more studies are needed for AOTs optimization.

Considering the scale up of a treatment option, these processes can be expensive both in the implementation and maintenance. Therefore, it is of major importance to perform life cycle assessment and cost effectiveness analysis for these processes under a common base of comparison. A market placement analysis will be important for cost estimation of a commercial real-scale unit, assessing the economic viability of the selected treatment process and the market acceptability. A benchmark evaluation considering efficiency, price and placement of the novel technology, and a CAPEX/OPEX analysis for the respective investment, should be performed taking as reference conventional solutions already implemented.

From the results obtained in Chapters 5 and 6, despite the high microbial inactivation, including ARB&ARGs, both culture-dependent and -independent methods demonstrated that part of the UWW microbiota was viable and able to regrow, and the potential risk of this regrowth still has to be assessed. Regardless of the AOT tested, the selection of some bacterial occurred. Moreover, given the ubiquity and genome plasticity of these bacteria, the results obtained suggest that disinfection processes may have implications on the microbiological quality of the disinfected water. However, further studies regarding the bacterial community composition should consider the dilution effect after effluent discharge in a given water compartment. Thus, the proper understanding of the potential disturbance of the water bacterial communities by the applications of AOTs may have relevant ecology implications and should be considered in the design of these technologies, even for those with very high mineralization rates.

As a possible solution to increase the removal efficiency of biological pollutants, an ultrafiltration system could be coupled to the photocatalytic/ozonation reactor, since a physical barrier will probably be the best solution to avoid bacterial regrowth (including reactivation of genes) in treated water.

Regarding the photocatalytic treatment of OMPs under continuous mode using gCN_T coated glass rings (Chapter 7) additional research is needed, including the optimization of the column length, the amount of catalyst immobilized on the support, the size and number of gCN_T coated glass rings per unit volume, and the light distribution in the reactor. The efficiency of gCN_T photocatalysis on the removal of biological pollutants should be also investigated. Moreover, metagenomics analysis should be a common practice to better understand the changes on the resistome of treated UWW.

In fact, considering the regrowth of potentially harmful microorganisms and genes as one of the major challenges that we found in the present work, further studies should also verify if the by-products of the applied AOTs (such as assimilable organic carbon) are the main regrowth drivers in UWW treatment. This could be accomplished by resuspending the surviving cells in an environment with a very low DOC content, simulating an oligotrophic environment. If regrowth still occurs at these conditions, the potential disturbance of the water bacterial communities should be considered even when severe processes are applied. The results of such a study are expected to contribute to re-think water treatment targets, and to design proper advanced UWW treatments at full scale.

Appendices

Appendix A

Fast mineralization and detoxification of amoxicillin and diclofenac by photocatalytic ozonation and application to an urban wastewater

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Fast mineralization and detoxification of amoxicillin and diclofenac by photocatalytic ozonation and application to an urban wastewater

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ABSTRACT

The degradation of two organic pollutants (amoxicillin and diclofenac) in 0.1 mM aqueous solutions was studied by using advanced oxidation processes, namely ozonation, photolysis, photolytic ozonation, photocatalysis and photocatalytic ozonation. Diclofenac was degraded quickly under direct photolysis by artificial light (medium-pressure vapor arc, $\lambda_{exc} > 300$ nm), while amoxicillin remained very stable. In the presence of ozone, regardless of the type of process, complete degradation of both organic pollutants was observed in less than 20 min. Photolysis or ozonation on their own led to modest values of total organic carbon (TOC) removal (<6% or 41%, respectively in 180 min), while for photocatalysis (no ozone present) a significant fraction of nonoxidizable compounds remained in the treated water (~15% after 180 min). In the case of photolytic ozonation, the kinetics of TOC removal was slow.

In contrast, a relatively fast and complete mineralization of amoxicillin and diclofenac (30 and 120 min, respectively) was achieved when applying the photocatalytic ozonation process. The absence of toxicity of the treated waters was confirmed by growth inhibition assays using two different microorganisms, *Escherichia coli* and *Staphylococcus aureus*. Photocatalytic ozonation was also applied to an urban wastewater spiked with both amoxicillin and diclofenac. The parent pollutants were easily oxidized, but the TOC removal was only as much as 68%, mainly due to the persistent presence of oxamic acid in the treated sample. The same treatment allowed the effective degradation of a wide group of micropollutants (pesticides, pharmaceuticals, hormones and an industrial compound) detected in non-spiked urban wastewater.

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1. Introduction

The today large production of drugs over the world (ca. 4000 types in the order of 100–200,000 tons annually) is one of the main causes for the progressive increase of pharmaceutical contaminants in water (Rehman et al., in press; Pastrana-Martínez et al., 2012). The large amount of drugs that is prescribed and consumed by humans and animals, and subsequently excreted by them, are ending in urban wastewater treatment plants (WWTPs). It is of general interest to reduce the emission of these pollutants since they can be very toxic to ecosystems even when appearing at very low concentrations. However, many of these active pharmaceutical ingredients (APIs) are purposely made to be stable and to remain biologically active with the aim of being effective in health care. Therefore, the abatement of these drugs is very difficult by the conventional processes employed in WWTPs, only a fraction of them being destroyed (Mohammadi et al., 2012).

As consequence, increasing concentrations of these emerging organic micropollutants have been detected in aquatic environments and even in drinking water during the last years. US EPA directives define emerging contaminants as chemical compounds that are not regulated and whose impact on the environment and human health is poorly understood yet (Deblonde et al., 2011). Even if the impact of this type of pollution on living beings is not yet clear, a serious indirect problem is associated to the spreading of antibiotic resistance among bacteria (Watkinson et al., 2007; Vaz-Moreira et al., 2014). Therefore, antibiotics must be completely eliminated before reaching natural waters.

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Advanced oxidation processes (AOPs), such as photocatalysis and ozonation, are seen as alternative technologies to combat the above mentioned problems. AOPs are conceptually based on the generation of highly reactive free radicals that can rapidly oxidize organic compounds in water or act as disinfection species. In literature, titanium dioxide (TiO_2) supplied by Evonik Degussa GmbH (P25) is the most employed photocatalyst for water treatment. When this semiconductor is irradiated with energy equal (or above) its band-gap energy (ca. 3.2 eV), reactive surface excited electrons and holes are generated, and then the pollutants are oxidized by the free radicals that are formed (such as $HO^•$ and $O_2^{•-}$) or by direct oxidation with photogenerated holes (Mohammadi et al., 2012; Agustina et al., 2005; Marques et al., 2013). Regarding the ozonation process, ozone attacks preferentially aromatic molecules and unsaturated bonds. This oxidizing agent has the ability to attack organic compounds directly and selectively at low pH, or can undergo decomposition via a chain reaction mechanism that produces $HO^•$ radicals at high pH (Agustina et al., 2005; Kasprzyk-Hordern et al., 2003; Wang et al., 2002; Orge et al., 2011). However, it is well known that the use of ozone alone does not lead to complete mineralization of many organic contaminants in water, since some reaction intermediates, including specific carboxylic acids, are not easily destroyed by this oxidant or its derived oxidizing radicals.

The combination of ozone with photocatalysis (i.e. photocatalytic ozonation) is a more recent topic of research, and some review articles were recently published (Mehrjouei et al., 2015; Xiao et al., 2015). It is suggested in literature that free electrons (e^-) of the TiO_2 semiconductor adsorption layer can interact with ozone molecules forming ozonide radicals ($O_3^{•-}$) (Sánchez et al., 1998), or with oxygen generating $O_2^{•-}$ that can react with ozone molecules giving place to the same $O_3^{•-}$ radicals (Kopf et al., 2000), justifying the synergistic effect between ozonation and photocatalysis. In both cases, the $O_3^{•-}$ radicals generate $HO_3^•$ in aqueous solution (H^+), which can dissociate into $HO^•$ radicals and O_2 molecules.

The present work deals with the treatment of waters and wastewaters contaminated with an antibiotic (amoxicillin – AMX) and an anti-inflammatory drug (diclofenac – DFC) by using different AOPs, namely ozonation, photolysis, photolytic ozonation, photocatalysis and photocatalytic ozonation. The most efficient treatment was also tested for the removal of a group of micropollutants present at ng L^{-1} levels in an urban wastewater. AMX was selected as model pollutant because AMX is a beta-lactam antibiotic used to treat many different types of infections caused by a wide range of Gram-positive and Gram-negative bacteria in both humans and animals. In the past decades, AMX has been used to treat infections of the middle ear, tonsils, throat, larynx, pharynx, bronchi, lungs, urinary tract, skin and also to treat gonorrhea (Kaur et al., 2011, Andreozzi et al., 2005). In addition, DFC is a widely used anti-inflammatory non-steroidal drug with strong resistance to biodegradation. This compound is mostly used in its sodium salt form for medical care as analgesic, antiarthritic and antirheumatic (Yu et al., 2013; Bagal and Gogate, 2014; García-Araya et al., 2010).

2. Materials and methods

2.1. Chemicals and materials

AMX ($C_{16}H_{19}N_3O_5S$, >97%) and DFC ($C_{14}H_{11}Cl_2NO_2$) sodium salt, chosen as model organic pollutants, were provided by Fluka and Cayman Chemical Company, respectively. Oxalic acid (>99%), sodium nitrate (>99%), sodium chloride (>99%) and magnesium sulfate (>99.5%) were obtained from Sigma–Aldrich, whereas oxamic acid (>96%), sodium nitrite (>99%) and ammonium sulfate (>98%) were purchased from Fluka. TiO_2 (80% anatase and 20% rutile crystalline phases) was obtained from Evonik Degussa GmbH (P25).

2.2. Experimental set-up and procedure

All experiments were performed in the same lab-scale reactor, filled with 250 mL of an AMX or DFC aqueous solution (0.1 mM) or with an urban wastewater from the secondary biological treatment of a WWTP (located in the North region of Portugal). Urban wastewater samples were collected in September 2014 and spiked with both pollutants (AMX and DFC) and also collected in July 2015 to study a wider range of micropollutants (non-spiked). The reaction media was magnetically stirred at 400 rpm. In the experiments with ozone, this oxidant was produced from pure oxygen in a BMT 802X ozone generator at a constant ozone flow rate (150 Ncm^3 min^{-1}) and inlet concentration (50 g Nm^{-3}). The concentration of ozone in the gas phase was monitored with a BMT 964 ozone analyzer. The ozone leaving the reactor in the gas phase was removed using gas washing bottles filled with potassium iodide solution. In photolysis, the ozone containing stream was replaced by the same flow rate (150 Ncm^3 min^{-1}) of oxygen, and a Heraeus TQ 150 medium-pressure mercury vapor lamp (UV/Vis >300 nm with dominant emission lines at 314, 366, 405, 436, 546 and 578 nm) was held in a DURAN 50® glass immersion tube with water recirculation, to maintain a room temperature in the experiments. Photolytic ozonation experiments were performed as in the case of photolysis, but replacing oxygen by an ozone–oxygen mixture. Photocatalytic experiments were also similar to photolysis, but adding the TiO_2 photocatalyst (0.5 g L^{-1} load) to the solution in slurry operation. An ozone–oxygen gas mixture was bubbled instead of oxygen in photocatalytic ozonation experiments. Samples were taken from the reactor and centrifuged to remove any possible suspended particles before analysis. The removal of a set of micropollutants found in urban wastewater samples (non-spiked) was evaluated by photocatalytic ozonation, under the experimental conditions described above and testing other two different inlet dosages of ozone (70 and 90 g Nm^{-3}), all in triplicate. Ozonation and photocatalysis were also tested using the previously described experimental conditions.

2.3. Analytical techniques

AMX and DFC concentrations were determined by high performance liquid chromatography (HPLC) using a Hitachi Elite Lachrom apparatus equipped with a diode array detector (DAD) and a YMC Hydrosphere C18 column (250 mm × 4.6 mm) working at room temperature under isocratic elution. The mobile phase (flow rate of 1 mL min^{-1}) consisted of a 95% phosphate buffer solution ($pH = 2.8$) and 5% acetonitrile for AMX analysis, and 30% of the phosphate buffer solution ($pH = 2.8$) with 70% methanol in the case of DFC. The oxalic and oxamic acid concentrations were also determined by HPLC, but using a Altech AO-1000 column (300 mm × 6.5 mm) operating under isocratic elution with 0.5 mL min^{-1} of 5 mM H_2SO_4.

A fully validated method of Solid Phase Extraction–Ultra High Performance Liquid Chromatography with tandem Mass Spectrometry (SPE–UHPLC–MS/MS) was used to assess the concentrations of the target micropollutants in samples from the urban WWTP, before and after the application of AOPs at lab-scale, namely DFC, ibuprofen, naproxen, tramadol, azithromycin, clarithromycin, erythromycin, sulfamethoxazole, trimethoprim, fluticasone propionate, montelukast, warfarin, clopidogrel, metoprolol, propranolol, hydrochlorothiazide, atorvastatin, bezafibrate, simvastatin, carbamazepine, citalopram, fluoxetine, norfluoxetine, venlafaxine, diphenhydramine, 17-α-ethinylestradiol, 17-β-estradiol, estrone, alachlor, atrazine, simazine, diuron, isoproturon, chlorfenvinphos, pentachlorophenol, clofibric acid and perfluorooctanesulfonic acid. All reference and isotopically labeled internal standards (>98% purity) were acquired from Sigma–Aldrich (Steinhein, Germany). Ethanol (HPLC grade) was purchased from Fisher Scientific UK Limited (Leicestershire, UK) and ultrapure water was supplied by a Milli-Q water system. The wastewater samples were spiked with a solution of isotopically labeled internal standards and pre-concentrated by SPE using Oasis® HLB (Hydrophilic-Lipophilic-Balanced) (150 mg, 6 mL), purchased from Waters (Milford, Massachusetts, USA). For UHPLC–MS/MS analyses, a Shimadzu Corporation apparatus (Tokyo, Japan) was used, consisting of a Nexera UHPLC equipment, coupled to a LCMS-8040 triple quadrupole mass spectrometer detector with an electrospray ionization source operating in both positive and negative ionization modes. The column Kinetex™ 1.7 μm XB-C18 100 Å (100 × 2.1 mm i.d.) (Phenomenex, Inc., California, USA) was operated under reversed mode with a mobile phase consisting of a mixture of ethanol and water (50/50, v/v). Quantification of each compound was carried out by internal calibration method and selected reaction monitoring (SRM), through the evaluation of the two most abundant SRM transitions between the precursor and the fragment ions.

The total organic carbon (TOC) was measured in a Shimadzu TOC-5000A analyzer. Ion chromatography analyses were performed in a Metrohm 881 Compact IC Pro apparatus, equipped with a Metrosep C4 Cationic Exchange Column (250 mm × 4.0 mm) for quantification of ammonium and a Metrosep A Supp 7 Anionic Exchange Column (250 mm × 4.0 mm) for quantification of nitrates, nitrites, sulfates and chlorides. The pH values were measured using a WTW InoLab equipment.

2.4. *Growth inhibition assay for samples containing AMX or DFC and thereof oxidation products*

Samples collected over the photocatalytic ozonation treatment of AMX or DFC (0.1 mM) at 0 (negative control), 15, 30, 60 and 120 min were filter sterilized (0.2 μm pore size syringe-filters) and stored at 4 °C for no more than 24 h. Growth inhibition assays were carried out in 96-well microtiter plates using *Escherichia coli* DSM 1103 and *Staphylococcus aureus* DSM 1104 as test strains. In each well, 200 μL of sample was supplemented with 2 g L^{-1} of sterile yeast extract, and inoculated with a suspension of *E. coli* or *S. aureus* at an initial optical density (OD) of 0.08 at 610 nm. Sterile distilled water was used instead of sample in controls. Controls included cell-free medium (blank) and inoculated medium (positive control). Each condition was tested in quadruplicate. Absorbance was determined at 610 nm every 30 min, using a *Synergy HT Multi-Mode Microplate Reader* (Biotek Instruments, USA) at 37 °C under continuous shaking at medium speed. The specific growth rate (μ, h^{-1}) was calculated, for each well, by adjusting a first-order kinetic model to the experimental data of the exponential growth phase, and the biomass yield (Y) was determined by the difference between the maximum OD achieved and the initial OD. Then, the values were normalized by the positive control.

3. Results and discussion

3.1. *AMX and DFC removal by different AOPs*

Fig. 1a shows the normalized AMX and DFC concentrations (C/C_0), in aqueous solutions, for ozonation (O_3), photolysis (UV/Vis), photolytic ozonation (O_3 + UV/Vis), photocatalysis (UV/Vis + TiO_2) and photocatalytic ozonation (O_3 + UV/Vis + TiO_2).

AMX was very stable (<5% decrease in concentration) in the experiment of direct photolysis. This result was expected since no overlapping is observed between the AMX absorption spectra (<280 nm) and the effective near UV/Vis irradiation reaching the aqueous solution (>300 nm), as shown in Fig. 1b. In fact, Elmolla and Chaudhuri (2010) studied the degradation of different antibiotics by photolysis (365 nm) and also observed a very slight decrease in the AMX concentration (2.9% after 300 min), since this antibiotic absorbs light only below the wavelength of the employed irradiation. Pereira et al. (2014) concluded also that AMX is very stable under photolysis (with cutoff below 280 nm), with no significant mineralization being observed for a reaction time of 215 min.

However, AMX was easily oxidized in the experiments where ozone was employed (ozonation, photolytic ozonation and photocatalytic ozonation), or even in photocatalysis (Fig. 1). Therefore, at these conditions, single photolysis is not adequate for the treatment of waters containing AMX, but all the processes implementing ozone and/or TiO_2 seem to be good alternatives for AMX removal. In single ozonation, direct attack of the pollutants by molecular ozone is expected to be the main degradation mechanism (instead of the oxidation of these pollutants through $HO^{\bullet}$ radicals), due to the acidic pH of the initial aqueous solutions (4.0 ± 0.2) in the present work.

Regarding DFC, this compound was easily removed (less than 15 min) in all the experiments performed (Fig. 1a), including photolysis, the initial pH being near 4.5 in this case. Fig. 1b shows that DFC absorbs light at higher wavelengths (up to ca. 325 nm) than AMX, overlapping in some extent with the UV–Vis spectrum of the irradiation reaching the aqueous solution (as shown in the

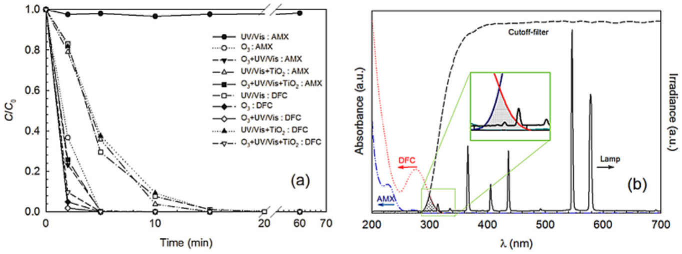


Fig. 1. (a) Normalized concentration (C/C_0) of AMX or DFC in aqueous solution (C_0: 0.1 mM) as a function of time, for photolysis (UV), ozonation (O_3), photolytic ozonation (O_3 + UV/Vis), photocatalysis (UV/Vis + TiO_2) and photocatalytic ozonation (O_3 + UV/Vis + TiO_2). Conditions: natural pH, 50 g O_3 Nm^{-3} in ozonation; 0.5 g L^{-1} of TiO_2 in photocatalytic experiments. (b) Absorption spectra of DFC and AMX in aqueous solution. Medium-pressure Hg-vapor lamp emission and cutoff filtering resulting from the DURAN 50® glass jacket.

inset of Fig. 1b). Pérez-Estrada et al. (2005) also observed a DFC degradation by solar photolysis, even if its mineralization never occurred. This fact was justified by the tail in DFC absorption spectrum, well above 300 nm. Aguinaco et al. (2012) studied the DFC removal by photocatalytic ozonation and verified a complete degradation of DFC after 6 min of reaction. Since DFC has a nucleophilic site (an amino group), it is expected a fast reaction with an electrophilic agent, such as ozone. García-Araya et al. (2010) also studied the DFC removal in water, by using ozone and TiO_2 photocatalysis separately, the results confirming that DFC is completely eliminated in both cases.

3.2. TOC removal by different AOPs

The normalized TOC content (TOC/TOC_0) as a function of time is shown in Fig. 2a for DFC ($TOC_0 = 16.8$ mg L^{-1}) and Fig. 2b for AMX ($TOC_0 = 19.2$ mg L^{-1}), for all AOPs tested. The TOC evolution was quite similar for the two model pollutants when comparing the same AOP. For instance, a very low TOC decrease was observed for both pollutants in photolysis, the TOC removal never reaching more than 6% in 180 min. The mineralization was higher for single ozonation, but never exceeding 41% in 180 min. Although ozone can be a powerful oxidant of aromatic compounds, it reacts slowly with other species, leading to the accumulation of many by-products, such as carboxylic acids and carbonyl compounds, that cannot be easily oxidized by molecular ozone (Agustina et al., 2005). Therefore, in the present work, single ozonation was a good process for elimination of the parent compounds, but not for the respective reaction by-products.

Additionally, a considerable fraction of nonoxidizable compounds (~15% after 180 min) was generated in photocatalysis, and a slow TOC removal was observed in the case of photolytic ozonation even if high TOC removals (ca. 95%) were obtained in 300 min. Regarding the photocatalytic ozonation process, it was possible to achieve a complete TOC abatement for AMX and DFC in 30 and 120 min, respectively. Therefore, it is possible to predict a synergistic effect when the photocatalytic and ozonation treatments are carried out simultaneously, as reported by Wang et al. (2002) when studying the degradation of formic acid; in the present work the initial reaction rate for photocatalytic ozonation was more than 2 times higher than the sum of the initial reaction rates for ozonation and photocatalysis. García-Araya et al. (2010) also observed a low DFC mineralization employing ozone or TiO_2 photocatalysis separately (ca. 50% and 80% respectively), while Aguinaco et al. (2012) studied photolysis, photocatalysis and photocatalytic ozonation of DFC solutions concluding that the last process leads to the highest TOC removal (ca. 90%), as is the case of the present work. This process has also proved to improve the treatment performance by decreasing the ozone demand and energy requirements to completely remove other emerging contaminants (Quiñones et al., 2015).

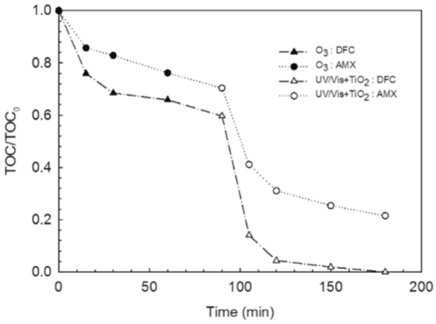


Fig. 3. Normalized TOC content (TOC/TOC_0) for DFC or AMX in aqueous solution (C_0: 0.1 mM) as a function of time, during a sequential combination of ozonation (O_3) followed by photocatalysis (UV/Vis + TiO_2). Conditions: natural pH, 50 g O_3 Nm^{-3} in ozonation; 0.5 g L^{-1} of TiO_2 in photocatalytic experiments.

Additional experiments (one with AMX and another with DFC) were performed by applying photocatalysis after 90 min of the ozonation process (Fig. 3), namely by closing the stream of ozone after 90 min, adding TiO_2 to the solution and turning on the lamp immediately. It can be observed that DFC was completely mineralized in 180 min, probably due to the presence of an enough amount of residual ozone in the liquid phase when photocatalysis was initiated. However, a significant fraction of TOC remained in the treated solution when AMX was employed as model pollutant. Therefore, simultaneous application of these processes (i.e., photocatalytic ozonation) seems to be more efficient than the sequential approach.

3.3. Evolution of low molecular weight carboxylic acids

For the experiments with AMX, the concentrations of oxalic and oxamic acids over time (which are typical low molecular weight carboxylic acids formed in AOPs) are shown in Fig. 4a and b, respectively. Regarding photolysis, a negligible oxalic acid concentration was detected, while oxamic acid was not detected above

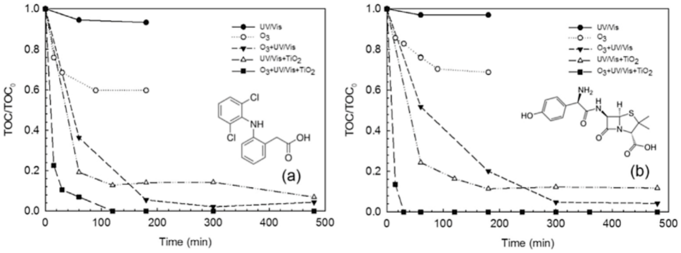


Fig. 2. Normalized TOC content (TOC/TOC_0) for (a) DFC or (b) AMX in aqueous solution (C_0: 0.1 mM) as a function of time, for photolysis (UV/Vis), ozonation (O_3), photolytic ozonation (O_3 + UV/Vis), photocatalysis (UV/Vis + TiO_2) and photocatalytic ozonation (O_3 + UV/Vis + TiO_2). Conditions: natural pH, 50 g O_3 Nm^{-3} in ozonation; 0.5 g L^{-1} of TiO_2 in photocatalytic experiments.

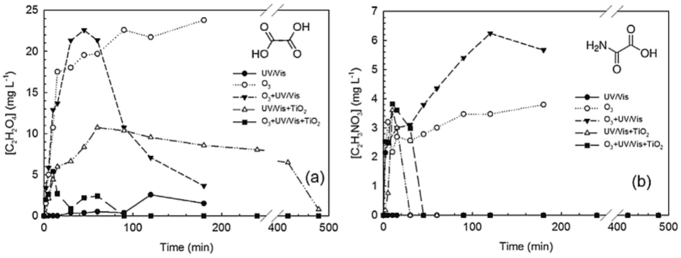


Fig. 4. Evolution of the (a) oxalic and (b) oxamic acids concentrations as a function of time, for photolysis (UV), ozonation (O_3), photolytic ozonation (O_3 + UV/Vis), photocatalysis (UV/Vis + TiO_2) and photocatalytic ozonation (O_3 + UV/Vis + TiO_2) in experiments performed with AMX in aqueous solution (C_0: 0.1 mM). Conditions: natural pH, 50 g O_3 Nm^{-3} in ozonation; 0.5 g L^{-1} of TiO_2 in photocatalytic experiments.

the detection limit of the analytical technique. These results are in agreement with the high stability of AMX (and respective high TOC content) at such conditions. For single ozonation, the concentration of both acids increased over the experiment, mainly in the initial 30 min, indicating that the degradation of these organic compounds is difficult by using this process. This might be explained by the selective attack of ozone at low pH, where the aromatic compounds are primarily attacked and low molecular weight carboxylic acids are of more difficult degradation by single ozonation.

For photolytic ozonation, the concentration of oxalic acid increased (up to ca. 22.5 mg L^{-1} in 60 min) and then decreased (close to 3.6 mg L^{-1} in 180 min). Oxamic acid was a more refractory by-product in photolytic ozonation, since a accumulation of this compound (up to 6 mg L^{-1}) was observed. Interestingly, the opposite behavior was observed in photocatalysis, i.e. oxalic acid was more refractory than oxamic acid, justifying in some extent why the TOC removal was higher when both processes were combined (photocatalytic ozonation) than when applied separately, i.e. ozone might be more selective to some organic compounds and photocatalysis to others. In fact, for photocatalytic ozonation, the oxalic and oxamic acid concentrations increased in the first 10 min and immediately started to decrease. In any case, it is important to note that the oxamic acid concentrations were quite low, never exceeding 7 mg L^{-1} (1.9 mg L^{-1} of TOC) and thus with a low contribution on the TOC content.

Fig. 5a and b show the oxalic and oxamic acid concentrations, respectively, as a function of time in experiments with DFC. As described for AMX, these low molecular weight carboxylic acids were not detected in photolysis and formed in the case of ozonation. Both acids were refractory to oxidation in ozonation, as reported in literature (Faria et al., 2008), whereas oxalic acid was readily oxidized when ozone was combined with UV/Vis (at variance with oxamic acid). Photocatalysis or photocatalytic ozonation are the best performing processes since both were able to remove these by-products in a relatively short period of time (near 45 min). Comparing the TOC removals with the abatement of low molecular weight carboxylic acids, it is also possible to conclude that the significant fraction of nonoxidizable compounds (~15% after 180 min, Fig. 2a) remaining in the aqueous solution after the photocatalytic treatment of DFC is not due to oxalic or oxamic acids, since they were degraded to values below the detection limits by photocatalysis (Fig. 5).

3.4. Evolution of ions

The concentration of nitrates, ammonium, sulfates and chlorides were also analyzed and shown in Table 1 for the experiments with AMX or DFC. As expected, the values in the initial solutions were below the detection limits of the analyses. Nitrites (not shown) were below the detection limit regardless of the time and experiment performed, as expected, since nitrites (if formed) are readily transformed into nitrates. A very small amount of nitrates was detected by the end of single ozonation, photolysis and photolytic ozonation, while more significant amounts were

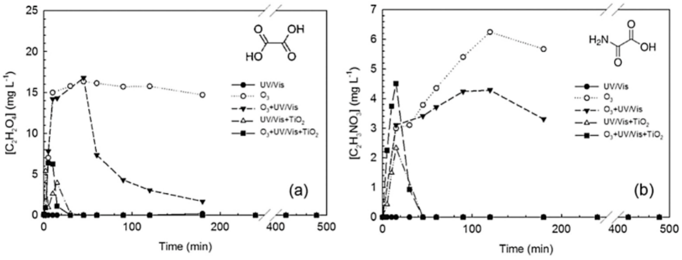


Fig. 5. Evolution of the (a) oxalic and (b) oxamic acids concentrations as a function of time, for photolysis (UV), ozonation (O_3), photolytic ozonation (O_3 + UV/Vis), photocatalysis (UV/Vis + TiO_2) and photocatalytic ozonation (O_3 + UV/Vis + TiO_2) in experiments performed with DFC in aqueous solution (C_0: 0.1 mM). Conditions: natural pH, 50 g O_3 Nm^{-3} in ozonation; 0.5 g L^{-1} of TiO_2 in photocatalytic experiments.

Table 1
Nitrates, ammonium, sulfates and chlorides concentrations (mM) for different experiments performed with AMX and DFC.

	Process	AMX		DFC	
		60 min	180 min	60 min	180 min
Nitrates (mM)	O_3	[a]	0.008	[a]	0.005
	UV	[a]	0.015	[a]	[a]
	O_3 + UV/Vis	0.014	0.033	[a]	0.038
	UV/Vis + TiO_2	[a]	[a]	[a]	[a]
	O_3 + UV/Vis + TiO_2	0.094	0.167	0.055	0.054
Ammonium (mM)	O_3	[a]	0.063	0.030	0.006
	UV/Vis	[a]	[a]	[a]	[a]
	O_3 + UV/Vis	0.063	0.079	0.032	0.009
	UV/Vis + TiO_2	0.182	0.216	0.073	0.080
	O_3 + UV/Vis + TiO_2	0.097	0.077	0.058	0.046
Sulfates (mM)	O_3	[a]	[a]	[a]	[a]
	UV/Vis	[a]	[a]	[a]	[a]
	O_3 + UV/Vis	[a]	0.022	[a]	[a]
	UV/Vis + TiO_2	0.093	0.096	[a]	[a]
	O_3 + UV/Vis + TiO_2	0.088	0.095	[a]	[a]
Chlorides (mM)	O_3	[a]	[a]	0.168	0.169
	UV/Vis	[a]	[a]	0.190	0.186
	O_3 + UV/Vis	[a]	[a]	0.179	0.175
	UV/Vis + TiO_2	[a]	[a]	0.165	0.201
	O_3 + UV/Vis + TiO_2	[a]	[a]	0.201	0.209

[a] < Detection Limit (Nitrates: 0.005 mM; Ammonium: 0.005 mM; Sulfates: 0.009 mM; Chlorides: 0.03 mM).

detected with photocatalytic ozonation, confirming that the last process is more efficient in the oxidation of the parent compounds (and respective intermediates) into final reaction products. Ammonium ions were detected in more considerable amounts, since the early beginning of the experiments (60 min), notoriously for photocatalysis, which seems to be more selective to the generation of ammonium ions than nitrates. As expected, sulfates were also detected in experiments with AMX and chlorides in those performed with DFC, since sulfur and chlorine are elements of the respective parent compounds.

Table 2 shows nitrogen (in nitrates, oxamic acid and ammonium) and sulfur (sulfates) balances in experiments performed with AMX for a reaction time of 180 min. The difference between nitrogen in AMX and in identified nitrogen-containing compounds (i.e., non-identified nitrogen: N_{NI}), as well as the difference between sulfur in AMX and in identified sulfur-containing compounds (i.e., non-identified sulfur: S_{NI}), is also shown. In general, these results are in agreement with those obtained for the TOC removal. In particular, for photocatalytic ozonation, 81% of nitrogen was identified as ammonium and nitrates, while 19% as non-identified nitrogen species (for instance some nitrogen may have been converted to N_2). In addition, 95% of the sulfur present in AMX resulted into sulfates.

Table 2
Nitrogen and sulfur balance (180 min) in experiments performed with AMX. Nitrogen (N) in AMX, oxamic acid, ammonium, nitrates and non-identified species (N_{NI}). Sulfur (S) in AMX, sulfates and non-identified species (S_{NI}).

Process	% N or S (180 min)				
	O_3	UV	O_3 + UV	UV + TiO_2	O_3 + UV + TiO_2
N_{AMX}	0	95	0	0	0
$N_{oxamic\ acid}$	14	[a]	21	[a]	[a]
$N_{NH_4^+ + NO_3^-}$	24	5	37	72	81
N_{NI}	62	0	42	28	19
S_{AMX}	0	100	0	0	0
$S_{SO_4^{2-}}$	[a]	[a]	22	96	95
S_{NI}	100	0	78	4	5

[a] < Detection Limit.

Table 3
Nitrogen and chlorine balance (180 min) in experiments performed with DFC. Nitrogen (N) in DFC, oxamic acid, ammonium, nitrates and non-identified compounds (N_{NI}). Chlorine (Cl) in DFC, chlorides and non-identified compounds (Cl_{NI}).

Process	% N or Cl (180 min)				
	O_3	UV	O_3 + UV	UV + TiO_2	O_3 + UV + TiO_2
N_{DFC} (mM)	0	0	0	0	0
$N_{oxamic\ acid}$ (mM)	64	[a]	37	0	0
$N_{NH_4^+ + NO_3^-}$	5	[a]	47	82	100
N_{NI} (mM)	31	100	16	18	0
Cl_{DFC} (mM)	0	0	0	0	0
C_{Cl^-} (mM)	84	93	87.5	100	100
Cl_{NI} (mM)	16	7	12.5	0	0

[a] < Detection Limit.

The balance for nitrogen and chlorine in experiments with DFC for different reactions (in 180 min) is shown in Table 3. These results are once again in agreement with the TOC removals verified for the different processes. For the particular case of photocatalytic ozonation, all nitrogen was detected as ammonium and nitrates, while chlorine as chlorides.

3.5. *Growth inhibition assays for samples containing AMX or DFC and by-products thereof*

The normalized bacterial growth rate and biomass yield values of *S. aureus* and *E. coli*, for samples resulting from the AMX or DFC photocatalytic ozonation treatment (presenting the highest TOC removal), are shown in Fig. 6. The main objective of this test was to verify if AMX and DFC have toxic properties and if compounds more toxic than the parent pollutants were formed during photocatalytic ozonation. As expected, the initial AMX solution inhibited the growth of both strains tested (Fig. 6a and c). This result was expected since AMX is an antibiotic and it was present in a concentration (0.1 mM = 36.5 mg L^{-1}) above the minimal inhibitory concentration for the test organisms (4.0 and 0.25 mg_{AMX} L^{-1}, for *E. coli* and *S. aureus*, respectively) (Aguinaco et al., 2012). In opposition, bacterial growth occurred in all the samples collected over the treatment (15, 30, 60 and 120 min). Thus, 15 min of reaction was sufficient to inactivate the AMX antimicrobial properties, allowing *S. aureus* and *E. coli* to grow. Moreover, by-products of AMX photocatalytic ozonation did not inhibit the growth of these organisms. Therefore, it is possible to conclude that photocatalytic ozonation is a good process to treat waters contaminated with AMX, allowing its complete mineralization without the formation of toxic compounds to these microbiological strains.

In opposition to AMX, both tested organisms grew not only in the presence of the DFC by-products but also in the presence of the parent compound (Fig. 6b and d). These results can be explained by the fact that DFC is not an antimicrobial compound. Indeed, according to Dutta et al. (2007), DFC presents bactericidal effect only at higher concentrations (between 50 and 200 mg_{DFC} L^{-1} for *S. aureus* and 50 to ≥800 mg_{DFC} L^{-1} for *E. coli*) than those tested in the present work (0.1 mM = 29.6 mg L^{-1}). Nevertheless, *S. aureus* seems to be more sensitive to DFC and its oxidation products than *E. coli*, since lower normalized growth rates and biomass yields were registered for this Gram-positive organism than for *E. coli* in samples collected over photocatalytic ozonation (Fig. 6b and d).

Overall, since the AMX and DFC concentrations tested in these experiments are higher than those found in urban wastewaters, these parent compounds at ng L^{-1} (as well as their respective reaction intermediates) in urban wastewaters are not expected to have any effect on the microorganisms tested in this work.

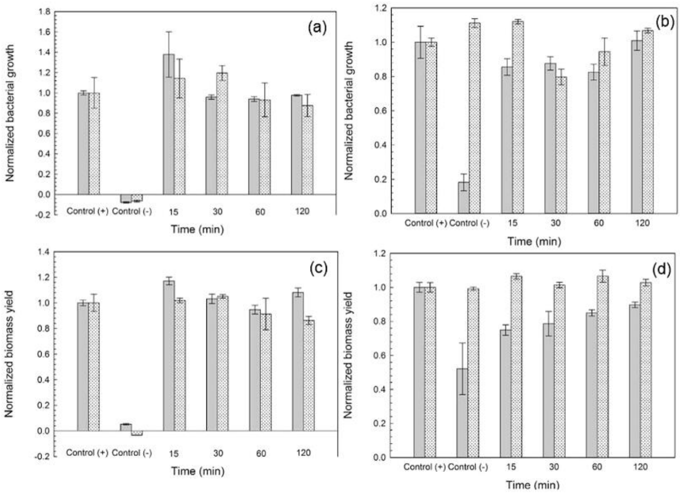


Fig. 6. *S. aureus* (gray columns) and *E. coli* (white-dotted columns) normalized bacterial growth rate (a, b) and normalized biomass yield (c, d) for samples resulting from the treatment of AMX (a, c) or DFC (b, d), in aqueous solution (C_0: 0.1 mM) by photocatalytic ozonation, at different reaction times and compared to the positive control (Control (+)).

3.6. Photocatalytic ozonation of AMX and DFC in a spiked urban wastewater

In order to verify the matrix effect on the photocatalytic ozonation efficiency, a real wastewater from an urban WWTP was collected and spiked with AMX and DFC simultaneously, at the same concentrations used for experiments with distilled water (i.e., 0.1 mM each). The parent compounds were degraded in less than 20 min (not shown), as in the case of distilled water as matrix. Fig. 7a shows the evolution of oxalic and oxamic acid concentrations. In opposition to experiments performed with distilled water (Figs. 4b and 5b), degradation of oxamic acid was not observed; the concentration of this low molecular weight acid increased in the first 45 min (up to ca. 40 mg L^{-1}), and then remained constant until the end of the experiment. Therefore, oxamic acid seems to be very refractory to the photocatalytic ozonation treatment when urban wastewater is used as matrix. Conversely, oxalic acid did not accumulate and was easily degraded, as observed when using distilled water as matrix. Nitrites and ammonium were not detected in the spiked urban wastewater, while the concentrations of sulfates (0.783 mM), chlorides (2.469 mM) and nitrates (0.716 mM) were high and increased (up to 0.922, 2.702 and 1.006 mM, respectively) after photocatalytic ozonation, together with the concentration of ammonium (0.077 mM).

Fig. 7b shows a 68% decrease of the TOC content in 60 min of treatment (from ≈37 to 12 mg L^{-1}), but a fraction of nonoxidizable organic compounds remained in the wastewater (32% of the initial TOC). The concentration of oxalic and oxamic acids (Fig. 7a) were used to determine the theoretical TOC value resulting from these two species. Interestingly, the fraction of TOC determined experimentally from 60 to 180 min, exactly corresponds to the theoretical TOC due to oxamic acid (Fig. 7b). It is important to note that the

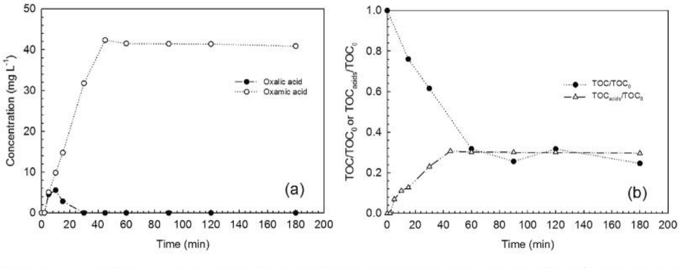
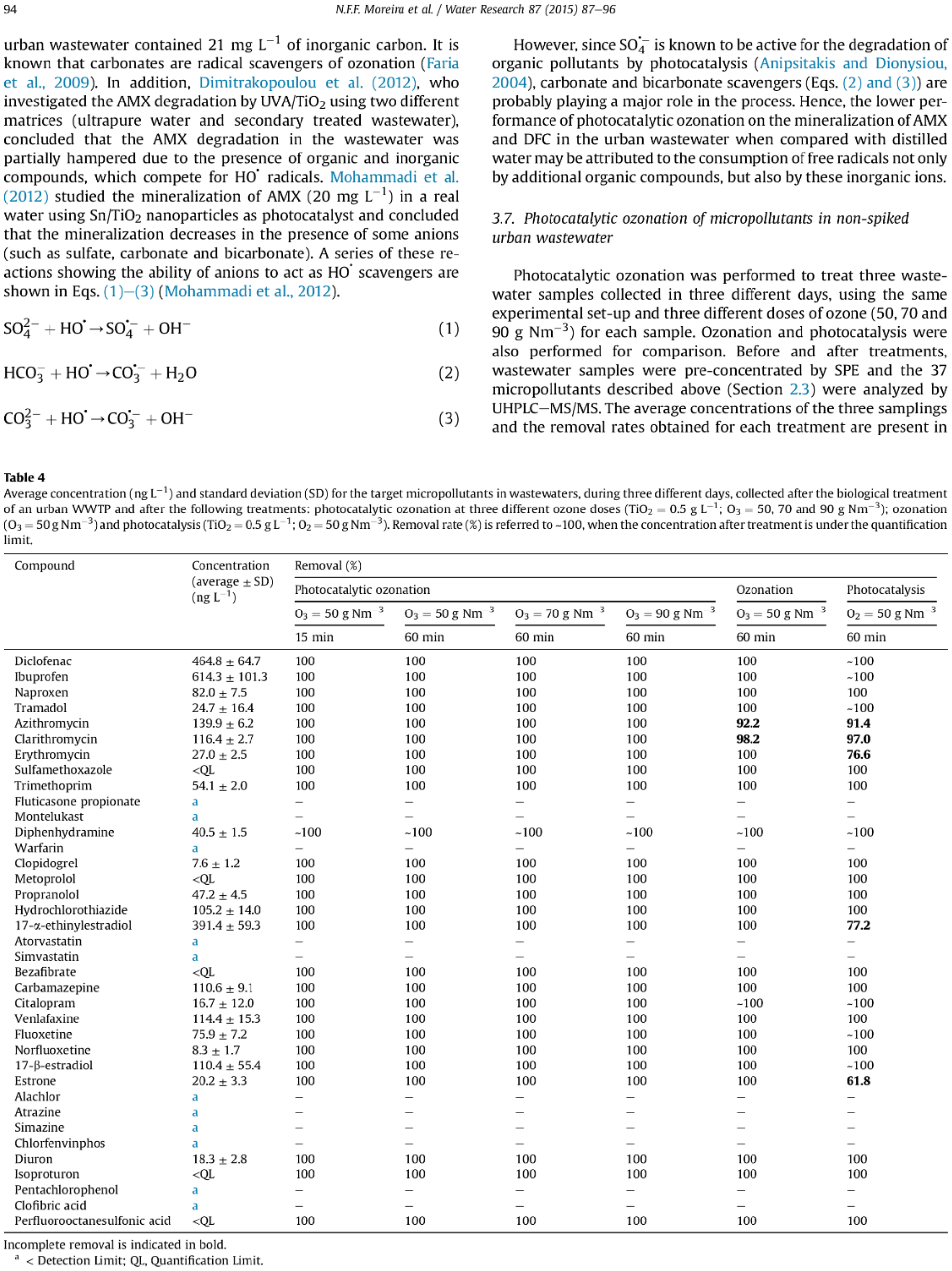


Fig. 7. Photocatalytic ozonation (O_3 + UV/Vis + TiO_2) of an urban wastewater spiked with both AMX and DFC at 0.1 mM, using 0.5 g L^{-1} of TiO_2 and 50 g O_3 Nm^{-3}: (a) Evolution of oxalic and oxamic acid concentrations; (b) normalized TOC content (TOC/TOC_0; and theoretical TOC determined from the oxalic and oxamic acid concentrations (TOC_{acids}) normalized by the initial TOC content of the sample (TOC_{acids}/TOC_0), as a function of time ($TOC_0 \approx 37$ mg L^{-1}; $TOC_{0,AMX + DFC} = 29$ mg L^{-1}; $TOC_{0,urban\ wastewater} \approx 8$ mg L^{-1}).



urban wastewater contained 21 mg L^{-1} of inorganic carbon. It is known that carbonates are radical scavengers of ozonation (Faria et al., 2009). In addition, Dimitrakopoulou et al. (2012), who investigated the AMX degradation by UVA/TiO_2 using two different matrices (ultrapure water and secondary treated wastewater), concluded that the AMX degradation in the wastewater was partially hampered due to the presence of organic and inorganic compounds, which compete for $HO^{\bullet}$ radicals. Mohammadi et al. (2012) studied the mineralization of AMX (20 mg L^{-1}) in a real water using Sn/TiO_2 nanoparticles as photocatalyst and concluded that the mineralization decreases in the presence of some anions (such as sulfate, carbonate and bicarbonate). A series of these reactions showing the ability of anions to act as $HO^{\bullet}$ scavengers are shown in Eqs. (1)–(3) (Mohammadi et al., 2012).

$$SO_4^{2-} + HO^{\bullet} \rightarrow SO_4^{\bullet-} + OH^- \quad (1)$$

$$HCO_3^- + HO^{\bullet} \rightarrow CO_3^{\bullet-} + H_2O \quad (2)$$

$$CO_3^{2-} + HO^{\bullet} \rightarrow CO_3^{\bullet-} + OH^- \quad (3)$$

However, since $SO_4^{\bullet-}$ is known to be active for the degradation of organic pollutants by photocatalysis (Anipsitakis and Dionysiou, 2004), carbonate and bicarbonate scavengers (Eqs. (2) and (3)) are probably playing a major role in the process. Hence, the lower performance of photocatalytic ozonation on the mineralization of AMX and DFC in the urban wastewater when compared with distilled water may be attributed to the consumption of free radicals not only by additional organic compounds, but also by these inorganic ions.

3.7. Photocatalytic ozonation of micropollutants in non-spiked urban wastewater

Photocatalytic ozonation was performed to treat three wastewater samples collected in three different days, using the same experimental set-up and three different doses of ozone (50, 70 and 90 g Nm^{-3}) for each sample. Ozonation and photocatalysis were also performed for comparison. Before and after treatments, wastewater samples were pre-concentrated by SPE and the 37 micropollutants described above (Section 2.3) were analyzed by UHPLC–MS/MS. The average concentrations of the three samplings and the removal rates obtained for each treatment are present in

Table 4
Average concentration (ng L^{-1}) and standard deviation (SD) for the target micropollutants in wastewaters, during three different days, collected after the biological treatment of an urban WWTP and after the following treatments: photocatalytic ozonation at three different ozone doses ($TiO_2 = 0.5$ g L^{-1}; $O_3 = 50$, 70 and 90 g Nm^{-3}); ozonation ($O_3 = 50$ g Nm^{-3}) and photocatalysis ($TiO_2 = 0.5$ g L^{-1}; $O_2 = 50$ g Nm^{-3}). Removal rate (%) is referred to ~100, when the concentration after treatment is under the quantification limit.

Compound	Concentration (average ± SD) (ng L^{-1})	Removal (%)					
		Photocatalytic ozonation				Ozonation	Photocatalysis
		$O_3 = 50$ g Nm^{-3}	$O_3 = 50$ g Nm^{-3}	$O_3 = 70$ g Nm^{-3}	$O_3 = 90$ g Nm^{-3}	$O_3 = 50$ g Nm^{-3}	$O_2 = 50$ g Nm^{-3}
		15 min	60 min	60 min	60 min	60 min	60 min
Diclofenac	464.8 ± 64.7	100	100	100	100	100	~100
Ibuprofen	614.3 ± 101.3	100	100	100	100	100	~100
Naproxen	82.0 ± 7.5	100	100	100	100	100	100
Tramadol	24.7 ± 16.4	100	100	100	100	100	~100
Azithromycin	139.9 ± 6.2	100	100	100	100	**92.2**	**91.4**
Clarithromycin	116.4 ± 2.7	100	100	100	100	**98.2**	**97.0**
Erythromycin	27.0 ± 2.5	100	100	100	100	100	**76.6**
Sulfamethoxazole	<QL	100	100	100	100	100	100
Trimethoprim	54.1 ± 2.0	100	100	100	100	100	100
Fluticasone propionate	a	–	–	–	–	–	–
Montelukast	a	–	–	–	–	–	–
Diphenhydramine	40.5 ± 1.5	~100	~100	~100	~100	~100	~100
Warfarin	a	–	–	–	–	–	–
Clopidogrel	7.6 ± 1.2	100	100	100	100	100	100
Metoprolol	<QL	100	100	100	100	100	100
Propranolol	47.2 ± 4.5	100	100	100	100	100	100
Hydrochlorothiazide	105.2 ± 14.0	100	100	100	100	100	100
17-α-ethinylestradiol	391.4 ± 59.3	100	100	100	100	100	**77.2**
Atorvastatin	a	–	–	–	–	–	–
Simvastatin	a	–	–	–	–	–	–
Bezafibrate	<QL	100	100	100	100	100	100
Carbamazepine	110.6 ± 9.1	100	100	100	100	100	100
Citalopram	16.7 ± 12.0	100	100	100	100	~100	~100
Venlafaxine	114.4 ± 15.3	100	100	100	100	100	100
Fluoxetine	75.9 ± 7.2	100	100	100	100	100	~100
Norfluoxetine	8.3 ± 1.7	100	100	100	100	100	100
17-β-estradiol	110.4 ± 55.4	100	100	100	100	100	~100
Estrone	20.2 ± 3.3	100	100	100	100	100	**61.8**
Alachlor	a	–	–	–	–	–	–
Atrazine	a	–	–	–	–	–	–
Simazine	a	–	–	–	–	–	–
Chlorfenvinphos	a	–	–	–	–	–	–
Diuron	18.3 ± 2.8	100	100	100	100	100	100
Isoproturon	<QL	100	100	100	100	100	100
Pentachlorophenol	a	–	–	–	–	–	–
Clofibric acid	a	–	–	–	–	–	–
Perfluorooctanesulfonic acid	<QL	100	100	100	100	100	100

Incomplete removal is indicated in bold.

[a] < Detection Limit; QL, Quantification Limit.

Table 4. The higher concentrations were observed for pharmaceuticals in the following order (in ng L^{-1}): ibuprofen (614.3 ± 101.3) > DFC (464.8 ± 64.7) > 17-α-ethinylestradiol (391.4 ± 59.3) > azithromycin (139.9 ± 6.2) > clarithromycin (116.4 ± 2.7) > venlafaxine (114.4 ± 15.3) > carbamazepine (110.6 ± 9.1) > hydrochlorothiazide (105.2 ± 14.0). The natural hormone 17-β-estradiol was found at the same magnitude of concentration (110.4 ± 55.4 ng L^{-1}). It is noteworthy that some of these compounds are included in the watch list of the Decision 495/2015 of European Commission (Decision 495, 2015). Regarding the removal efficiency of each AOP, the micropollutants found in the wastewaters were completely removed by photocatalytic ozonation, independently of the reaction time or the ozone dose employed. The macrolide antibiotics azithromycin and clarithromycin were not totally degraded by either ozonation or photocatalysis. In addition, erythromycin, also a macrolide antibiotic, and the natural hormone estrone were not completely removed by photocatalysis. These results remark the effectiveness of photocatalytic ozonation to completely remove organic micropollutants, even when they are present at very low concentrations, while ozonation or photocatalysis alone are less efficient processes, in agreement with the results presented in Section 3.2.

As the complete removal of this set of micropollutants is not indicative of a complete mineralization of the organic content of the wastewaters, the ions (not shown) as well as oxalic and oxamic acids (Fig. 8a and b, respectively) were analyzed. Concerning the ions, while nitrites were not detected, nitrates were quantified in the wastewaters samples, the amount of nitrates increasing in the photocatalytic ozonation experiments. In addition, as higher the ozone inlet concentration, higher the formation of such species, suggesting that the organic pollutants containing nitrogen are being more rapidly oxidized when implementing higher ozone doses.

Regarding the low molecular weight carboxylic acids, oxalic acid (Fig. 8a) was formed during the first 15 min of photocatalytic ozonation (90 g Nm^{-3}) and its concentration started to decrease afterwards, as observed before when both distilled water and the spiked urban wastewater were used as matrix. The production of this acid was also ozone dose dependent, its formation being slightly slower when applying ozone doses of 70 or 50 g Nm^{-3}, in agreement with the concentration of ions. For ozonation and photocatalysis alone, the oxalic acid formation rate is much slower than in photocatalytic ozonation, and oxalic acid accumulates until the end of the experiment (up to 0.07 mg L^{-1} and 0.14 mg L^{-1} for photocatalysis and ozonation, respectively), which seems to be related to the lower efficiency of the single processes.

Similar trends were observed for oxamic acid (Fig. 8b), but in this particular case the concentration of the acid increased in the first 30 min and then remained similar (ca. 0.04 mg L^{-1}) until the end of the photocatalytic ozonation treatment, in perfect agreement with the results obtained with the spiked urban wastewater (Fig. 7). Thus, these results confirm that regardless of the type and concentration of the organic pollutants present in the urban wastewater (spiked or non-spiked), the oxamic acid formed (by degradation of the organic pollutants and/or other organic matter present) was refractory to the photocatalytic ozonation treatment. Having into account the results obtained by photocatalytic ozonation with distilled water as matrix (i.e., fast oxamic acid removal, Figs. 4b and 5b), one possible option to achieve the complete mineralization is to implement a pre-treatment of the urban wastewater in order to remove inorganic ions that can affect the photocatalytic ozonation performance.

4. Conclusions

The photocatalytic ozonation process was an efficient solution for fast (5 min) breakdown of AMX and DFC into small low weight compounds and, more important, for their complete mineralization (in 30 and 120 min, respectively), which is of major relevance for developing effective water/wastewater treatment technologies.

Ozonation, photolysis, photolytic ozonation and photocatalysis were not efficient due to the slow TOC removal and/or the formation of nonoxidizable organic compounds.

Oxalic and oxamic acids were identified as the low molecular weight reaction intermediates of AMX and DFC degradation, but they were readily degraded (in less than 20 min) by photocatalytic ozonation.

The nitrogen present in the parent AMX and DFC molecules was converted into nitrates and ammonium as final reaction products. Similarly, sulfur was converted into sulfates in experiments with AMX and chlorine into chlorides in those performed with DFC.

In photocatalytic ozonation of AMX solutions, no activity against *E. coli* and *S. aureus* was determined after 15 min. In addition, for both compounds (AMX and DFC) no toxic by-products were detected for these two microorganisms.

Experiments performed with an urban wastewater from a WWTP, non-spiked or spiked with AMX and DFC, showed that the intermediate oxamic acid, generated in these conditions is much more refractory to oxidation, in disparity with the results obtained with distilled water, probably due to the presence of oxoanions. Even so, all the identified parent micropollutants (pesticides, pharmaceuticals, hormones and an industrial compound) were removed by photocatalytic ozonation of the non-spiked urban wastewater.

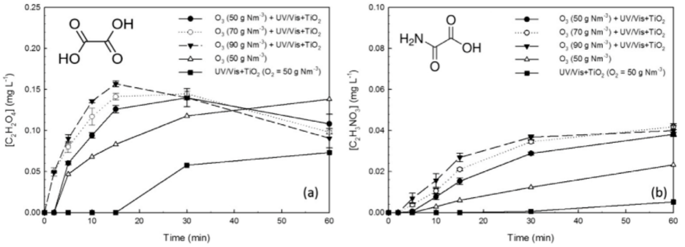


Fig. 8. Evolution of (a) oxalic and (b) oxamic acid concentrations (mg L^{-1}) for ozonation (O_3) with an inlet concentration of 50 g O_3 Nm^{-3}, photocatalysis (UV/Vis + TiO_2) with 0.5 g L^{-1} of TiO_2 and 50 g Nm^{-3} of O_2 and photocatalytic ozonation (O_3 + UV/Vis + TiO_2) using 0.5 g L^{-1} of catalyst load and different ozone inlet dosages (50, 70 and 90 g O_3 Nm^{-3}), in experiments performed with non-spiked (as received) urban wastewater samples.

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Appendix B

Photocatalytic ozonation of urban wastewater and surface water using immobilized TiO_2 with LEDs: Micropollutants, antibiotic resistance genes and estrogenic activity

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Photocatalytic ozonation of urban wastewater and surface water using immobilized TiO_2 with LEDs: Micropollutants, antibiotic resistance genes and estrogenic activity

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Cytotoxicity

ABSTRACT

Photocatalytic ozonation was employed for the first time in continuous mode with TiO_2-coated glass Raschig rings and light emitting diodes (LEDs) to treat urban wastewater as well as surface water collected from the supply area of a drinking water treatment plant (DWTP). Different levels of contamination and types of contaminants were considered in this work, including chemical priority substances (PSs) and contaminants of emerging concern (CECs), as well as potential human opportunistic antibiotic resistant bacteria and their genes (ARB&ARG). Photocatalytic ozonation was more effective than single ozonation (or even than TiO_2 catalytic ozonation) in the degradation of typical reaction by-products (such as oxalic acid), and more effective than photocatalysis to remove the parent micropollutants determined in urban wastewater. In fact, only fluoxetine, clarithromycin, erythromycin and 17-alpha-ethinylestradiol (EE2) were detected after photocatalytic ozonation, by using solid-phase extraction (SPE) pre-concentration and LC-MS/MS analysis. In surface water, this treatment allowed the removal of all determined micropollutants to levels below the limit of detection (0.01–0.20 ng L^{-1}). The efficiency of this process was then assessed based on the capacity to remove different groups of cultivable microorganisms and housekeeping (16S rRNA) and antibiotic resistance or related genes (*intI1*, *blaTEM*, *qnrS*, *sul1*). Photocatalytic ozonation was observed to efficiently remove microorganisms and ARGs. Although after storage total heterotrophic and ARB (to ciprofloxacin, gentamicin, meropenem), fungi, and the genes 16S rRNA and *intI1*, increased to values close to the pre-treatment levels, the ARGs (*blaTEM*, *qnrS* and *sul1*) were reduced to levels below/close to the quantification limit even after 3-days storage of treated surface water or wastewater. Yeast estrogen screen (YES), thiazolyl blue tetrazolium reduction (MTT) and lactate dehydrogenase (LDH) assays were also performed before and after photocatalytic ozonation to evaluate the potential estrogenic activity, the cellular metabolic activity and the cell viability. Compounds with estrogenic effects and significant differences concerning cell viability were not observed in any case. A slight cytotoxicity was only detected for Caco-2 and hCMEC/D3 cell lines after treatment of the urban wastewater, but not for L929 fibroblasts.

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1. Introduction

The environmental occurrence of priority substances (PSs), contaminants of emerging concern (CECs), human pathogens and antibiotic resistance genes (ARGs) is a serious issue affecting ecosystem services (such as drinking water supplies) and urban wastewater treatment plants (WWTPs) are hot spots for such type of contamination (Berendonk et al., 2015; Ribeiro et al., 2015a). For instance, conventional WWTPs are not specifically designed to eliminate PSs and CECs occurring at trace concentrations, ending into the environment. Among these, specific pesticides, endocrine disrupting compounds, industrial compounds, flame retardants, polycyclic aromatic hydrocarbons, dioxins and dioxin-like compounds, have already been identified as PSs in Directive 2013/39/EU (Directive, 2013). Furthermore, a watch list of 10 substances/groups of substances that should be monitored within the European Union was recently included in Decision 495/2015/EU (Decision, 2015), namely pharmaceuticals (diclofenac, 17-alpha-ethinylestradiol (EE2) and three macrolide antibiotics), two natural hormones (17-beta-estradiol (E2) and estrone (E1)), some pesticides, an UV filter and a food additive.

Besides the chemical contamination, the biological pollution is also a matter of concern in aquatic environments, in particular in human-impacted areas, calling for adequate responses from WWTPs (Dunlop et al., 2015; Ferro et al., 2015a; Michael et al., 2013; Rizzo et al., 2014). Conventional WWTPs, although able to remove the bacterial loads up to 2 logarithmic cycles, do not contribute to significant reductions of antibiotic resistance prevalence and, in some cases, can even contribute to its increase (Michael et al., 2013). It is estimated that urban conventional WWTPs using distinct types of biological treatments, and operating to achieve the recommended quality thresholds, release at least 10^9 antibiotic resistant coliforms per minute to the surrounding environment (Vaz-Moreira et al., 2014).

Therefore, the spread of PSs, CECs and antibiotic resistant bacteria and their genes (ARB&ARG) emitted from WWTPs contributes for the contamination of surface and groundwater, existing the possibility that some of these pollutants may reach the drinking water systems (Vaz-Moreira et al., 2014; Xi et al., 2009). This scenario makes also vulnerable the quality of treated wastewater and, thus, its reuse in activities such as irrigation in agriculture when this wastewater is not properly treated (Ferro et al., 2015b). In this context, new treatment options should be capable of eliminating PSs and CECs and reducing ARGs to levels at which further dissemination will be improbable (Berendonk et al., 2015; Dodd, 2012).

Chemical oxidation technologies (COTs) and advanced oxidation processes (AOPs), such as Fenton-based processes, heterogeneous photocatalysis and ozonation-based processes, have been widely studied to eliminate PSs and CECs from different types of wastewater, but most studies are focused on single compounds, normally at concentrations higher than those found in urban wastewater (Ribeiro et al., 2015a). In addition, photocatalysis (with or without H_2O_2) and the photo-Fenton process were already tested for the inactivation of an antibiotic resistant *Escherichia coli* strain (Dunlop et al., 2015; Ferro et al., 2015a; Rizzo et al., 2014). However, more studies are still needed to better understand the effect of the oxidative approaches on the control of ARB&ARG. For instance, photocatalytic ozonation has been studied for the removal of pharmaceuticals in effluents from urban WWTPs (Beltrán et al., 2012; Encinas et al., 2013; Espejo et al., 2013; Li et al., 2007; Márquez et al., 2014; Moreira et al., 2015; Quiñones et al., 2015; Rey et al., 2014; Tanaka et al., 2001), and a couple of reviews were published last year (Mehrjouei et al., 2015; Xiao et al., 2015), but not much is known about the potential of photocatalytic ozonation to remove ARB&ARGs from urban wastewater. Much less information can be found regarding surface water. In fact the literature is still scarce, less than 300 publications being found in Scopus for "photocatalytic ozonation", against more than 35,500 for "photocatalysis" and 12,000 for "ozonation or ozonolysis", in January 2016. The high efficiency of the photocatalytic ozonation treatment has been explained by a synergistic effect between ozonation and photocatalysis. The photogenerated electrons can react with ozone molecules generating ozonide radicals while decreasing the possible recombination of electron–hole pairs (Mehrjouei et al., 2015; Sánchez et al., 1998).

Based on this background information, the present study aimed at performing a comprehensive evaluation of the capacity of photocatalytic ozonation to treat wastewater collected after the secondary treatment of an urban WWTP. These experiments were performed in continuous mode implementing an innovative approach with TiO_2-coated glass Raschig rings (Sampaio et al., 2013) and light emitting diodes (LEDs). The same process was tested for surface water treatment, aiming at producing water fulfilling the requirements of drinking water. Thus, different levels of contamination and different types of contaminants were considered in this work, including a set of chemical PSs and CECs, some selected from Directive, 2013/39/EU (Directive, 2013) and Decision 495/2015/EU (Decision, 2015), as well as potential human opportunistic pathogens and ARGs. Yeast estrogen screen (YES), thiazolyl blue tetrazolium reduction (MTT) and lactate dehydrogenase (LDH) assays were also performed before and after photocatalytic ozonation to evaluate biological effects, including the potential estrogenic activity, the cellular metabolic activity and the cell viability in fresh and treated waters. Because biological contaminants have the potential to regrow, when the stress conditions imposed by the water treatment processes are relieved, microbiological indicators were also monitored after storage of treated water.

2. Materials and methods

2.1. Chemicals and materials

TiO_2 (80% anatase and 20% rutile crystalline phases) was provided by Evonik Degussa GmbH (P25). Ultrapure water (resistivity > 18 MΩ cm) was supplied by a Milli-Q water system. Methanol and acetonitrile (MS grade) were acquired from VWR International (Fontenay-sous-Bois, France) whereas methanol and ethanol (HPLC grade) as well as orthophosphoric acid were purchased from Fisher Scientific UK Limited (Leicestershire, UK). Sulphuric acid was purchased from Merck (Darmstadt, Germany). Oxamic acid (>96%) was supplied by Fluka (Buchs, Switzerland).

Anhydrous sodium dihydrogen phosphate, sodium chloride and oxalic acid (>99%) were purchased from Sigma–Aldrich (Steinhein, Germany), as well as all reference standards for liquid chromatography (>98% purity): diclofenac sodium, ibuprofen sodium, naproxen, tramadol hydrochloride, azithromycin dihydrate, clarithromycin, erythromycin, sulfamethoxazole, trimethoprim, fluticasone propionate, montelukast sodium, warfarin, clopidogrel hydrogen sulphate, metoprolol tartrate, propranolol hydrochloride, hydrochlorothiazide, atorvastatin calcium salt trihydrate, bezafibrate, simvastatin, carbamazepine, citalopram hydrobromide, fluoxetine hydrochloride, norfluoxetine oxalate, venlafaxine hydrochloride, diphenhydramine, 17-alpha-ethinylestradiol (EE2), 17-beta-estradiol (E2), estrone (E1), alachlor, atrazine, simazine, diuron, isoproturon, chlorfenvinphos, pentachlorophenol, clofibric acid and perfluorooctanesulfonic acid. Stock solutions of each individual compound (approximately 1000 mg L^{-1}) were prepared in methanol, ethanol or acetonitrile, depending on their solubility,

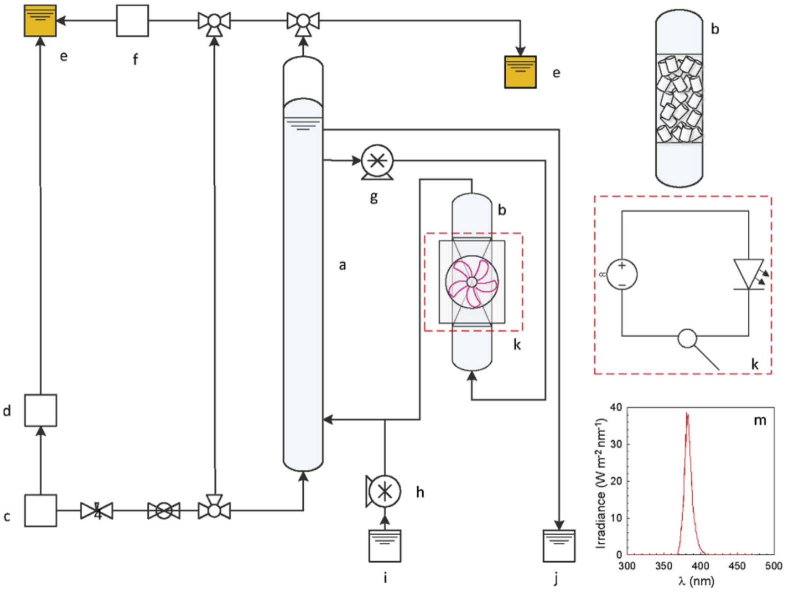

Fig. 1. Experimental set-up of the homemade photocatalytic reactor able to work in continuous mode: (a) Column for gas–liquid contact; (b) Column for liquid–solid contact (Raschig rings); (c) Ozone generator; (d) Mass flow controller; (e) Ozone gas destroyer; (f) Ozone gas monitor; (g) Peristaltic pump for recirculation; (h) Peristaltic pump; (i) Inlet solution; (j) Outlet solution; (k) LEDs; (m) LEDs irradiance spectrum.

and a working solution (200 μg L^{-1}) was prepared by diluting these solutions in ethanol. The deuterated compounds used as internal standards were also purchased from Sigma–Aldrich (Steinhein, Germany), namely: ketoprofen–d3, ofloxacine–d3, propanolol–d7 solution, fluoxetine–d5 solution, E2–d5 solution and atrazine–d5. A working solution containing 10 mg L^{-1} of all internal standards was prepared by diluting individual stock solutions (1000 mg L^{-1}) in ethanol.

For cell culture experiments, dimethyl sulfoxide (≥99.9%), Dulbecco's phosphate buffered saline (PBS) pH 7.4, Triton™ X-100, thiazolyl blue tetrazolium (MTT) and trypan blue powder were purchased from Sigma–Aldrich. Dulbecco's modified eagle media (DMEM; ref: 31966-021), heat inactivated fetal bovine serum (FBS), penicillin-streptomycin (PenStrep) and trypsin-EDTA were purchased from Gibco® by Life Technologies™ (UK). EndoGRO™ – MV complete media kit was obtained from Merck Millipore and LDH Cytotoxicity Detection Kit was acquired from Takara Bio Inc. (Shiga, Japan). Caco-2 cell line was purchased to the American Type Culture Collection (ATCC, Wesel, Germany) and used between passage number 35 and 42. hCMEC/D3 cell line was kindly supplied by the Institut National de la Santé et de la Recherche Médicale (INSERM, Paris, France) and used between the passage number 31 to 34. Murine fibroblasts L929 were also obtained from ATCC.

2.2. *Experimental set-up and procedure*

A bubble column reactor (Fig. 1a; 2.2 I.D. × 60 cm height) equipped with a loop column (Fig. 1b; 2.2 I.D. × 15 cm height) was used for all the experiments.

This reactor was designed for ozonation experiments operating in continuous mode (Restivo et al., 2012a) and equipped with LEDs in the present work. Two 10 W UV high intensity LEDs (15.5 mm × 23 mm), with dominant emission line at 382 nm and long service life (intensity remains above 70% after 10,000 h work) (Fig. 1k and m), were placed perpendicularly to each other,

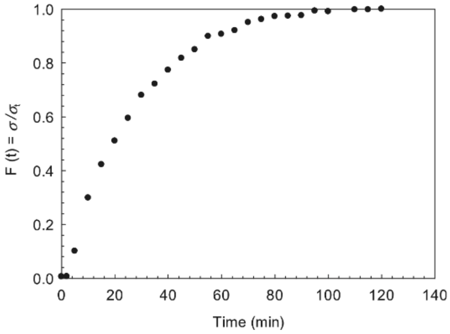

Fig. 2. Normalized conductivity (σ/σ_t) of the outlet solution, introducing an inlet NaCl solution (2000 mg L^{-1}). σ is the conductivity of the outlet solution and σ_t the conductivity of the tracer injected in the column.

Table 1
Conditions used in quantitative PCR assays.

Gene	Primers	Reference	Efficiency (%)	Conditions	Reference
16S rRNA	1114F (CGGCAACGAGCGCAACCC) 1275R (CCATTGTAGCACGTGTGTAGCC)	*Escherichia coli* ATCC 25922	100	95 °C for 10 min (1 cycle) 95 °C for 15 s, 55 °C for 20 s and 72 °C for 10 s (35 cycles) Other: 1a	(Denman and McSweeney, 2006)
blaTEM	blaTEM-F (TTCCTGTTTTTGCTCACCCAG) blaTEM-R (CTCAAGGATCTTACCGCTGTTG)	*Escherichia coli* (A2FCC14)	96	95 °C for 10 min (1 cycle) 95 °C for 15 s, 60 °C for 30 s and 72 °C for 10 s (40 cycles) Other: 2a	(Bibbal et al., 2007)
intI1	intI1-F (CCTCCCGCACGATGATC) intI1-R (TCCACGCATCGTCAGGC)	*Escherichia coli* (A2FCC14)	94	95 °C for 10 min (1 cycle) 95 °C for 15 s, 55 °C for 30 s and 72 °C for 10 s (40 cycles) Other: 1a	(Goldstein et al., 2001)
qnrS	qnrSrtF11 (GACGTGCTAACTTGCGTGAT) qnrSrtR11 (TGGCATTGTTGGAAACTTG)	*Enterobacter cloacae* (S1+)	95	95 °C for 5 min (1 cycle) 95 °C for 15 s, 60 °C for 1 min (40 cycles) Other: 2c	(Marti and Balcázar, 2013)
sul1	sul1-FW (CGCACCGGAAACATCGCTGCAC) sul1-RV (TGAAGTTCCGCCGCAAGGCTCG)	*Achromobacter* sp.	94	95 °C for 5 min (1 cycle) 95 °C for 15 s, 60 °C for 1 min (40 cycles) Other: 2b	(Pei et al., 2006)
vanA	vanA3FP (CTGTGAGGTCGGTTGTGCG) vanA3RP (TTTGGTCCACCTCGCCA)	*Enterococcus faecalis* (H1EV23)	98	95 °C for 5 min (1 cycle) 95 °C for 15 s, 60 °C for 1 min (40 cycles) Other: 2a	(Volkmann et al., 2004)

1) KAPA SYBR® FAST ABI Prism® qPCR Master Mix; 2) SYBR® Select Master Mix; a) 200 nM of primer; b) 400 nM of primer; c) 600 nM of primer.

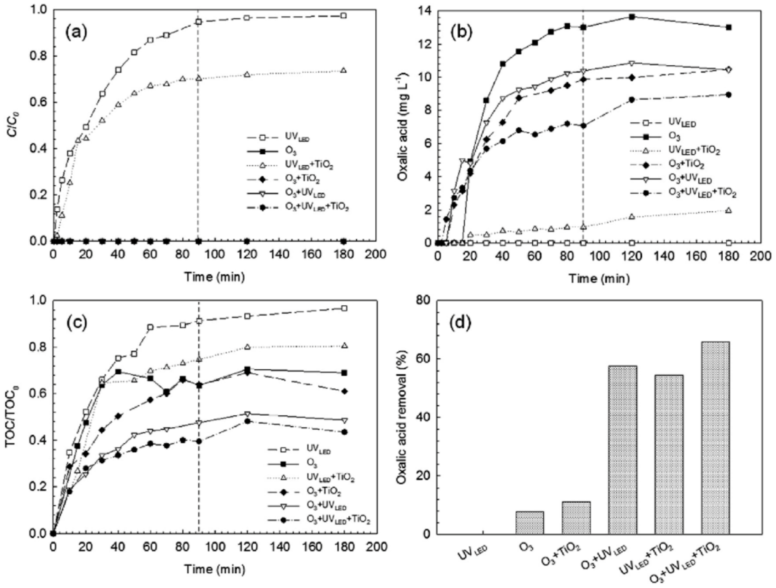

Fig. 3. (a) Normalized concentration (C/C_0) of diclofenac in ultrapure water ($C_0 = 32$ mg L^{-1}), (b) evolution of its oxalic acid by-product concentration, and (c) respective normalized TOC content (TOC/TOC_0) under different advanced processes. (d) Removal of oxalic acid when used as feed ($C_0 = 90$ mg L^{-1}). Experiments started with the column filled with ultrapure water only (without pollutant at t = 0 min), the steady state was obtained at ca. 90 min and the retention time is 26 min.

irradiating the stream (distance of 5 cm) passing through the loop column, by setting a recirculation flow rate of 60 mL min^{-1} (Fig. 1g), as performed in a previous work dealing with catalytic ozonation (Restivo et al., 2012b). The wavelength around 382 nm was chosen

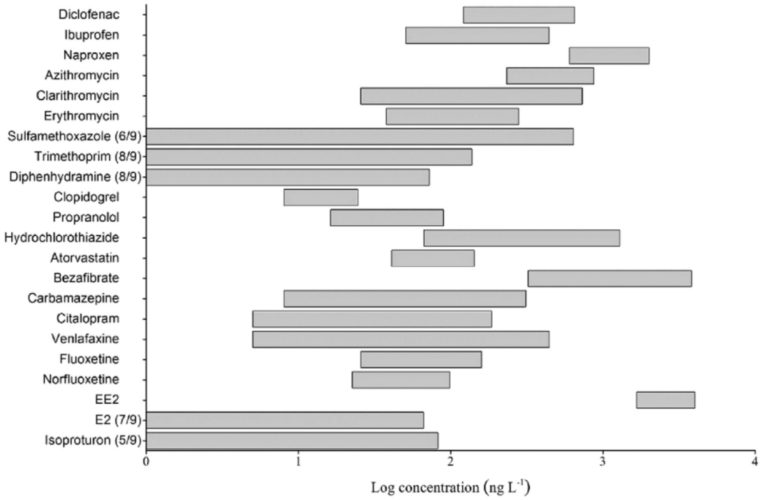

Fig. 4. Logarithmic range of concentrations (ng L^{-1}) of the detected micropollutants in WWTP samples collected after the secondary biological treatment. The frequency of occurrence was 100% (9/9) for all compounds, except when indicated.

considering a compromise between the cost of LEDs (increasing when the emission wavelength decreases) and the possibility to achieve an overlapping between the LEDs main emission wavelength and the TiO_2 absorption spectrum. LEDs emitting in UV-C wavelengths can be up to 10 times more expensive than those emitting in near visible wavelengths. In addition, operation at lower wavelengths demands more energy, the LEDs lifetime decreases significantly (Lui et al., 2014), and energy is wasted in the form of heat. For these reasons, the wavelength near 382 nm was seen as the best option to the system under study, in this way using the minimum energy to activate the TiO_2 photocatalyst.

The loop column was packed with seventy three TiO_2-coated glass Raschig rings (Fig. 1b) that were prepared as described elsewhere (Sampaio et al., 2013), from a procedure adapted from literature (Quici et al., 2010). Uncoated Raschig rings were used in non-catalytic assays. In photolysis and photocatalytic experiments, the LEDs were switched on at the beginning of the experiment, with a constant oxygen flow rate (15 Ncm^3 min^{-1}). In ozonation and photocatalytic ozonation assays, a BMT 802X ozone generator (Fig. 1c) 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}). A BMT 964 ozone analyzer (Fig. 1f) was used to control the concentration of ozone in the gas phase. Gas washing bottles filled with potassium iodide solution were used to remove the ozone leaving the reactor in the gas phase (Fig. 1e).

Regardless of the type of test performed, the reactor was always filled with ultrapure water and the experiment started ($t = 0$ min) when the water to be treated (wastewater or surface water) (Fig. 1i) was pumped to the reactor (Fig. 1h), i.e., being diluted until achieving the steady state. Thus, since the reactor was fully filled with ultrapure water before starting the experiment with a realistic matrix, some dilution occurred up to 90 min in every run. This reactor is designed to operate in continuous mode, with a continuous entrance of realistic matrix at the bottom of the biggest column (liquid flow rate of 15 mL min^{-1}), a continuous outlet stream of treated surface water/wastewater at the top of the same column, and a continuous recirculation (60 mL min^{-1}) between the biggest column and the smaller column holding the photocatalyst, in this way simulating a perfectly mixed reactor. Thus, the removal efficiency was inferred when the steady state was achieved, i.e. when the outlet solution had always the same concentration and there was no more dilution effect. For this reason, preliminary studies on hydraulic retention time (HRT) were performed using NaCl as tracer (inlet concentration of 2000 mg L^{-1}) and uncoated Raschig glass rings were placed randomly inside the loop column, measuring the conductivity with a conductimeter Crison GLP 31 (Barcelona, Spain). The gas and liquid streams were maintained at 15 Ncm^3 min^{-1} and 15 mL min^{-1}, having into account preliminary ozonation experiments with different ozone flow rates (using oxalic acid as prove molecule) and that these conditions were optimized for catalytic ozonation in a previous work (Restivo et al., 2012b). The HRT studies allowed to determine the time needed to achieve the steady state and the time spent by the fluid inside the reactor (retention time). Fig. 2 shows the evolution of the normalized conductivity (σ/σ_t) where σ is the conductivity of the outlet solution and σ_t the conductivity of the tracer injected in the column. The time needed to achieve the steady state is ca. 90–100 min and the retention time (26 min) was determined from the area under the representation of 1-(σ/σ_t) *vs.* time. Other run was performed with vertically aligned uncoated glass rings but the results were similar to those obtained with randomly distributed glass rings. Having into account these results, the next experiments were performed during 180 min with randomly distributed Raschig glass rings (retention time = 26 min).

The first set of experiments with chemical pollutants was performed with ultrapure water spiked with diclofenac (32 mg L^{-1}; pH = 4.5) and the removal of this pharmaceutical was followed over time by analysing the treated effluent (Fig. 1j). In a second set of experiments, wastewater samples collected after the activated sludge biological treatment of an urban WWTP located in Northern

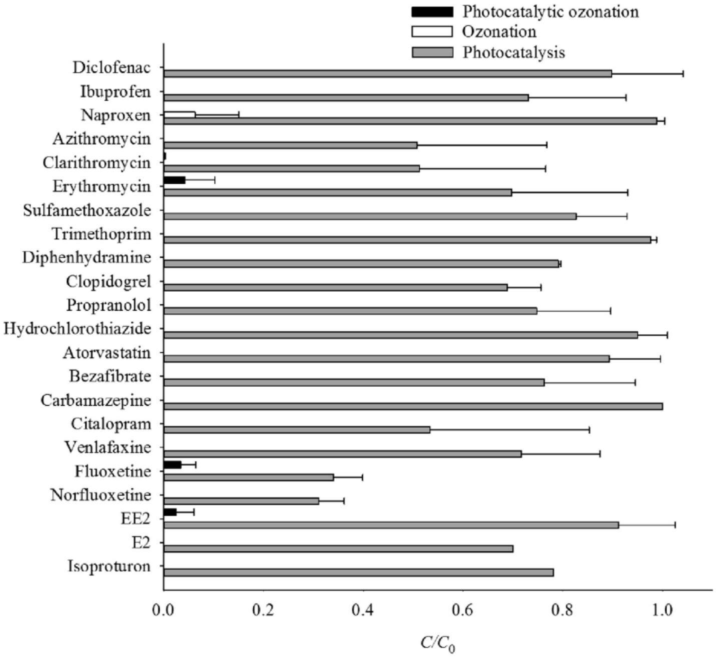

Fig. 5. Normalized concentration of micropollutants (C/C_0) in the effluents from urban WWTP treated by photocatalysis, ozonation and photocatalytic ozonation; C_0 refers to the concentration before treatment and C to that after treatment (retention time: 26 min); compounds with C_0 below the LOD before treatment are not shown.

Portugal were treated by ozonation, photocatalysis and photocatalytic ozonation ($n = 3$, i.e. a total of three samples for each treatment, each sample collected in a different day). For the catalytic treatments, coated Raschig glass rings were replaced between experiments. The sampling (April–May 2015) was performed using pre-rinsed amber glass bottles (2.5 L). Surface water samples were collected (May–June 2015) in the supply area of a drinking water treatment plant (DWTP) and treated by photocatalytic ozonation ($n = 3$). All samples were transported at 4 °C to the laboratory and processed immediately.

2.3. Materials characterization

TiO_2-coated glass rings were characterized before and after the photocatalytic ozonation treatment. Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS) were performed in a FEI Quanta 400 FEG ESEM/EDAX Genesis X4M instrument. The materials hydrophilicity was assessed by measuring the water contact angle in an Attension equipment (Theta model) which allows image acquisition and data analysis. The measurements were performed at room temperature using the sessile drop method in five different locations over glass rings. Thermogravimetric (TG) analysis was performed using a STA 490 PC/4/H Luxx Netzsch thermal analyzer by heating the samples from room temperature until 800 °C at 10 °C min^{-1} under helium flow, holding this temperature for 7 min and finally changing to air atmosphere during 13 min.

2.4. Chemical analysis

High performance liquid chromatography with diode array detection (HPLC-DAD) was performed to analyze the removal of diclofenac, using a Hitachi Elite Lachrom apparatus equipped with a YMC Hydrosphere C18 column (250 mm × 4.6 mm i.d.), working at room temperature under isocratic elution. The mobile phase consisted in a mixture of phosphate buffer solution (pH 2.8) and methanol (30/70, v/v). The flow rate was 1 mL min^{-1} and the volume of injection was 15 μL. A pH meter pHenomenal® pH 1100L (VWR, Germany) was used for all pH adjustments. The oxalic and oxamic acid concentrations were determined by HPLC-UV, using an Altech AO-1000 column (300 mm × 6.5 mm i.d.) operating under

Table 2
Concentration of the micropollutants detected in surface water collected during three sampling campaigns, between May and June 2015, and normalized concentration after treatment (C/C_0).

	C_0 (ng L^{-1})	C/C_0	C_0 (ng L^{-1})	C/C_0	C_0 (ng L^{-1})	C/C_0
Clarithromycin	4.13	0	1.36	0	2.11	0
Fluoxetine	5.72	0	<LOQ	0	<LOQ	0
Norfluoxetine	7.66	0	<LOD	n.a.	<LOD	n.a.
Carbamazepine	<LOQ	0	35.1	0	56.2	0
E2	<LOQ	0	<LOQ	0	<LOQ	0
EE2	<LOD	n.a.	<LOQ	0	<LOQ	0
Isoproturon	<LOQ	0	24.3	0	21.3	0

LOD: limit of detection; LOQ: limit of quantification; n.a.: not applicable.

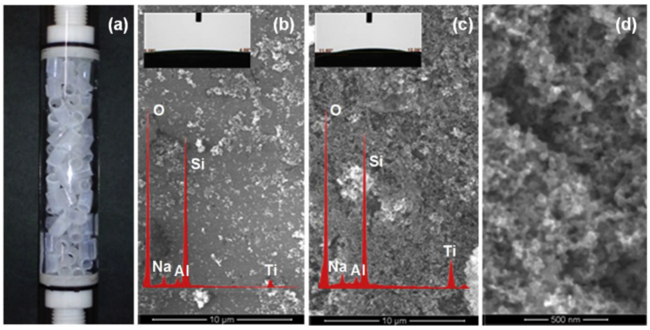

Fig. 6. (a) TiO_2-coated glass Raschig rings and respective (b, c, d) SEM micrographs, EDS and contact angles (b) before and (c, d) after photocatalytic ozonation.

isocratic elution with 5 mM H_2SO_4 at 0.5 mL min^{-1}. The total organic carbon (TOC) content was determined using a Shimadzu TOC-5000A analyzer. The concentrations of the target micropollutants (listed in section 2.1) were determined in urban wastewater and surface water samples, before and after the treatment by the advanced oxidation processes, using a fully validated method of solid phase extraction (SPE) and ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) in a Shimadzu Corporation apparatus (Tokyo, Japan), as described elsewhere (Ribeiro et al., 2015b). Wastewater (100 mL) and surface water (250 mL) samples were acidified to pH 3 with sulphuric acid, followed by extraction of the micropollutants using Oasis® HLB (Hydrophilic-Lipophilic-Balanced sorbent, 150 mg, 6 mL) cartridges (Waters, Milford, Massachusetts, USA). For internal calibration, isotopically labeled internal standards were added to the samples before SPE.

2.5. Microbiological characterization, DNA extraction and qPCR analysis

Wastewater and surface water samples were characterized for the abundance of different groups of cultivable microorganisms, quantification of selected antibiotic resistance and related genes (herein referred to ARG), before and immediately after photocatalytic ozonation, and after three days of water storage at room temperature. For each photocatalytic ozonation experiment, all assays were performed in triplicate. The membrane filtration method was used for the enumeration of cultivable microorganisms. Briefly, adequate serial dilutions of water samples were filtered through cellulose membrane filters (0.22 μm porosity; Whatman, UK) and incubated on culture media targeting different microbial groups: Plate Count Agar (PCA) (Merck, 30 °C, 1–7 days) for total heterotrophs; membrane Fecal Coliforms (Difco, 37 °C, 1 day) for enterobacteria; m-Enterococcus agar (Difco, 37 °C, 2 days) for enterococci; and Rose Bengal Chloramphenicol agar (VWR International, 30 °C, 7 days) for fungi. Additionally, PCA supplemented with ciprofloxacin (4 mg L^{-1}), gentamicin (16 mg L^{-1}) or meropenem (4 mg L^{-1}) were used to assess resistance prevalence in surface water before and after treatment. These antibiotics were selected because resistance to carbapenems (e.g., meropenem) and aminoglycosides (e.g., gentamicin) is commonly found in drinking water bacteria or because selection of fluoroquinolone resistance (e.g., ciprofloxacin) is sometimes observed in aquatic environments (Vaz-Moreira et al., 2014). For culture-independent assays, total DNA was extracted from water samples with volumes ranging from 100 mL to 1 L, after filtration through polycarbonate membranes (0.22 μm porosity; Whatman, UK), using the commercial kit PowerWater® DNA Isolation (MO BIO Laboratories, Inc., USA). DNA extracts were cryopreserved at −20 °C until the analyses of the abundance of ARG. Quantitative PCR (qPCR) was used according to the conditions indicated in Table 1, as described elsewhere (Narciso-da-Rocha et al., 2014).

2.6. Biological assays

2.6.1. Yeast estrogen screen (YES) assay

The YES assay was performed using the commercially available kit XenoScreen YES/YAS assay (Xenometrix, Hégenheim, France). Water samples (wastewater and surface water) collected before and after photocatalytic ozonation were filtered through 0.45 μm hydrophilic membranes and directly analyzed, without any preconcentration. The assay and potential estrogenic activity calculations were carried out following the manufacturer's instructions. Briefly, samples were transferred to a 96-well microplate, mixed with assay medium and inoculated with the transformed yeast cells. Assay mixture consisted of growth medium containing the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG). The natural estrogen E2, at concentrations between 10^{-6}–10^{-9} M, was used as positive control and standard. Ultrapure water was used as negative control. A solvent blank was also assayed. The plate was incubated for 48 h at 31 °C with orbital shaking. After incubation, spectrophotometric measurements were performed in a microplate reader (Synergy HT, Bio-Tek Instruments, Winooski, USA) at 690 nm for growth and at 570 nm for expression of β-galactosidase. The potential estrogenic activity was determined through the calculation of the parameters growth factor (G) and induction ratio (IR). The G parameter was calculated as the ratio of absorbance at 690 nm for the sample $(A_{690})_{sample}$ and the absorbance at 690 nm for the solvent $(A_{690})_{solvent}$. The IR parameter was calculated as $(1/G) \times ((A_{570} - A_{690})_{sample}/(A_{570} - A_{690})_{solvent})$.

2.6.2. Thiazolyl blue tetrazolium reduction (MTT) and lactate dehydrogenase (LDH) assays

Thiazolyl blue tetrazolium reduction (MTT) assay evaluates cellular metabolic activity while lactate dehydrogenase (LDH) assay provides information about cell viability through evaluation of membrane integrity. Cell culture procedures are described in detail elsewhere (Ferreira et al., 2015). Briefly, samples were diluted 1:2.5 in culture medium. Afterwards, 100 μL of diluted sample were

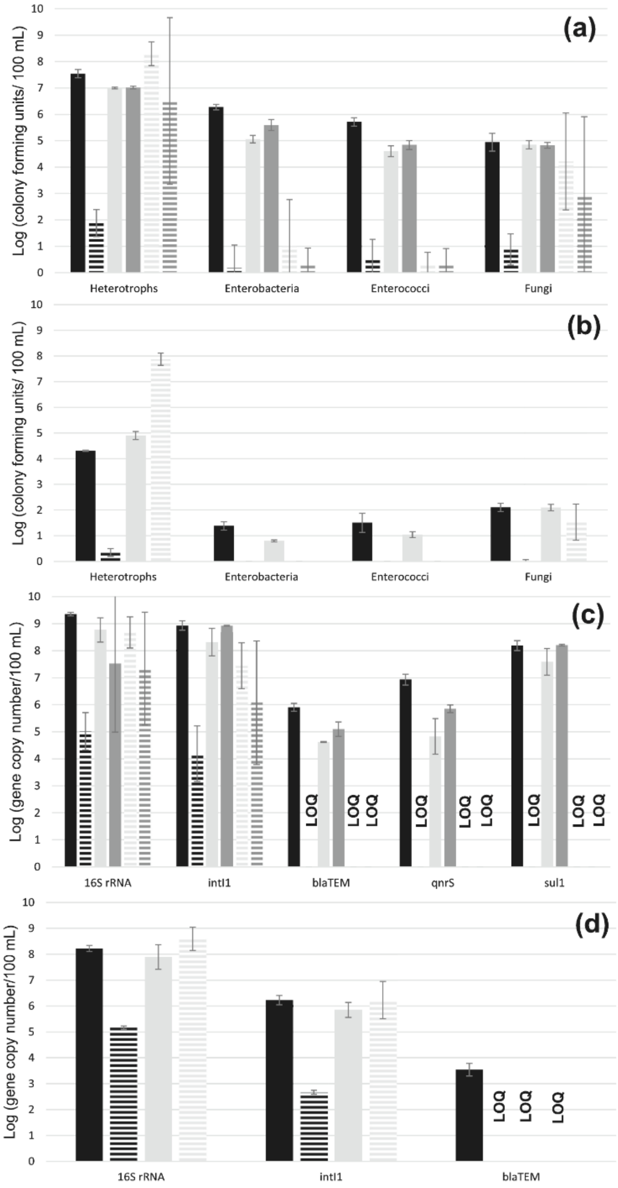

Fig. 7. Microbial inactivation by photocatalytic ozonation in continuous reaction performed on wastewater effluents (a, c) and surface water (b, d) was assessed based on the quantification of selected microbial groups using culture-dependent methods (a, b) and qPCR of selected genes (c, d), in non-treated control samples (filled bars) and after a treatment retention time of 26 min (striped bars), at the initial time (black) and after 3-days storage at room temperature with light (grey) or in the dark (dark grey). LOQ, below or close to the limit of quantification (at least more than half of the quantification reactions were below the quantification limit and none was more than 3 times above that value).

added to cell layers or suspension, containing 100 μL of culture medium. After 24 h, the supernatant was removed for LDH assay, while the remaining content was used for MTT assay. For MTT assay, absence of cytotoxicity (100%) was evaluated by replacing test sample by culture medium. For LDH assay, absence of cell viability (100%) was evaluated by replacing test sample by 1% (v/v) Triton X-100 solution in PBS.

3. Results and discussion

3.1. Degradation of organic micropollutants

The first set of experiments with chemical pollutants was performed using ultrapure water spiked with diclofenac as model compound, due to the known high occurrence of this pharmaceutical in the environment, its recalcitrance, and inclusion in the watch list for European Union monitoring in the field of water policy (Decision, 2015). The evolution of the normalized concentration of diclofenac is shown in Fig. 3a, the time needed to achieve the steady state in all the experiments being around 90–100 min, as concluded from HRT studies (Fig. 2). Fig. 3a shows that complete removal of this compound was achieved in all the experiments where ozone was employed (ozonation, photolysis assisted ozonation, catalytic ozonation, and photocatalytic ozonation). For (non-catalytic) photolysis, the outlet and inlet diclofenac concentrations were practically the same in steady state. This result was expected since this model compound absorbs maximally radiation at wavelengths below 325 nm while the radiation peak of the used LEDs was centered at 382 nm, above 367 and below 410 nm (Fig. 1m). In the case of TiO_2-coated glass rings photocatalysis, a modest diclofenac removal in steady state was observed (ca. 26%; i.e. $C/C_0 = 0.74$).

Oxalic and oxamic acids are well known reaction products during ozonation of larger molecules (Faria et al., 2008), and thus they were also analyzed, but only oxalic acid was detected above the limit of detection – LOD (Fig. 3b). As expected, oxalic acid was not formed during photolysis, while photocatalysis originated a low amount of this acid in steady state (ca. 2.0 mg L^{-1}), which is probably related to the poor efficiency of this process for the degradation of diclofenac. In contrast, ozonation alone led to the largest amount of oxalic acid (ca. 13.0 mg L^{-1}), followed by photolysis assisted ozonation and catalytic ozonation (ca. 10.5 mg L^{-1}), and then by photocatalytic ozonation (ca. 9.0 mg L^{-1}).

TOC analysis was performed to account for the presence of other organic compounds that can be formed during the degradation of diclofenac (Fig. 3c). Once again, photolysis and photocatalysis were the least effective treatments, with TOC removals of ca. 3 and 20% in steady state, respectively, while photolysis assisted ozonation and photocatalytic ozonation were the best performing options in terms of TOC removal (ca. 51% and 56%, respectively).

Since oxalic acid was the main by-product detected from diclofenac degradation, additional experiments were performed having oxalic acid only (90 mg L^{-1}) in ultrapure water as feed (Fig. 3d). Oxalic acid was completely recalcitrant in the photolysis assay, while low removal values were obtained with ozonation (8%) and catalytic ozonation (11%). All the other processes (photocatalysis, photolysis assisted ozonation and photocatalytic ozonation) were quite effective in the removal of oxalic acid (55%, 58% and 66%, respectively). Even so, photocatalytic ozonation was more efficient than photolysis assisted ozonation for the degradation of oxalic acid, probably due to the overlap between the absorption spectrum of TiO_2 and the emission spectrum of the LEDs, enough to get the minimum energy needed to surpass the energy band gap of TiO_2. In batch conditions (Moreira et al., 2015), it was already observed that oxalic acid (one of the main reaction by-products in ozonation-based processes) is oxidized efficiently by photocatalytic ozonation, while photolysis assisted ozonation was not able to remove the oxalic acid accumulated. In addition, it is known that UV at 382 nm (far from UV-C) is inefficient for disinfection in the absence of a photocatalyst (Nelson et al., 2013). Thus, considering the targeted simultaneous treatment of both chemical and biological contaminants, and since a photocatalyst can increase the process disinfection efficiency by the formation of highly reactive radicals, photocatalytic ozonation was applied to treat wastewater samples collected after the secondary biological treatment of an urban WWTP. Ozonation and photocatalysis experiments were also performed in order to assess the individual contribution of each process on their own.

The micropollutants (pharmaceuticals, a metabolite, hormones, pesticides and an industrial compound listed in section 2.1) were analyzed (before and after the treatment) by UHPLC-MS/MS, after pre-concentration of the samples by SPE. Fig. 4 shows the concentration range for the detected micropollutants in the effluent of the biological treatment used in the urban WWTP during the sampling campaign (April–May 2015). The highest concentrations were observed for the following compounds with a frequency of detection of 100% (9/9): the anti-inflammatory naproxen, the diuretic hydrochlorothiazide, the lipid regulator bezafibrate and the synthetic hormone EE2 included in the watch list of the Decision 495/2015/EU (Decision, 2015). Diclofenac, azithromycin, clarithromycin and erythromycin, also included in the watch list, as well as ibuprofen, clopidogrel, propranolol, atorvastatin, carbamazepine, fluoxetine and norfluoxetine, were quantified in all samples. Citalopram and venlafaxine were also detected, but not always above the limit of quantification (LOQ), whereas sulfamethoxazole, trimethoprim, diphenhydramine, E2 and isoproturon were not detected in some samples.

Fig. 5 shows that the higher efficiency to remove the detected micropollutants was found for ozonation and photocatalytic ozonation. Photocatalysis was comparatively less effective in the degradation of these micropollutants. Only fluoxetine, clarithromycin, erythromycin and EE2 were detected in the outlet solution after photocatalytic ozonation. While both antibiotics were only detected in the final effluent of one of the triplicate experiments, fluoxetine and EE2 were detected twice. Naproxen was only quantified in one replicate assay. It is also important to note that samples were collected daily for each experiment. Regarding the TOC removal, the initial TOC of ca. 25 mg L^{-1} decreased to 19 mg L^{-1} in steady state conditions of photocatalytic ozonation. The initial and final pH values were quite similar (7.4 and 7.2, respectively) while the low weight carboxylic acids and ions were below the LOQs. Thus, both single ozonation and photocatalytic ozonation treatments were quite effective to remove the chemical organic micropollutants (Fig. 5), but it should be also taken into account that ozonation is typically much less effective than photocatalytic ozonation in the mineralization (Fig. 3c) and degradation of typical reaction by-products (Fig. 3b). Among different treatments described elsewhere in batch conditions (Moreira et al., 2015), photocatalytic ozonation was considered the best performing process: a fast mineralization of amoxicillin and diclofenac was observed in spiked urban wastewater; toxic intermediate products for *Escherichia coli* and *Staphylococcus aureus* were not detected; and the parent micropollutants determined were removed from an urban wastewater.

Photocatalytic ozonation, the most efficient process for the degradation and mineralization of chemical pollutants in urban wastewater, was also tested to treat surface water samples collected near a DWTP. The micropollutants detected after pre-concentration of the samples by SPE were: clarithromycin, fluoxetine, norfluoxetine, carbamazepine, EE2, E2 and isoproturon (Table 2). The

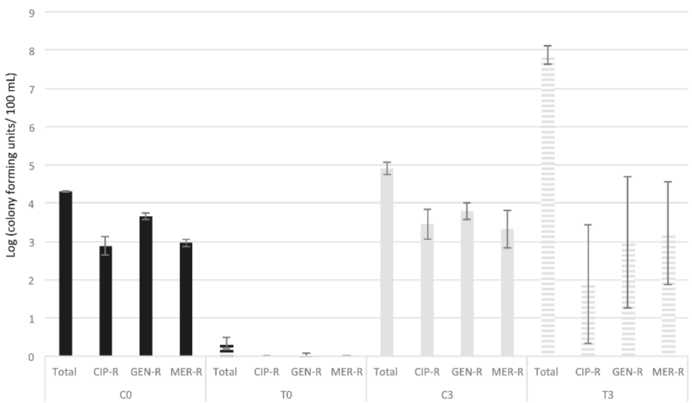

Fig. 8. Total, ciprofloxacin resistant (CIP-R), gentamicin resistant (GEN-R) and meropenem resistant (MER-R) heterotrophic counts before (C0), after photocatalytic ozonation treatment (T0) and after 3-days incubation of untreated surface water (C3) and treated surface water (T3) at dark conditions for microbial reactivation assessment.

Table 3
Results (percentage) from MTT[a] and LDH[b] assays before and after treatment of urban wastewater sample.

Cell line	MTT assay		LDH assay[c]	
	Before treatment	After treatment	Before treatment	After treatment
Caco-2	92 ± 20	59 ± 11	55.3 ± 3.1 (51.5 ± 4.6)	49.0 ± 3.3 (49.5 ± 4.0)
hCMEC/D3	106 ± 10	58 ± 21	61.1 ± 3.7 (66.6 ± 7.0)	60.3 ± 4.2 (67.4 ± 5.5)
L929	120 ± 19	91 ± 10	50.7 ± 2.5 (51.6 ± 3.0)	55.7 ± 3.2 (56.7 ± 3.3)

[a] Values for culture media were 100% (RSD < 20%) and between 1.3 and 10% for Triton X-100 (total disruption of cells).
[b] Values for Triton X-100 (total disruption of cells) were 100% (RSD < 8%).
[c] Values in parentheses correspond to blank values obtained in culture media only (intact cells).

antibiotic clarithromycin, recently included in the watch list of substances for monitoring (Decision, 2015), was quantified at trace concentrations (up to 4.13 ng L^{-1}). Fluoxetine was also detected in all samples, but only quantified in one sample (at 5.72 ng L^{-1}), while its human metabolite norfluoxetine was detected once in the same sample, with a higher concentration (7.66 ng L^{-1}) than the parent compound. The highest levels were obtained for the anti-epileptic carbamazepine (up to 56.2 ng L^{-1}) and for the pesticide isoproturon (up to 24.3 ng L^{-1}), the last considered as PS (Directive, 2013). All these micropollutants were completely removed by photocatalytic ozonation. Although not quantifiable, EE2 and E2 were detected before photocatalytic ozonation and completely removed. Therefore, photocatalytic ozonation was observed as an efficient treatment for the degradation of organic micropollutants in both urban wastewater and surface water.

3.2. TiO_2-coated glass rings characterization

The TiO_2-coated glass rings (shown in Fig. 6a) were characterized by different techniques before and after photocatalytic ozonation of the urban wastewater samples. A negligible weight loss (WL) was obtained by TG analysis of the fresh TiO_2-coated glass rings (not shown), regardless of the atmosphere employed (helium or air). In the case of the rings recovered after photocatalytic ozonation, desorption of water occurred below 100 °C under helium atmosphere while 4% of the weight decreased when the gas phase was shifted from helium to air at 800 °C, most probably due to the oxidation of some organic matter remaining in the surface of these rings after photocatalytic ozonation. SEM micrographs, EDS spectra and contact angles of water droplets over the glass rings are shown in Fig. 6b and c, respectively before and after the photocatalytic ozonation treatment. TiO_2 presence is evident in both samples, with Fig. 6d showing a higher SEM magnification of the TiO_2 particles. It is also possible to conclude that TiO_2-coated glass rings are highly hydrophilic since very low contact angles were obtained (<15°). Overall, no significant differences were observed between the fresh and used samples, even considering the high matrix complexity of effluents from urban WWTPs.

3.3. Microbial inactivation and reactivation

The photocatalytic ozonation of secondarily treated wastewater showed the reduction of enterococci, enterobacteria, and fungi from ~$10^5 - 10^6$ colony forming units (CFU) per 100 mL to values around or below ~10^1 CFU/100 mL. In contrast, total heterotrophs that may comprise bacteria with different levels of endurance, such as endospore producers, presented lower reductions, with about ~10^2 CFU/100 mL being detected after the advanced treatment (Fig. 7a). These results were confirmed in the assays testing the regrowth capacity, with significantly higher counts of total heterotrophic populations and fungi than of the other microbial groups analyzed. Regrowth may have resulted from the combination of treatment survival and capacity to use any available carbon and energy sources. Indeed, the occurrence of microbial regrowth after water treatment has been attributed to the presence of assimilable organic carbon, generated during the oxidation of recalcitrant organic matter (Thayanukul et al., 2013; Zhao et al., 2014). The comparison of the regrowth capacity in the presence of light or in the dark, suggested that light may have facilitated the recovery of cell injuries, mainly in bacterial heterotrophs and fungi (Fig. 7a), as previously described (Hijnen et al., 2006).

Since most of the microorganisms in water are not cultivable, and advanced water treatment may enhance the fraction of non-cultivable populations, the use of culture-independent methods was necessary in this study. In spite of their stress status, some bacteria maintain viability, being able to regrow when the disinfection stress is relieved. From some of these bacterial cells, DNA extraction may be not possible immediately after surface water/wastewater treatment, therefore the measurement of the same target genes after three days of incubation was a way to assess the abundance of bacteria fitted to survive after disinfection. Of the analyzed genes, 16S rRNA, *intI1*, *blaTEM*, *qnrS* and *sul1* were above the LOQ in the secondarily treated wastewater samples analyzed. The gene *vanA* was below the LOD (Fig. 7c). Photocatalytic ozonation led to a significant reduction of total prokaryotic cells (assessed based on the abundance of the 16S rRNA gene) and of the gene *intI1*, associated with mobile gene cassettes, also with widespread distribution in different bacterial groups. In addition, also the antibiotic resistance genes *blaTEM*, *qnrS* and *sul1* were significantly reduced after the photocatalytic ozonation. These genes, although highly prevalent in human impacted environments were originally at lower abundance than 16S rRNA and *intI1* genes, and were reduced to levels below or close the LOQ (~10 gene copies per mL) after photocatalytic ozonation. For the genes 16S rRNA and *intI1*, the reduction observed after treatment was apparently a transient effect. After storage in the dark or presence of light, the gene copy numbers per 100 mL of 16S rRNA and *intI1* genes were about 1 logarithmic cycle below the initial value (Fig. 7c). The other analyzed genes did not yield a significant increase in their relative abundance during storage (Fig. 7c). These results were also useful to measure the effectiveness of the treatment process, with the regrowth capacity representing a good indication of the fraction of live cells remaining immediately after treatment.

Regarding surface water, the photocatalytic ozonation treatment produced similar results to those obtained for the secondarily treated wastewater. Except for total heterotrophs, the photocatalytic ozonation treatment led to the removal of the analyzed microbial groups (Fig. 7b) to values close to or below the LOD (10^{-2} CFU/100 mL). However, regrowth was observed for fungi and was particularly enhanced for total heterotrophs. In addition, in this case, the analysis of specific potential antibiotic resistant populations suggested that bacteria with acquired antibiotic resistance traits may have the capacity to regrow in photocatalytic ozonated water up to values found in non-treated surface water (Fig. 8). Similar conclusions were retrieved in a study where the effect of chlorination and H_2O_2/sunlight processes on the regrowth of a multidrug *Escherichia coli* strain was investigated (Fiorentino et al., 2015).

In surface water the genes *qnrS*, *vanA* and *sul1* were below the LOQ, while *blaTEM* presented 10^4 gene copy number per 100 mL (Fig. 7d). Photocatalytic ozonation treatment led to reductions of around 3 logarithmic cycles of the 16S rRNA and *intI1* genes, and the gene *blaTEM* was reduced to levels close to the LOQ. As for wastewater, for 16S rRNA and *intI1* genes, the inactivation effect was apparently transient (Fig. 7d), corroborating the cultivable-dependent data. Although these findings may suggest a potential increase of microbiological risks, it would be necessary to study the diversity of the populations and/or the prevalence of resistance genes among them to have a better estimation of potential risks.

3.4. Biological effects

YES assay results revealed that no agonistic estrogenic activity was found for samples collected before and after photocatalytic ozonation treatment of the WWTP effluent, along with no growth inhibition. The IR values determined for both samples were between 0.804 and 1.074, which is below IR10 (1.487 for E2). These observations indicate that the proposed treatment strategy did not originate compounds with estrogenic effects. Furthermore, cytotoxicity and cellular viability assays using cell models for digestive epithelium (Caco-2), blood–brain barrier (hCMEC/D3), and skin (L929) showed no significant difference concerning cell viability (Table 3, LDH assay). MTT assay is a suitable tool for assessment of cytotoxic effects from wastewater, surface water and drinking water samples as reported before (Trintinaglia et al., 2015; Žegura et al., 2009). Slight cytotoxicity was observed for Caco-2 and hCMEC/D3 cell lines after treatment, but not for L929 fibroblasts (Table 3, MTT assay). The first two cell lines are human-derived, while L929 fibroblasts were originated in mouse, accounting for their different susceptibility. Furthermore, as reported before (Trintinaglia et al., 2015), the exposure to water testing sample mixed with culture media can originate different results, depending upon the final growth media composition.

Regarding the surface water, no agonistic estrogenic activity was found for these samples when using the YES assay, along with no growth inhibition. The IR values determined for both samples were between 0.681 and 0.962, which is also below IR10 (1.487 for E2). These observations corroborate that the proposed treatment strategy did not originate compounds with estrogenic effects. Similar results were obtained when applying recombinant yeasts expressing estrogen receptor alpha to evaluate the estrogenic activity of wastewater, bottled waters and tap water in Finnish samples (Omoruyi and Pohjanvirta, 2015). For LDH assay, cellular viability was maintained after treatment for both Caco-2 and hCMEC/D3 cells, providing values similar to those obtained for culture media.

4. Conclusions

Among different oxidative treatments tested in continuous mode, photocatalytic ozonation using TiO_2-coated glass Raschig rings with LEDs irradiation was the most efficient for the removal of PSs and CECs from urban wastewater. In these experiments, fluoxetine, clarithromycin, erythromycin and EE2 were more refractory to oxidation than the other compounds. The same process completely removed all the micropollutants detected in surface water (carbamazepine, isoproturon, clarithromycin, norfluoxetine, fluoxetine, E2 and EE2). In addition, the efficiency to remove different groups of cultivable microorganisms and housekeeping (16S rRNA) and antibiotic resistance or related genes (*intI1*, *blaTEM*, *qnrS*, *sul1*) was studied, with photocatalytic ozonation showing high efficiency on the removal of microbial loads, including ARG, from both urban wastewater and surface water. The tested ARGs were successfully removed to levels below/close to the threshold of quantification. Moreover, after the 3-days storage of treated surface water/wastewater at room temperature, there was no evidence of regrowth of the host, as inferred from the absence of an increase in the relative abundance of the respective ARG. However, both culture-dependent and -independent methods demonstrated that part of the surface water/wastewater microbiota was viable, able to regrow, and the potential risk of this regrowth is still to be assessed. From YES, MTT and LDH assays it follows that compounds with estrogenic effects were not formed and that differences concerning cell viability were not statistically significant for both urban wastewater and surface water. Therefore, photocatalytic ozonation proved to be a potential solution for simultaneous removal of organic micropollutants, different microbial groups of potential human pathogens, ARB and ARG present in wastewater or surface water. However, more studies are still required as well as the evaluation of the process associated costs.

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Appendix C

Solar treatment (H_2O_2, TiO_2-P25 and GO-TiO_2 photocatalysis, photo-Fenton) of organic micropollutants, human pathogen indicators, antibiotic resistant bacteria and related genes in urban wastewater

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Solar treatment (H_2O_2, TiO_2-P25 and GO-TiO_2 photocatalysis, photo-Fenton) of organic micropollutants, human pathogen indicators, antibiotic resistant bacteria and related genes in urban wastewater

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ABSTRACT

Solar-driven advanced oxidation processes were studied in a pilot-scale photoreactor, as tertiary treatments of effluents from an urban wastewater treatment plant. Solar-H_2O_2, heterogeneous photocatalysis (with and/or without the addition of H_2O_2 and employing three different photocatalysts) and the photo-Fenton process were investigated. Chemical (sulfamethoxazole, carbamazepine, and diclofenac) and biological contaminants (faecal contamination indicators, their antibiotic resistant counterparts, 16S rRNA and antibiotic resistance genes), as well as the whole bacterial community, were characterized.

Heterogeneous photocatalysis using TiO_2-P25 and assisted with H_2O_2 (P25/H_2O_2) was the most efficient process on the degradation of the chemical organic micropollutants, attaining levels below the limits of quantification in less than 4 h of treatment (corresponding to $Q_{UV} < 40$ kJ L^{-1}). This performance was followed by the same process without H_2O_2, using TiO_2-P25 or a composite material based on graphene oxide and TiO_2.

Regarding the biological indicators, total faecal coliforms and enterococci and their antibiotic resistant (tetracycline and ciprofloxacin) counterparts were reduced to values close, or beneath, the detection limit (1 CFU 100 mL^{-1}) for all treatments employing H_2O_2, even upon storage of the treated wastewater for 3-days. Moreover, P25/H_2O_2 and solar-H_2O_2 were the most efficient processes in the reduction of the abundance (gene copy number per volume of wastewater) of the analysed genes. However, this reduction was transient for 16S rRNA, *intI1* and *sul1* genes, since after 3-days storage of the treated wastewater their abundance increased to values close to pre-treatment levels. Similar behaviour was observed for the genes *qnrS* (using TiO_2-P25), $bla_{CTX\text{-}M}$ and bla_{TEM} (using TiO_2-P25 and TiO_2-P25/H_2O_2). Interestingly, higher proportions of sequence reads affiliated to the phylum *Proteobacteria* (*Beta-* and *Gammaproteobacteria*) were found after 3-days storage of treated wastewater than before its treatment. Members of the genera *Pseudomonas*, *Rheinheimera* and *Methylotenera* were among those with overgrowth.

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1. Introduction

Conventional urban wastewater treatment plants (UWWTPs) are not specifically designed for the removal of organic micropollutants, and in many cases neither for effective disinfection (removals of bacterial loads up to 2 log cycles) which considers the inactivation of bacteria that can contribute to the spread of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARG) into the environment. These contaminants can reach the natural waters, such as surface and ground waters that are serving as drinking water sources (Fatta-Kassinos et al., 2011; Manaia et al., 2016). Moreover, the continuous disposal of antibiotics and related products into the environment can lead to the development and proliferation of ARB, decreasing the efficiency of these antibiotics when supplied to human and animals (Ferro et al., 2016; Rizzo et al., 2013). Since high bacterial density can be found in effluents of UWWTPs (i.e., bacterial cells are close to each other), horizontal gene transfer and selection of ARB can be considered important mechanisms for ARG enrichment (Davison, 1999). The dissemination of these contaminants urges the development of new technologies able to improve the simultaneous removal of organic micropollutants and microorganisms of concern.

Advanced oxidation processes (AOPs) are conceptually based on the generation of the highly reactive hydroxyl radicals ($HO^{\bullet}$), but other reactive species can also be formed. AOPs, such as UVC/H_2O_2 and ozonation, have been widely studied for the removal of hazardous organic chemical compounds and disinfection, and they have been applied as viable solutions to enhance the quality of secondary effluents before discharged or reused (Ribeiro et al., 2015). However, several drawbacks have been associated to the aforementioned processes. For instance, ozonation-based treatments are known to produce bromate (from bromide), which is classified as a "possible human" carcinogen (Xiao et al., 2017), whereas the processes dependent on high/medium vapour pressure lamps (such as $UV_{254\ nm}$) have higher operating costs and they are less environmentally friendly than those implementing solar radiation. Furthermore, in a recent study (Becerra-Castro et al., 2016), we have shown that oxidation processes (such as $UV_{254\ nm}$, ozonation and photocatalytic ozonation) might have the potential to act selectively over some bacterial groups. In that bench-scale study, irrespective of the type of treatment used, it was observed a significant modification of the residual bacterial community after 3-days storage, which was characterized by higher proportions of *Proteobacteria* (*Gamma-* and *Betaproteobacteria*) than those observed in non-treated wastewater. This is an example of bacterial community disturbance induced by a disinfection treatment, which eventually may affect negatively the biological quality of the final stream if the reduction of the bacterial diversity and disequilibrium of the microbial ecosystem favour the possible selection of ubiquitous bacteria associated with acquirement and spread of virulence or ARGs (Becerra-Castro et al., 2016).

Among the different AOPs, those driven by natural sunlight have raised a great interest during last decades (i.e. natural solar radiation as source of photons), reducing the need to costly lamps or maintenance of systems. Photocatalysis with irradiated titanium dioxide (TiO_2) and the photo-Fenton process have demonstrated to be powerful wastewater treatment options. A number of experimental studies are available in literature recognizing the high potential of these solar techniques for water decontamination and disinfection of a wide range of water sources (Malato et al., 2009; Moreira et al., 2016; Pablos et al., 2013; Polo-López et al., 2014; Sousa et al., 2017; Yang et al., 2014). Especially interest for water disinfection has attracted the solar photo-assisted treatment with H_2O_2, which has achieved fast inactivation of several types of microorganisms in water (García-Fernández et al., 2012; Polo-López et al., 2011b; Sichel et al., 2009). In addition, the development of solar Compound Parabolic Collector (CPC) reactors has resulted in the development of technological solutions with increased efficiency for water treatment (Giannakis et al., 2016; Malato et al., 2009). However, the performance of these solar-based processes has not been investigated in terms of changes of the bacterial community composition and simultaneous removal of organic contaminants and ARB&ARG.

Thus, the present study aims at comparing the efficiency of different solar-driven AOPs on the simultaneous removal of micropollutants and disinfection of a secondary effluent of an UWWTP. Solar-H_2O_2, heterogeneous photocatalysis (with and/or without the addition of H_2O_2) and the photo-Fenton process were tested using a pilot-plant CPC solar photoreactor. For the first time, the performance of each process was assessed based on the removal efficiency of organic contaminants and ARB&ARG, as well as on the changes of the bacterial community composition. Microbial characterization was performed before treatment, after 5 h of treatment and after 3-days storage of treated wastewater at room temperature. Thus, the simultaneous removal of chemical and biological contaminants by using solar-driven AOPs at pilot-scale and considering possible changes in the bacterial community, which can affect the water quality, is the main novelty of the present work.

2. Materials and methods

2.1. Chemicals and materials

Degussa (Aeroxide) TiO_2-P25 catalyst from Evonik Corporation (hereafter referred to as P25) and a composite consisting of TiO_2 and 4.0 wt% of graphene oxide (GO-TiO_2) – respective preparation method and characterization described elsewhere (Pastrana-Martínez et al., 2012) – were used to conduct heterogeneous photocatalytic experiments. For comparative purposes, bare TiO_2 was also prepared following the same method used for GO-TiO_2, but without the addition of GO (hereafter referred to as TiO_2). The H_2O_2 30% (w/v) solution, analytical grade sulphuric acid (H_2SO_4, 98%), bovine liver catalase and ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) were obtained from Riedel-de Haën (Germany), Merk (Germany), Sigma-Aldrich (USA) and PANREAC (Spain), respectively. Carbamazepine (CBZ), sulfamethoxazole (SMX) and diclofenac (DFC) were all high-purity grade (>99%), and purchased from Sigma-Aldrich (Spain). Stock solutions were prepared by dissolving the individual compounds ($2.5\ g\ L^{-1}$) in methanol due to water solubility limitations. Required volumes of the stock solutions were directly added to the urban wastewater samples into the CPC pilot reactor to obtain the initial concentration of $100\ \mu g\ L^{-1}$ of each organic micropollutant. Methanol (J.T.Baker) and acetonitrile (Sigma-Aldrich) were HPLC-grade. Ultrapure water was supplied by a Milli-Q water system.

2.2. Municipal wastewater treatment plant samples

All solar-driven treatments were carried out using urban wastewater samples collected (every day in batches of 60 L) after the secondary treatment from the UWWTP of El Bobar (Almería, Spain), and stored at 4 °C no more than 2 h before solar experiments. The UWWTP uses conventional activated sludge plus decantation as secondary treatment. In 2015 (year of the sampling campaign), it produced an average of secondary effluent daily flow of ca. 33,000 m^3, with a capacity of 100,000 inhabitant-equivalent. The main physicochemical characteristics of the effluent, including turbidity, pH, conductivity, dissolved inorganic carbon (DIC), dissolved organic carbon (DOC) and inorganic ions concentration, are listed in Table S1 (Supplementary Information).

2.3. Pilot scale CPC photoreactor and solar experiments

All the solar-driven oxidation processes were performed in a pilot-scale CPC photoreactor, at Plataforma Solar de Almeria (PSA), Spain (37°84′N and 2°34′W), on sunny days between June and August 2015, with a duration of 5 h. The configuration of the CPC photoreactor is described elsewhere (Rodríguez-Chueca et al., 2014). The CPC photoreactor tube module, tilted at an angle of 37° relative to the horizontal plane, is connected to a recirculation tank and a centrifugal pump. The water flow rate was set at 10 L min^{-1}. The total volume of the photoreactor was 20 L, while the illuminated volume and the irradiated collector surface area were 15 L and 1 m^2, respectively. Target chemical and biological contaminants were simultaneously monitored in the same experiment.

In heterogeneous photocatalysis (P25, TiO_2 and GO-TiO_2) a catalyst load of 200 mg L^{-1} was used. In photo-Fenton (Fe^{2+}/H_2O_2), $FeSO_4 \cdot 7H_2O$ was used as source of 10 mg L^{-1} of Fe^{2+}. In H_2O_2 assisted processes (H_2O_2, Fe^{2+}/H_2O_2, P25/H_2O_2 and GO-TiO_2/H_2O_2), the initial concentration of H_2O_2 was 20 mg L^{-1} reached by adding the H_2O_2 30% (w/v) solution. Different amounts of H_2O_2 added to the system may have different effect on the removal efficiencies (Cai and Hu, 2017; Polo-López et al., 2014). The catalyst and H_2O_2 concentrations were selected considering the optimization done in our previous studies with solar-driven oxidation processes (Fernández-Ibáñez et al. 1999; Polo-López et al., 2014). Immediately after the collection of a sample, residual H_2O_2 was eliminated by adding a freshly prepared solution of bovine liver catalase (0.1 g L^{-1}) in a ratio 0.1/5.0 (v/v). Photolysis (Blank) assays were performed to study the effect of solar radiation without the addition of any catalyst or reactant.

Besides the experimental time, the accumulated solar UVA energy received in the solar reactor per unit of treated water volume ($Q_{UV,\ kJ\ L^{-1}}$) was considered for comparison of the treatment efficiencies (Malato et al., 2009) and calculated using Eq. (1):

$$Q_{UV,n} = Q_{UV,n-1} + \frac{\Delta t_n \overline{UV}_{G,n} A_r}{V_t};\ \Delta t_n = t_n - t_{n-1} \qquad (1)$$

where $UV_{G,n}$ is the global UV irradiance (W m^{-2}) averaged during exposure time; t_n the exposure time (s); V_t the total reactor volume (L); and A_r the illuminated reactor surface (m^2).

A global UVA (300–400 nm, Model CUV4, Kipp & Zonen, Netherlands) pyranometer tilted 37° was used to measure the solar radiant UVA rate incident (W m^{-2}) as described elsewhere (Rodríguez-Chueca et al., 2014). The average value of solar UVA irradiance was 40 W m^{-2}.

Before treatment, the CPC photoreactor was covered by an opaque sheet while the wastewater and reagents were added to the reactor and recirculated during 15 min to guarantee homogenisation. Samples were collected each hour over the treatment. During this period, temperature (Checktemp, Hanna instruments, Spain) and pH (multi 720, WTW, Germany) were measured. The temperature of the wastewater varied from 16.6 ± 1.5 °C to 43.2 ± 4.2 °C.

2.4. Chemical analysis

High performance liquid chromatography (HPLC) was used to analyze the evolution of the target organic micropollutants using an apparatus from Agilent Technologies (series 1260, Palo Alto, CA, USA), equipped with a diode array detector (UV-DAD) and a C-18 column. The mobile phase consisted of 90% formic acid aqueous solution at 25 mM and 10% acetonitrile. A linear gradient was used from 10% to 85% of acetonitrile during 13 min at a flow rate of 1 mL min^{-1}. The injection volume was set at 100 μL. Before injection, samples were filtered through a 0.2 μm syringe-driven filter, and afterwards the filter was washed with 1 mL of acetonitrile to remove the adsorbed compounds. CBZ, SMX and DFC were selected as model organic micropollutants since they have been frequently found in aquatic environments, including wastewater, surface and groundwater and even in drinking water (Barbosa et al., 2016). The detection wavelength values were 267 nm for both CBZ and SMX, and 273 nm for DFC. The limits of quantification (LOQ) were 4.7, 6.2 and 4.1 μg L^{-1} for CBZ, SMX and DFC, respectively.

DX-600 and DX-120 (Dionex Corporation, Sunnyvale, CA) equipments were used for quantification of anions and cations, respectively. DOC and DIC measurements were performed using a 5050 A TOC (Shimadzu Corporation, Kyoto, Japan) analyzer, after sample filtration using 0.2 μm syringe filters. Turbidity was determined with a turbidimeter (Model 2100N, Hach, USA). The H_2O_2 concentration was determined by a spectrometric method, as described elsewhere (Polo-López et al., 2011a), and the Fe^{2+} concentration by the ISO 6332 method. Natural iron was not detected in any of the urban wastewater samples by using this method, i.e. a spectrophotometric technique with phenanthroline/acetic acid (UV–Vis measurements, limit of detection 0.05 mg L^{-1}).

2.5. Microbiological cultivation, DNA extraction, qPCR and bacterial community analysis

To assess the disinfection efficiency, cultivable faecal indicator bacteria, targeted ARB and selected ARGs were quantified before and immediately after the 5 h-treatment, and after 3-days of storage of treated wastewater at room temperature. The abundance of faecal coliforms, enterococci and their tetracycline and ciprofloxacin resistant counterparts was assessed by using the membrane filtration method. Faecal coliforms and enterococci are used worldwide as indicators of faecal contamination, in particular to assess the microbiological water quality (APHA, 2005; ISO9308-1, 2000). In addition, the ubiquity of these bacteria in human impacted environments and their high persistence in the environment, as well as genome plasticity, make of faecal coliforms and enterococci important tracers to assess the antibiotic resistance status of environmental samples. Furthermore, in these groups high resistance prevalence has been observed for antibiotics such as tetracycline and ciprofloxacin in aquatic environments, including wastewater (Vaz-Moreira et al., 2014). After serial 10-fold dilutions in sterile saline solution (0.85% NaCl), 100 mL of each dilution was filtered through cellulose membrane filters (0.22 μm porosity; Whatman, UK). The filtering membranes were incubated on selective media for each target bacterial groups - membrane Faecal Coliforms agar (m-FC) (Difco, 30 °C, 24 h) for faecal coliforms, and Slanetz & Bartley agar (S&B) (Difco, 30 °C, 48 h) for enterococci. In addition, the prevalence of ARB was assessed in the same media supplemented with tetracycline (Fluka, 16 mg L^{-1}) and ciprofloxacin (Applichem, 1 mg L^{-1}). The antibiotic stock solutions were sterilized by filtration (0.2 μm syringe driven-filters).

The use of adequate indicators of antibiotic resistance in both culturable and non-culturable bacteria has been recommended (Berendonk et al., 2015). For culture-independent assays, a volume of 250 mL of each sample (before and after the 5 h treatment, and after 3-days of storage) was filtered through polycarbonate membranes (0.22 μm porosity; Whatman, UK). DNA was extracted using the commercial kit PowerWater® DNA Isolation (MO BIO Laboratories, Inc., USA) and stored at −20 °C. These extracts were used for the quantification of the house keeping gene 16S rRNA, *intI1* (related with ARG mobilization) and a set of ARGs encoding resistance to different classes of antibiotics frequently detected in wastewater (Vikesland et al., 2017) by quantitative PCR (qPCR) of samples collected before treatment, immediately after treatment, and after 3-days of storage. In addition, the same extracts were also used for the bacterial community analysis, in this case except for the samples collected immediately after treatment, due to DNA scarcity. The qPCR (StepOne™ Real-Time PCR System; Life Technologies, Carlsbad, CA) assays were performed according to the conditions shown in Table S2 - Supplementary Information (Bibbal et al., 2007; Denman and McSweeney, 2006; Goldstein et al., 2001; Marti and Balcázar, 2013; Marti et al., 2014; Pei et al., 2006; Volkmann et al., 2004), and as described elsewhere (Narciso-da-Rocha et al., 2014).

The bacterial community composition was analysed based on the hypervariable V3/V4 region (forward primer Bakt_341F 5′-CCTACGGGNGGCWGCAG-3′ and reverse Bakt_805R 5′-GACTACHVGGGTATCTAATCC-3′) of 16S rRNA gene Illumina sequencing (Genoinseq, Cantanhede, Portugal). Nucleotide sequencing data were processed and analysed using the QIIME pipeline (Caporaso et al., 2010). Briefly, sequences shorter than 250 bp and with average quality scores lower than 25 were eliminated, and bases with average quality lower than 25 in a window of 5 bases, were trimmed using the software PRINSEQ (Schmieder and Edwards, 2011). Chimeric sequences were identified and removed using USEARCH v6.1 (Edgar, 2010). Free-chimeric sequences were further grouped into operational taxonomic units (OTUs) using USEARCH v6.1 (Edgar, 2010) with a phylotype threshold of ≥97% sequence similarity and were taxonomically assigned using QIIME default values. The sequences comprising each OTU were aligned using PyNAST (Caporaso et al., 2010) and were taxonomically classified using Greengenes Database version 13_8 (updated: August 2013) (DeSantis et al. 2006). As a variable number of sequences was obtained between samples, the alpha diversity indices Shannon, phylogenetic diversity (PD) whole tree, and Simpson were calculated after rarefying to 54,771 sequences *per* sample (value of the smallest dataset) (Faith, 1992; Shannon and Weaver, 1963; Simpson, 1949). An alpha diversity index represents the diversity within a particular community and it is affected by its size, in particular by the number of different species and the abundance of each one (Whittaker, 1972). The cumulative sum scaling (CSS) normalization procedure was applied to the sequence data to assess the beta diversity patterns (Paulson et al., 2013). The weighted UniFrac metric distances (Lozupone and Knight, 2005) were calculated in the QIIME pipeline and the results shown as Principal Coordinates Analysis (PCoA) biplots that include the position of the ten most prevalent bacterial classes. Correlations between the relative abundance of populations at different taxonomic levels were analysed using the statistics software STAMP v2.1.3 (Parks et al., 2014).

3. Results and discussion

3.1. Degradation of organic micropollutants

The evolution of the concentrations of targeted organic micropollutants (i.e., CBZ, SMX and DFC) spiked in urban wastewater under solar-driven oxidation treatments are shown in Fig. 1. Control experiments were performed under the same conditions but without the addition of any catalyst or H_2O_2 (i.e., Blank = photolysis). CBZ and SMX were very resistant upon irradiation in the absence of a catalyst or H_2O_2 (only 20 ± 6% and 17 ± 4% of removal, respectively). In contrast, DFC was efficiently removed by photolysis after 4 h. This result was expected since the DFC absorption spectrum (not shown) has a tail entering well above the 300 nm (Moreira et al., 2015).

The heterogeneous photocatalysts (P25, TiO_2 and GO-TiO_2), without the addition of H_2O_2, converted the targeted organic micropollutants in the following order of decreasing efficiency: P25 > GO-TiO_2 > TiO_2 (Fig. 1). The high photocatalytic efficiency of GO-TiO_2 on the degradation of different types of organic micropollutants in synthetic matrices has been already demonstrated in our previous studies, namely for: diuron, alachlor, isoproturon and atrazine – pesticides classified by the EU as priority pollutants

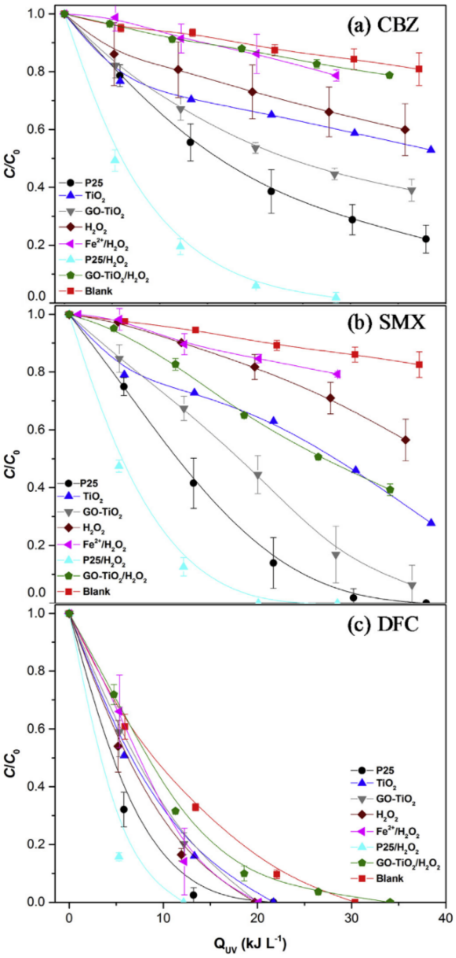

Fig. 1. Normalized concentration (C/C_0) of (a) CBZ, (b) SMX and (c) DFC spiked in urban wastewater as function of accumulated energy (Q_{UV}) using different solar-driven treatments. Except for TiO_2, values are the average of four (P25), three (GO-TiO_2, H_2O_2, Fe^{2+}/H_2O_2) and two (P25/H_2O_2, GO-TiO_2/H_2O_2, Blank) independent assays. Error bars represent standard deviations.

(Cruz et al., 2017); microcystin-LA – cyanotoxin (Sampaio et al., 2015); bisphenol A – xenoestrogen (Maroga Mboula et al., 2013); diphenhydramine – antihistamine pharmaceutical, and methyl orange – azo dye (Pastrana-Martínez et al., 2012). The GO-TiO_2 photocatalyst has been much more active than P25 under Vis-light illumination, but under near UV–Vis radiation the activity of this type of photocatalysts depends on the target pollutant. The better performance of GO-TiO_2 in comparison to bare TiO_2 has been attributed to the good assembly and interfacial coupling between TiO_2 and GO sheets as well as the respective quenching of photoluminescence (inhibiting charge recombination) (Pastrana-Martínez et al., 2012). In the present study, P25 was the most efficient photocatalyst for the degradation of the organic micropollutants, most probably, because of the better performance of P25 under UV radiation.

The addition of H_2O_2 to the P25 photocatalytic system, increased the degradation efficiency of the targeted micropollutants. H_2O_2 captures and reacts with the photoinduced surface electrons (supressing the electron/hole recombination) and it also reacts with the superoxide radical anions, both pathways leading to the formation of additional hydroxyl radicals (Kositzi et al., 2004; Pablos et al., 2013). There is markedly a competing process, which may be mediated by the active surface of the photocatalyst. Interestingly, the removal efficiency decreased when H_2O_2 was added to the photocatalytic system containing GO-TiO_2. Degradation of GO-TiO_2 may eventually occur, for instance by the H_2O_2 attack to the underlying C-C bonds in the superficial defect sites of GO (Xing et al., 2014).

Solar-H_2O_2 and photo-Fenton processes (Fe^{2+}/H_2O_2) also led to very modest removals of CBZ and SMX (Fig. 1a and b, respectively). One of the downsides of photo-Fenton is the formation of iron sludge due to the precipitation of iron hydroxide at neutral pH. In this work, the pH was adjusted to a circumneutral value (5.5) by using H_2SO_4, which could explain the low photo-Fenton efficiency that is known to be maximized at pH values around 3 (Agulló-Barceló et al., 2013; García-Fernández et al., 2012; Giannakis et al., 2017). In a previous work (Klamerth et al., 2010), solar photo-Fenton (also operated at mild pH conditions) was effective for the degradation of organic micropollutants, but the total inorganic carbon was removed by sample acidification since it competes with the organic contaminants for hydroxyl radicals (Klamerth et al., 2009).

Overall, P25/H_2O_2 followed by the P25 and GO-TiO_2 photocatalytic processes were the best performing treatments for removal of the targeted organic micropollutants. Regarding the mineralization of the organic matter present in the urban wastewater, P25/H_2O_2, solar-H_2O_2, and the photo-Fenton process were the most efficient treatments (DOC removals always around 23 ± 3%), other processes removing no more than ca. 10% of the initial DOC. However, due to the chemical complexity of this matrix, and the differences between the DOC (in the range of mg L^{-1}) and the concentration of the studied organic pollutants (in the range of μg L^{-1}), it is not possible to correlate the DOC and micropollutants removals.

3.2. Bacteria inactivation and reactivation

The performance of different solar-driven processes was assessed before and over the treatment, in terms of removal of total faecal coliforms (Fig. 2a), enterococci (Fig. 2c) and respective fraction of resistant populations (Fig. 2b and d). The reduction of the bacterial indicators loads was observed in all the treatments, with the highest inactivation rates leading to values bellow or close the LOD (1 CFU 100 mL^{-1}), for the processes where H_2O_2 was used (H_2O_2, Fe^{2+}/H_2O_2, P25/H_2O_2, GO-TiO_2/H_2O_2). Among these, and despite the iron precipitation, the photo-Fenton process was the most efficient treatment on the reduction of resistant and non-resistant faecal coliforms and enterococci (Fig. 2a–d, Fe^{2+}/H_2O_2). However, photo-Fenton showed similar disinfection profiles to solar-H_2O_2 for faecal coliforms (Fig. 2a and b) and to P25/H_2O_2 for enterococci (Fig. 2c and d). These three processes removed those bacteria for $Q_{UV} < 30$ kJ L^{-1}, suggesting that H_2O_2 plays a major role on disinfection, including in the case of solar-H_2O_2 and photo-Fenton that were not effective for the removal of all the organic micropollutants. In contrast, moderate inactivation rates were observed for photolysis (Fig. 2a, b and c, Blank), except for antibiotic resistant enterococci that were reduced to values below the LOD (Fig. 2d, Blank). Interestingly, P25/H_2O_2 showed high efficiency on the removal of organic micropollutants and resistant and non-resistant enterococci, whereas the efficiency for inactivation of resistant and non-resistant faecal coliforms was not so high in comparison with solar-H_2O_2.

The accepted hypotheses and mechanism that explain the inactivation of microorganisms by exposure to solar-H_2O_2 is based on the accumulated damages inside cells by internal cellular injures occurring under sunlight and accelerated in the presence of H_2O_2. It is well accepted that solar radiation produces internal damages affecting different intracellular vital components leading to bacterial death or lack of viability (Aguas et al., 2017). A recent study attributed bacterial inactivation during solar photolysis to the combined effect of intracellular production of reactive oxygen species by UV photons absorption and water temperature increase (Castro-Alférez et al., 2017). When H_2O_2 is added, it may diffuse inside bacteria cells promoting additional internal photo-reactions with naturally present iron and other metals via Fenton and Fenton–like reactions, activating, thus, a photo-Fenton cycle under sunlight at intracellular level also (Aguas et al., 2017). Both photo-effects act jointly producing an accelerated disinfection that has been also reported to be very efficient for other types of bacteria, viral indicators, and fungi including, *Escherichia coli* and *Enterococcus faecalis* (Rodríguez-Chueca et al., 2014), *Legionella jordanis* (Polo-López et al., 2017), F-specific RNA bacteriophage (Agulló-Barceló et al., 2013), *Fusarium* sp. (Polo-López et al., 2014; Sichel et al., 2009), *Phytophthora capsici* (Polo-López et al., 2013), *Curvularia* sp. (Aguas et al., 2017), and several antimicrobial resistant bacteria (Fiorentino et al., 2015).

Although H_2O_2 assisted processes performed better that non-assisted ones, heterogeneous photocatalysis without H_2O_2 also performed quite well on the inactivation of total or resistant populations of faecal coliforms and enterococci. Among these processes, the GO-TiO_2 composite was the most efficient catalyst for the removal of total and resistant populations of faecal coliforms (Fig. 2a and b, GO-TiO_2). The good efficiency of photocatalytic disinfection using this type of composites (but prepared by other methods) and under visible radiation only was already shown in previous studies (Cruz-Ortiz et al., 2017; Fernández-Ibáñez et al., 2015). Its high photocatalytic activity has been attributed to the improvement in charge separation since GO may promote the electron transfer with TiO_2 particles, acting as an electron bridge, and to the decrease of the bandgap energy of the composite catalysts as well as to an enhancement of the adsorptive properties. These authors concluded that hydrogen peroxide, hydroxyl radicals, and singlet oxygen were the main species involved in the disinfection process under UV–Vis irradiation and only singlet oxygen under visible illumination (Cruz-Ortiz et al., 2017). Moreover, very recently (Karaolia et al., 2018), the photocatalytic efficiency of P25 (in the absence of H_2O_2) was compared to that of a TiO_2-reduced GO composite on the removal of three organic micropollutants (including SMX) and *E. coli* resistant and non-resistant to antibiotics in urban wastewater. P25 performed better

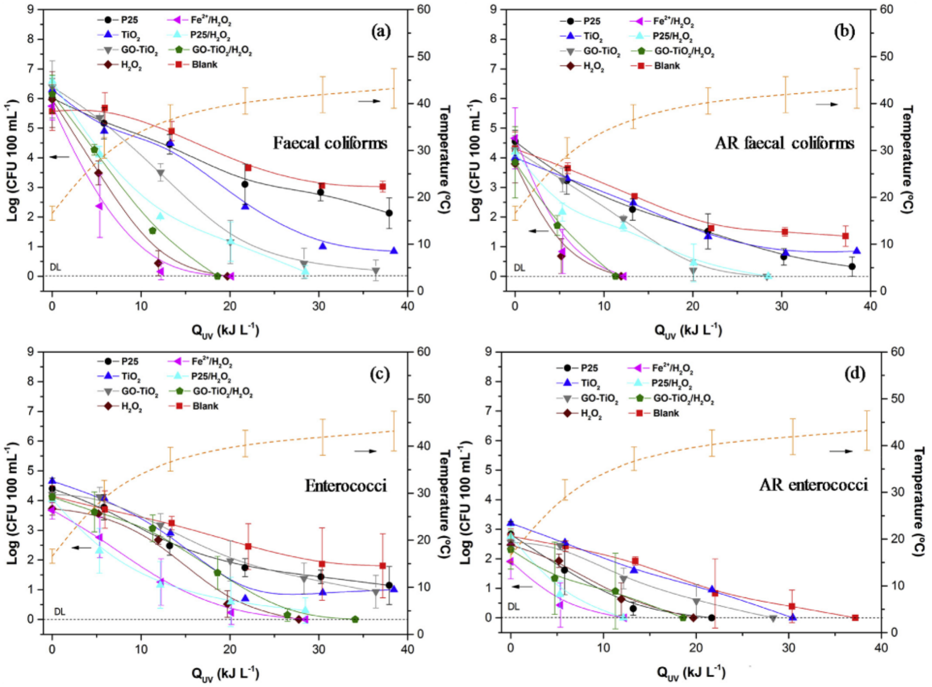

Fig. 2. Faecal coliforms (a) and enterococci (c) and their antibiotic resistant counterparts (b, d) inactivation in urban wastewater as function of accumulated energy (Q_{UV}) using different solar-driven treatments. Except for TiO_2, values are the average of four (P25), three (GO-TiO_2, H_2O_2, Fe^{2+}/H_2O_2) and two (P25/H_2O_2, GO-TiO_2/H_2O_2, Blank) independent assays. Error bars represent standard deviations. DL, detection limit.

than TiO_2-reduced GO for the photocatalytic degradation of SMX, but higher bacteria permanent inactivation was observed in the presence of the composite, in accordance with our results. However, in the same study, TiO_2-reduced GO performed better than P25 for the removal of erythromycin (i.e. the opposite of that observed for SMX), the photocatalytic efficiency thus depending on the target organic molecule. In our particular case, P25 was better than the GO-TiO_2 composite for the removal of all the studied organic micropollutants (CBZ, SMX and DFC).

Since microbial inactivation, monitored via culture-based methods, can be transient (Moreira et al., 2016; Spuhler et al., 2010; Zhao et al., 2014), further assays testing the regrowth capacity after 3-days storage of the treated wastewater at room temperature were performed. Bacterial reactivation is influenced by factors such as the storage conditions, temperature, availability of nutrients and the UV dose, among others (Giannakis et al., 2014; Ubomba-Jaswa et al., 2009). The bacterial loads before, after the treatment and after 3-days storage at room temperature are shown in Fig. 3a and b for faecal coliforms and Fig. 3c and d for enterococci. No regrowth was observed in stored wastewater treated by the H_2O_2-assisted processes, and total faecal coliforms and enterococci as well as their ARB counterparts were kept below the detection limit. In stored wastewater treated by heterogeneous photocatalysis without H_2O_2 (P25, TiO_2 and GO-TiO_2), the bacterial loads of these groups were 2–3 log values lower than before the treatment. Similar observations were registered for the control (Blank = photolysis). These results indicate the inability of these bacterial groups to recover to values close to those found in raw wastewater after the tested solar-driven processes. This can be also explained by the mode of action attributed to solar-H_2O_2 disinfection, where oxidative damages alter the bacteria viability at intracellular level, as proven in the literature (Polo-López et al., 2017). In such report, an EMA-qPCR method for the detection of membrane integrity damages of *L. jordanis* cells under two photo-oxidative processes was used, i.e. solar-H_2O_2 and P25 with solar radiation. It was confirmed the well-accepted mechanism of heterogeneous (P25) photocatalysis via oxidative attacks of the external cell membrane, whereas the mechanism for solar-H_2O_2 was based on internal photochemical reactions. This may explain the results reported in Fig. 3 on the non-recover capability of ARB when H_2O_2 was used in these treatment processes, while they can regrow when H_2O_2 is not in the media (Polo-López et al., 2017). In general, the selected solar-driven oxidation processes were effective in reducing faecal indicators, including those resistant to tetracycline and ciprofloxacin, from wastewater samples.

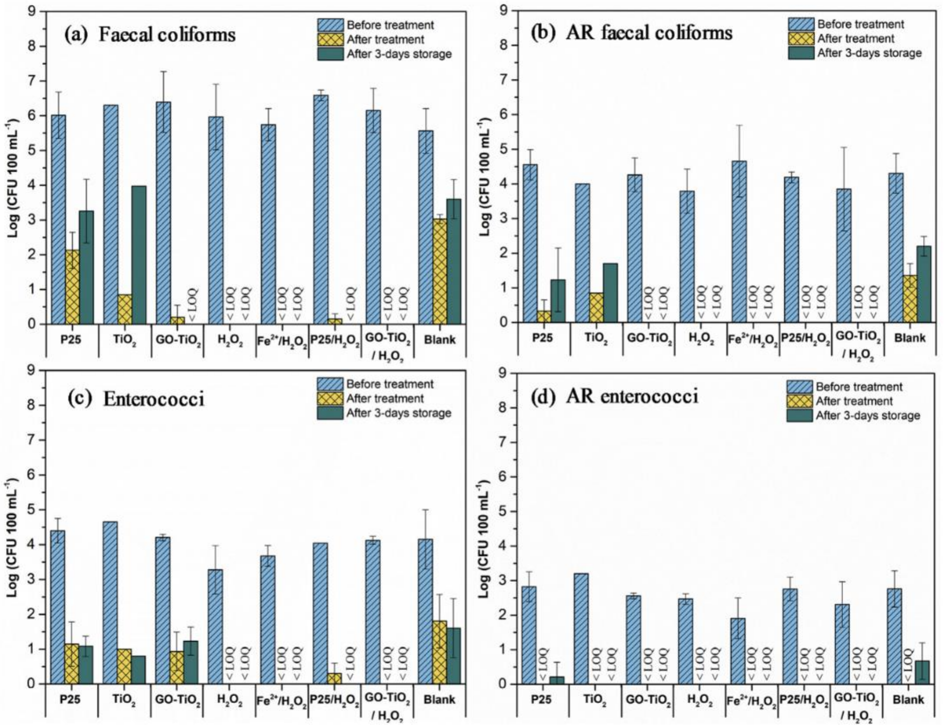

Fig. 3. (a) Faecal coliforms and (c) enterococci and their (b, d) antibiotic resistant counterparts counts before, after treatment and after 3-days storage using different solar-driven treatments. Except for TiO_2, values are the average of four (P25), three (GO-TiO_2, H_2O_2, Fe^{2+}/H_2O_2) and two (P25/H_2O_2, GO-TiO_2/H_2O_2, Blank) independent assays. Error bars represent standard deviations.

3.3. Effect of disinfection on ARGs and bacterial community composition

Considering that the bacterial community is much more diverse and complex than that assessed based on the cultivation methods used, and that some bacteria may be injured and hence, unable to grow, culture-independent methods were carried out to give additional insights on the disinfection effectiveness of the studied solar-driven processes. Since the main purpose of this work was the simultaneous removal of chemical and biological contaminants, the processes showing better performance on the degradation of organic micropollutants (i.e., P25/H_2O_2, P25 and GO-TiO_2 photocatalytic processes), disinfection (P25/H_2O_2 and solar-H_2O_2), and also the reference process (photolysis), were selected for further investigation based on culture-independent methods - Fe^{2+}/H_2O_2 was not chosen due to its bad performance for degradation of the organic micropollutants (Fig. 1).

Among the analysed genes (i.e., 16S rRNA, *intI1*, *qnrS*, *bla*$_{CTX-M}$, *sul1*, *bla*$_{TEM}$ and *vanA*), only *vanA* was below the LOD before treatment (not shown). For the other genes, P25/H_2O_2 photocatalysis and solar-H_2O_2 were the most efficient processes (i.e., lower abundance after treatment), both leading to log average reduction of 1 value, except for *bla*$_{CTX-M}$ (3 log reduction) (Fig. 4). However, after 3-days storage, regardless the treatment used, the abundance of 16S rRNA gene, a house keeping gene of prokaryotes, was close or even higher (up to 1 log for P25/H_2O_2) than those found before treatment, suggesting the ability of some bacteria to recover after the treatment. Similar results were observed for *intI1* and *sul1* genes, encoding integrase and conferring resistance to sulphonamides, respectively. Among the studied processes, only solar-H_2O_2 and GO-TiO_2 prevented the reactivation of *bla*$_{CTX-M}$ and *bla*$_{TEM}$ genes (encoding resistance to beta-lactams) above the pre-treatment levels. For the *qnrS* gene (a plasmid-mediated quinolone resistance gene), besides these two processes, also P25/H_2O_2 prevented its reactivation.

Other authors (Ferro et al., 2016, 2017) investigated the potential of UV/H_2O_2 process to control the potential spread of ARB into the environment. Despite of a successful inactivation of total coliforms, *E. coli* and antibiotic resistant counterparts, the genes *bla*$_{TEM}$, *qnrS* and *tetW* were still present in wastewater after 240 min of treatment, in accordance with the results of the present study.

Overall, the studied processes led to a slight reduction in the abundance of targeted genes immediately after treatment. Only *bla*$_{CTX-M}$, with a relatively low initial abundance, was reduced after

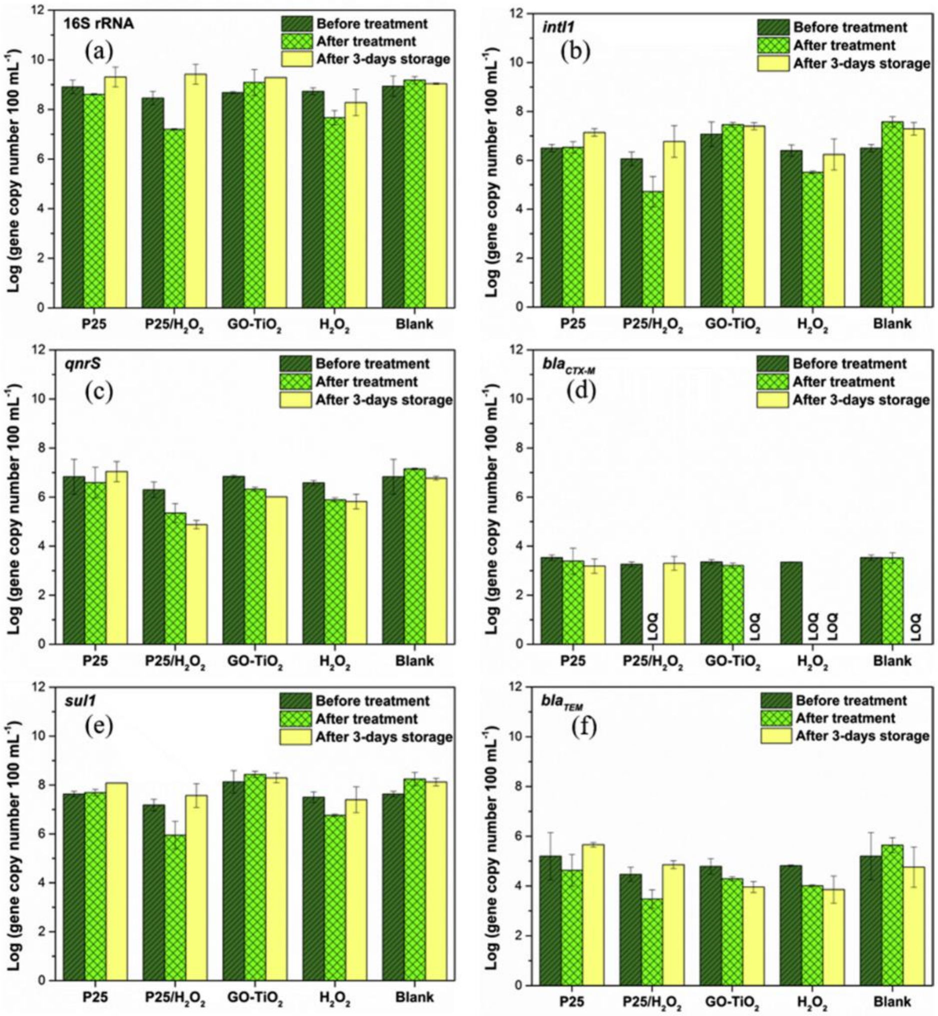

Fig. 4. Abundance of target genes before and after treatment, and after 3-days storage at room temperature using different solar-driven treatments: (a) 16S rRNA, (b) *intI1*, (c) *qnrS*, (d) *bla*$_{CTX-M}$, (e) *sul1* and (f) *bla*$_{TEM}$. Values are the average of two independent assays. Error bars represent standard deviations.

H_2O_2 associated treatments to values below the limit of quantification. Although these types of treatment were observed to cause higher reductions in the other genes analysed, none of these ARGs were reduced to levels below LOQ. A further investigation would be necessary to assess whether disinfection driven reductions of ARGs were due the loss of the ARG (for instance plasmid loss or ARG excision), with maintenance of the host cell viability or if such reductions were due to cell inactivation. However, the observation of reactivation after 3-days storage in most of the cases, suggest that at least part of the ARG removal was due to cell inactivation and not to ARG loss. These results suggest that at least some of the bacterial cells survived these treatments, being able to reproduce during storage. However, further research on this subject is needed to withdraw safe conclusions. Regarding the apparent inconsistency between data obtained based on qPCR analyses and cultivation methods, in fact it could be expected given the low fitness of the indicators of faecal contamination to grow in water. Moreover, it suggests that regrowth is due to other bacterial populations, as it can be confirmed below with the data based on the bacterial community analysis.

The effect of the different treatment processes on the bacterial communities was another aim of this study. Out of the 49 phyla found in freshly collected wastewater (before treatment), *Proteobacteria* (62 ± 7%) and *Bacteroidetes* (10 ± 2%) were, in average (n = 8), the most abundant (Fig. 5a, t_0). *Proteobacteria* comprised mainly members of the classes *Beta-* (28 ± 2%), *Gamma-* (20 ± 10%), *Alpha-* (7 ± 2%) and *Deltaproteobacteria* (5 ± 1%). Beside these groups, several other bacterial classes were detected in the freshly collected wastewater in relative abundances ranging from 2 ± 1% to 4 ± 2%; examples are "*Saprospirae*", *Flavobacteriia* and *Bacteroidia* (*Bacteroidetes*), *Clostridia* (*Firmicutes*), *Planctomycetia* (*Planctomycetes*), *ZB2* (*OD1*) and *TM7-1* (*TM7*). The other identified classes had abundances <1.3% (Fig. 5a, t_0).

Regardless of the solar driven process, treatment followed by storage at room conditions led to important bacterial community rearrangements that, in general, had the same pattern. The relative

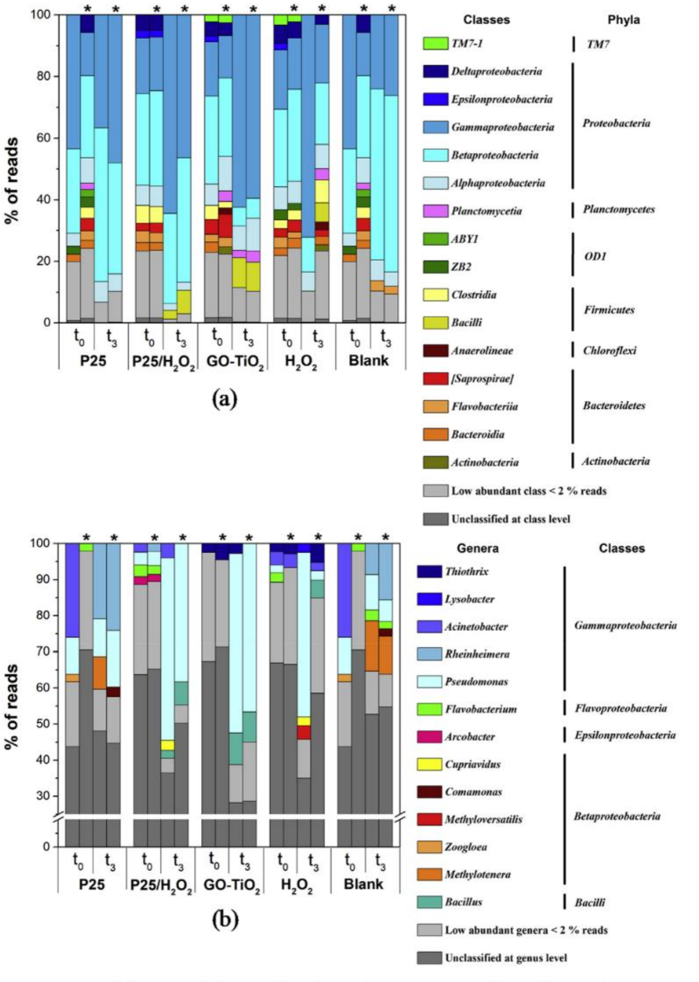

Fig. 5. Relative abundance of (a) classes and (b) genera before (t_0) and 3-days after treatment (t_3). *, indicates a second independent assay.

Table 1
Alpha diversity indices of the wastewater samples before (t_0) and after 3-days storage after treatment (t_3) calculated based on the average of 10 rarefaction OTU tables.

Experiment	Time	OTUs No.	Shannon	Simpson	PD whole tree
P25	t_0	3023	7.0	0.95	125.6
P25 [a]	t_0	3181	8.3	0.99	158.7
P25	t_3	3267	6.6	0.92	101.8
P25 [a]	t_3	4010	7.3	0.96	123.8
P25/H_2O_2	t_0	3353	7.8	0.98	156.7
P25/H_2O_2 [a]	t_0	3586	8.1	0.98	164.8
P25/H_2O_2	t_3	2462	6.1	0.93	59.4
P25/H_2O_2 [a]	t_3	2047	4.9	0.84	58.7
GO-TiO_2	t_0	3425	8.1	0.98	161.2
GO-TiO_2 [a]	t_0	2687	7.6	0.97	136.3
GO-TiO_2	t_3	2419	5.6	0.83	95.5
GO-TiO_2 [a]	t_3	2628	6.5	0.93	92.7
H_2O_2	t_0	3161	7.8	0.98	155.7
H_2O_2 [a]	t_0	3567	8.1	0.98	166.3
H_2O_2	t_3	2179	5.2	0.84	79.4
H_2O_2 [a]	t_3	2595	6.3	0.91	136.2
Blank	t_0	3023	7.0	0.95	125.6
Blank [a]	t_0	3181	8.3	0.99	158.7
Blank	t_3	3487	6.7	0.94	112.4
Blank [a]	t_3	3511	6.3	0.91	111.0

[a] indicates a second independent assay.

abundance of the members of *Proteobacteria* was higher ($p < 0.01$) in the stored treated wastewater than in the freshly collected wastewater samples, whereas it was lower ($p < 0.01$) for the majority of members of the other phyla (Fig. 5a, t_3). The lower values of the alpha-diversity indices of the stored treated wastewater samples when compared with those of freshly collected wastewater samples (Table 1) corroborate this loss of diversity and equitability. These rearrangements are well depicted in the PCoA biplot, where the bacterial communities of the freshly collected wastewater samples were separated from those treated and stored over axis PC1 (31.0%) (Fig. 6, squares and stars, respectively).

Differences on the structure and composition of the bacterial community of the stored treated wastewater samples were mainly based on the relative abundance of *Beta*- and *Gammaproteobacteria* and *Bacilli* (Fig. 5a, t3 and Fig. 6, stars). The structure and composition of the bacterial communities of treated stored water was similar. Nevertheless, the relative abundance of *Betaproteobacteria* was higher for photolysis > P25 > P25/H_2O_2 > solar-H_2O_2 > GO-TiO_2, varying between 57% and 7% (Fig. 5a). Consequently, the relative abundance of *Gammaproteobacteria* followed a kind of inverse order (i.e., GO-TiO_2 > P25/H_2O_2 > P25 > solar-H_2O_2 > photolysis), varying between 61% and 25% (Fig. 5a). Despite the lower values when compared with these proteobacterial classes, the relative abundance of *Bacilli* followed a similar order (8% and 0%, Fig. 5a). The ability of some members of class *Bacilli* to survive under harsh stressful conditions through the production of resistance forms (endospores) as well as their ability to withstand oxidative stress (Battistuzzi and Hedges, 2009; Mols and Abee, 2011) may explain their survival upon these treatments.

Altogether, the results obtained suggest that the solar-driven AOPs inactivated less efficiently *Beta*- and/or *Gammaproteobacteria* or that at least some bacteria belonging to these classes have higher capacity to regrow. Curiously, at the genus level, it is possible to observe that members of the ubiquitous genus *Pseudomonas* were, in general, the group with the sharpest increase during storage, followed by *Rheinheimera* and *Methilotenera* (Fig. 5b). Alterations in the composition and structure of the bacterial communities leading to higher proportions of *Proteobacteria* in stored water than before the treatment were reported in the literature for photolysis, ozonation and photocatalytic ozonation (Becerra-Castro et al., 2016), coupled biological and photocatalysis treatments (Chen et al., 2013) and ozonation coupled to a sequencing batch biofilm reactor (Esplugas et al., 2013).

4. Conclusions

Among the tested solar-driven oxidation processes, photo-Fenton at circumneutral pH was the worst performing one (quite similar to photolysis), whereas the combination of P25 and H_2O_2 was the most efficient approach for the removal of organic

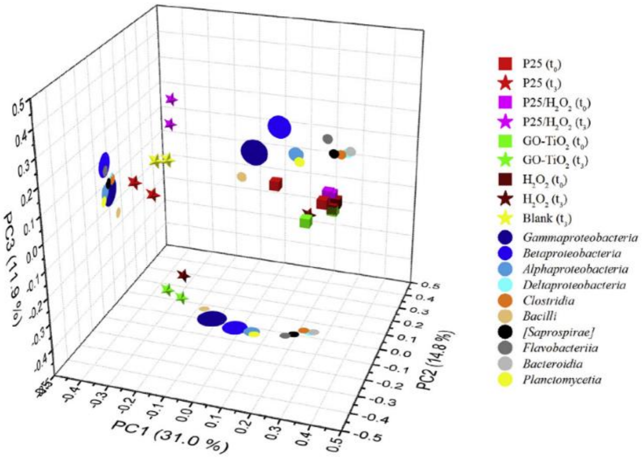

Fig. 6. Biplot of principal coordinates analysis (PCoA) based on weighted Unifrac distances of samples before (t_0 - squares) and 3-days storage after treatment (t_3 – stars).

micropollutants in the urban wastewater sampled.

Regarding the biological indicators, a decrease in the abundance of total faecal coliforms and enterococci and their antibiotic resistant counterparts was found for all the processes employing H_2O_2, which was permanent after 3-days storage of the treated wastewater. P25/H_2O_2 and solar-H_2O_2 were also able to reduce the total bacterial load, assessed based on the abundance of the 16S rRNA gene. Nevertheless, the abundance of total bacterial load increased after 3-days storage to values close or higher than those verified before treatment. Similar observations were found for the genes *intI1* and *sul1*. Hence, none of the studied processes was able to prevent bacterial reactivation, including antibiotic resistant populations.

Thus, among all the studied processes, P25/H_2O_2 seemed to be that achieving the best compromise for the removal of both organic micropollutants and biological contaminants, although not able to prevent bacterial reactivation.

Interestingly, regardless of the oxidation process studied, higher relative abundance of the phylum *Proteobacteria (Beta- and Gammaproteobacteria)*, namely of genera *Pseudomonas*, *Rheinheimera* and *Methylotenera*, was observed in treated wastewater after 3-days storage. Since within the phylum *Proteobacteria*, in particular of the classes *Beta- and Gammaproteobacteria*, it is possible to find diverse multidrug-resistant bacteria, the increase of this group of organisms in stored treated water may deserve further investigation. Moreover, the potential disturbance of the water bacterial communities may have relevant ecology implication and should be considered in the design of advanced oxidation technologies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.01.064.

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Appendix C1- Supplementary Information

Solar treatment (H_2O_2, TiO_2-P25 and GO-TiO_2 photocatalysis, photo-Fenton) of organic micropollutants, human pathogen indicators, antibiotic resistant bacteria and related genes in urban wastewater

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Table C-S6.1. Chemical characterization of the secondary wastewater from the UWWTP. Values are the average of four independent assays and errors represents standard deviations.

Secondary effluent characterisation			
Turbidity (NTU)	5.3 ± 3.1	PO_4^{3-} (mg L^{-1})	12.4 ± 6.1
pH	7.5 ± 0.2	SO_4^{2-} (mg L^{-1})	74.5 ± 15.6
Conductivity (µS cm^{-1})	1780 ± 63	Br^- (mg L^{-1})	2.7 ± 0.7
DIC† (mg L^{-1})	55 ± 30	Na^+ (mg L^{-1})	199 ± 6
DOC† (mg L^{-1})	19 ± 4	Cl^- (mg L^{-1})	368 ± 18
NH_4^+ (mg L^{-1})	35.3 ± 25.5	K^+ (mg L^{-1})	25.6 ± 1.8
NO_3^- (mg L^{-1})	68.6 ± 64.5	Mg^{2+} (mg L^{-1})	35.3 ± 2.6
NO_2^- (mg L^{-1})	1.8 ± 1.0	Ca^{2+} (mg L^{-1})	74.9 ± 3.2

†DIC: dissolved inorganic carbon; †DOC: dissolved organic carbon.

Table C-S6.2. Target genes and conditions used in qPCR assays.

Target gene	Primers (sequence) Reference	Conditions	Efficiency (%)	Reference
16S rRNA	1114F (CGGCAACGAGCGCAACCC) 1275R (CCATTGTAGCACGTGTGTAGCC) *Escherichia coli* - ATCC 25922	95 °C for 10 min (1 cycle) 95 °C for 15 s, 55 °C for 20 s and 72 °C for 10 s (35 cycles)	100	31
bla$_{\text{TEM}}$	blaTEM-F (TTCCTGTTTTTGCTCACCCAG) blaTEM-R (CTCAAGGATCTTACCGCTGTTG) *Escherichia coli* - A2FCC14	95 °C for 10 min (1 cycle) 95 °C for 15 s, 60 °C for 30 s and 72 °C for 10 s (40 cycles)	96	30
intI1	intI1-F (CCTCCCGCACGATGATC) intI1-R (TCCACGCATCGTCAGGC) *Escherichia coli* - A2FCC14	95 °C for 10 min (1 cycle) 95 °C for 15 s, 55 °C for 30 s and 72 °C for 10 s (40 cycles)	94	32
*qnr*S	qnrSrtF11 (GACGTGCTAACTTGCGTGAT) qnrSrtR11 (TGGCATTGTTGGAAACTTG) *Enterobacter cloacae* - S1+	95 ºC for 5 min (1 cycle) 95 ºC for 15 s and 60 ºC for 1 min (40 cycles)	95	33
*sul*1	sul1-FW (CGCACCGGAAACATCGCTGCAC) sul1-RV (TGAAGTTCCGCCGCAAGGCTCG) *Achromobacter sp.*	95 ºC for 5 min (1 cycle) 95 ºC for 15 s and 60 ºC for 1 min (40 cycles)	94	35
*van*A	vanA3FP (CTGTGAGGTCGGTTGTGCG) vanA3RP (TTTGGTCCACCTCGCCA) *Enterococcus faecalis* - H1EV23	95 ºC for 5 min (1 cycle) 95 ºC for 15 s and 60 ºC for 1 min (40 cycles)	98	36
bla$_{\text{CTX-M}}$	CTXM-FW (CTATGGCACCACCAACGATA) CTXM-RV (ACGGCTTTCTGCCTTAGGTT) *Escherichia coli* - A2FC14	95 ºC for 10 min (1 cycle), 95 ºC for 15 s and 60 ºC for 1 min (40 cycles)	94	34

Appendix D

Metal-free g-C_3N_4 photocatalysis of organic micropollutants in urban wastewater under visible light

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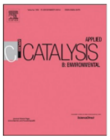

Metal-free g-C_3N_4 photocatalysis of organic micropollutants in urban wastewater under visible light

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ABSTRACT

Heterogeneous photocatalysis under visible light was employed for the degradation of organic micropollutants (MPs) found in the biologically treated effluents of an urban wastewater treatment plant (UWWTP). The irradiation source consisted in 4 light emitting diodes (LEDs) with a nominal power of 400–500 W m^{-2} at 417 nm. Metal-free exfoliated graphitic carbon nitride (gCN_T), which was synthetized through a simple thermal treatment using dicyandiamide as precursor, was employed as photocatalyst. The gCN_T material was characterized using different techniques: N_2 adsorption isotherms at 77 K, electron microscopy (scanning and transmission) and diffuse reflectance UV–Vis. The photocatalytic performance of the material was compared to the commercial TiO_2-P25, confirming that gCN_T led to a remarkable higher removal efficiency of the target MPs. Most of these MPs were removed in less than 10 min to levels below the limit of quantification (carbamazepine > isoproturon > clopidogrel > diclofenac > atenolol > bezafibrate > tramadol > venlafaxine > fluoxetine). Indirect photolysis, resulting from the secondary reactive oxidants generated during the irradiation of the aqueous matrix components, was found to partially contribute for the elimination of the occurring MPs. In addition, the gCN_T photocatalyst was immobilized on glass rings for use under continuous mode operation, a minimum residence time of 25 min being required to attain significant removal efficiencies. Phytotoxicity experiments showed that heterogeneous photocatalysis did not enhance the toxicity of the wastewaters.

1. Introduction

Organic micropollutants (MPs) are frequently found in the aquatic environment at concentrations levels between ng L^{-1} and μg L^{-1}. The difficult elimination of MPs by physicochemical and biological treatments conventionally applied in the urban wastewater treatment plants (UWWTPs) triggers the need of alternative approaches to remove them. Although discharge limits are not yet regulated for MPs, some recommendations have been launched by European Union (EU) in order to monitor harmful substances, namely the Directive 2013/39/EU [1], which indicates a group of 45 priority substances/groups of substances and recommends new treatment strategies, and the Decision 2015/495/EU [2], setting a Watch List of 17 contaminants of emerging concern (CECs) for EU-monitoring, which was very recently repealed by a new Watch List of 15 CECs [3].

Novel treatment tools have been sought such as advanced oxidation processes (AOPs), which have been largely studied to reach high degradation rates of MPs from UWWTP effluents [4]. However, only UV (mainly for disinfection) and ozonation have been implemented so far at full-scale [5–7], both approaches presenting some limitations but affordable costs when compared with most AOPs. Solar applications of heterogeneous photocatalysis are an interesting alternative to degrade a broad range of MPs [8]. Wide-bandgap optical semiconductors able to act as visible light photocatalysts require absorption edges above the UV limit since this only accounts for less than 5% of the solar spectrum reaching Earth's surface. Visible-light photocatalysts with a narrower bandgap, such as graphitic carbon nitride (g-C_3N_4 with a bandgap of 2.7 eV, corresponding to an absorption edge at 459 nm), have been receiving an increasing interest for a wide variety of photocatalytic applications (*e.g.*, CO_2 and NO_x reduction, degradation of organic pollutants, organic selective synthesis, hydrogen production and water splitting) [9–18]. The high photocatalytic activity of g-C_3N_4 materials on the degradation of different types of organic contaminants in water has been already demonstrated in previous studies, namely for methylene blue [19–22], rhodamine B [22–25], methyl orange [26–28], basic fuchsin, malachite green and crystal violet [29], acid orange 7

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[30], oxalic acid [31], tetracycline [32,33], phenol [34–36], atrazine [35,37], carbamazepine and sulfamethoxazole [35,38] and humic acids [39]. However, in the majority of these reports, the experiments are often carried out using ultrapure water, concentrations higher than those usually found in the environment, slurry (batch) systems, metal loaded g-C_3N_4 materials, and spiked matrices [40]. To the best of our knowledge, none of these studies report the treatment of an actual urban wastewater (*i.e.*, not spiked) employing g-C_3N_4 as metal-free photocatalyst.

g-C_3N_4 has been synthesized by thermal polymerization of several nitrogen-rich precursors, namely urea, thiourea, cyanamide, dicyandiamide or melamine [17,41]. Since its photocatalytic activity is struggled by the low surface area and by the fast recombination of electrons and holes, several approaches have been used to overcome these drawbacks, namely the chemical, mechanical and thermal exfoliation treatments of g-C_3N_4 [17,42]. Other procedures include the design of g-C_3N_4 based nanoarchitectures using templating approaches, doping with metals, and modification with other semiconductors, well discussed in the literature [41].

The chemical and thermal stability of the low-cost, metal-free g-C_3N_4 turns this visible light photocatalyst an outstanding option for solar-driven photocatalysis [9,43]. In addition, energy-efficient and low-cost visible light emitting diodes (LEDs) with a narrow emission band can be employed as light sources, as replacement of xenon or mercury lamps, and matching the absorption peak of the material for the economy of the process [43].

The novelty of the present study relies on the application of a highly-efficient photocatalytic process based on a metal-free g-C_3N_4 photocatalyst with enhanced activity towards degradation of a wide range of MPs in realistic urban wastewaters, by using a low-cost and energy-efficient visible light source (LED). The photocatalytic treatment was also performed in continuous mode, by employing an innovative approach with g-C_3N_4-coated glass rings. The phytotoxicity of the wastewater was evaluated, before and after photocatalytic treatment, to infer about the potential formation of reaction by-products toxic for plants, which could compromise water reuse. To the best of our knowledge, this is the first study on heterogeneous photocatalysis using g-C_3N_4 as a semiconductor for the removal of MPs in actual UWWTP effluents.

2. Materials and methods

2.1. Chemicals and materials

Dicyandiamide (99%) supplied from Sigma-Aldrich was used as precursor in the preparation of the bulk g-C_3N_4. Aeroxide® TiO_2-P25 ($\sim$80% anatase:20% rutile) powder was supplied by Evonik. Polyvinyl alcohol (PVA), reference standards for liquid chromatography (> 98%) and surrogate standards were purchased from Sigma–Aldrich, which include atenolol, bezafibrate, carbamazepine, clopidogrel, diclofenac, fluoxetine, isoproturon, tramadol venlafaxine, azithromycin-d3, atrazine–d5, diclofenac-d4 and fluoxetine–d5. Ethanol (99.5%) and 2-propanol (100%) were obtained from Fisher Scientific UK Limited. Acetonitrile (MS grade) was supplied by Panreac AppliChem. Formic (99.5%) and sulfuric (98%) acids were acquired from Merck (Darmstadt, Germany). Ultrapure water was produced in a Milli-Q water system (resistivity of 18.2 MΩ cm, at 25 °C). The Oasis® HLB (Hydrophilic-Lipophilic-Balanced) cartridges (150 mg, 6 mL) used for sample preparation, were purchased from Waters (Milford, MA, USA).

2.2. Catalysts preparation and characterization

The bulk g-C_3N_4 material was prepared by thermal decomposition of dicyandiamide, as previously described [17]. Briefly, a certain amount of dicyandiamide precursor was placed in a covered crucible and then heated to 550 °C in air atmosphere for 4 h. In a second step, thermal oxidative exfoliation of the bulk material was conducted at 500 °C. The resulting material was labelled as gCN_T. TiO_2 was used as received, for comparison purposes.

The specific surface area (S_{BET}) of the mentioned photocatalysts was calculated by using the Brunauer-Emmett-Teller (BET) method and N_2 adsorption isotherms obtained at −196 °C in a Quantachrome NOVA 4200e apparatus. The optical absorption was determined in the 220–800 nm range by diffuse reflectance UV–Vis (DRUV-Vis) in a JASCO V-560 spectrophotometer equipped with an integrating sphere. The results were recorded to equivalent absorption Kubelka–Munk units and used to obtain the optical bandgap.

Uncoated glass rings (diameter = 3 mm, length = 3 mm) were cleaned with an anionic detergent in water, under sonication. The washed rings were then immersed in 2-propanol and sonicated for 15 min. Before coating with gCN_T, the glass rings were immersed in a 2% (w/V) aqueous solution of PVA by dip-coating. This procedure was immediately followed by a second dip-coating immersion of the glass rings in a 5% (w/V) gCN_T:ethanol suspension, previously sonicated for 30 min.

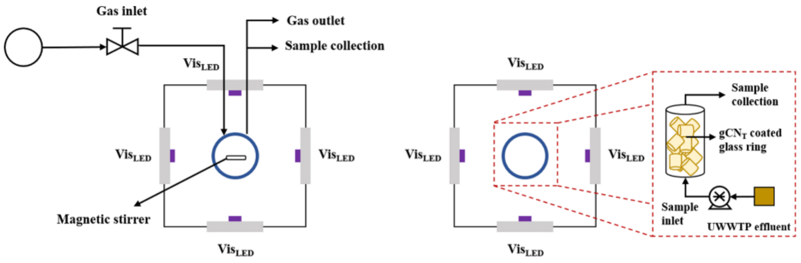
The surface morphology of bulk g-C_3N_4, gCN_T powders and gCN_T immobilized on glass rings was observed by scanning electron microscopy (SEM), using a FEI Quanta 400FEG ESEM/EDAX Genesis X4 M instrument. In the case of the immobilized gCN_T, the SEM apparatus was equipped with a sample holder, in which the broken glass rings were positioned to observe and measure the cross-section of the coated material, by using the respective SEM instrument software. Transmission electron microscopy (TEM) was performed on a JEM 220FS microscope (Jeol, Japan) with a LaB6 electron gun operating at 200 kV.

2.3. Experimental set-up

Wastewater samples from the secondary biological treatment of a UWWTP located in the North region of Portugal, were collected and separated in aliquots which were stored at – 4 °C. The photocatalytic performance of the gCN_T powder was studied in batch mode, using a borosilicate reactor containing 60 mL of UWWTP effluent, equipped with four high-power visible LEDs (λ_{max} = 417 nm, Full-Width Half-Maximum (FWHM) = 18.2 nm) perpendicularly placed to each other and equidistant (4.0 cm) from the reactor (Scheme 1a). The nominal irradiation of each LED varied between 400–500 W m^{-2}, as determined by UV-Vis spectroradiometry (USB2000+, OceanOptics, USA). The catalyst load was fixed at 1.0 g L^{-1} (selected in preliminary studies as optimal gCN_T load for the degradation of model organic pollutants). A dark period of 30 min was performed to establish the adsorption-desorption equilibrium under oxygenated conditions (air). Under these conditions, the removals of the 9 studied MPs never exceeded 6% after 30 min of adsorption period with either TiO_2 or gCN_T. Then, the suspension containing the UWWTP effluent and the photocatalyst was irradiated for 60 min, and samples were withdrawn regularly from the reactor.

Photolysis experiments were performed in UWWTP effluent and also in ultrapure water spiked with the MPs found in the UWWTP effluent and at similar concentrations, in this case under air and deoxygenated (argon) conditions. For that, the required volumes of each standard solution were added to a volumetric flask and the organic solvent was evaporated before filling it with the ultrapure water, avoiding the presence of the organic solvent and its possible scavenging effect. Control experiments using ultrapure water as matrix were also run in the dark, under oxygenated and deoxygenated conditions. All the experiments were carried out in triplicate.

Continuous flow experiments were performed using the gCN_T material immobilized on glass rings (Scheme 1b). A borosilicate cylindrical reactor (internal diameter = 2.7 cm, length = 7.0 cm) was packed with 115 gCN_T-coated glass rings. The filling volume after packing was approximately 31.5 mL. The reactor was continuously filled with UWWTP



a) Batch mode system b) Continuous mode system

Scheme 1. Photocatalytic experimental set-up under batch (a) and continuous (b) operation modes.

effluent using a peristaltic pump at constant flow rate (*Q*), and LEDs were then turned on after establishment of the adsorption-desorption equilibrium. Samples were regularly withdrawn after reaching the steady state. Different residence times (2, 5, 12, 25, 55 min) were tested, by changing the liquid flow rate (0.57, 1.26, 2.6, 6.3, 15.7 mL min^{-1}). Uncoated glass rings were used for photolysis experiments (residence time of 55 min) to maintain the hydrodynamic behaviour and light diffusion conditions, allowing the comparison with the photocatalytic experiments.

2.4. Analytical methods

The concentration of each organic MP in the UWWTP effluent was determined by ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) in a Shimadzu Corporation apparatus (Tokyo, Japan), consisting of a UHPLC equipment (Nexera) with two pumps (LC-30AD), an autosampler (SIL-30AC), an oven (CTO-20AC), a degasser (DGU-20 A 5R) and a system controller (CBM-20 A) with proper software (LC Solution Version 5.41SP1), coupled to a triple quadrupole mass spectrometer detector (Ultra-Fast Mass Spectrometry series LCMS-8040). Before UHPLC-MS analysis, a pre-concentration and clean up procedure was performed by solid phase extraction (SPE), adding isotopically labeled internal standards to the samples before SPE as described elsewhere [44]. A Cortecs™ C18+ column (100 × 2.1 mm i.d.; 1.6 μm particle diameter), supplied by Waters (Milford, MA, USA), was used with a mobile phase composed by ultrapure water and acetonitrile (20:80, *v/v*), both acidified with 0.1% formic acid. The chromatographic analytical method was performed at isocratic mode, using a flow rate of 0.3 mL min^{-1} and the column oven temperature set at 30 °C. The autosampler temperature was set at 4 °C and the injection volume was 5 μL. For quantification purposes, the selected reaction monitoring (SRM) transition between the precursor ion and the most abundant fragment ion was determined; whereas the second most intense fragment ion was used for confirmation of the identity of each target MP (SRM1/SRM2 ratio).

The dissolved organic carbon (DOC) was determined using a Shimadzu TOC-L apparatus (Shimadzu Scientific Instruments, Japan).

2.5. Phytotoxicity evaluation

PHYTOTOXKIT microbiotests (MicroBioTests Inc.) were used to assess the phytotoxicity, by comparing the germination of the seeds of 3 given plant species, as well as their root and shoot lengths, after exposure during 72 h to distilled water, non-treated and treated UWWTP effluents (after 10 and 60 min using TiO_2 and gCN_T-photocatalysts). The PHYTOTOXKIT microbiotest strictly adheres to ISO standard 18763. The plants were monocotyl *Sorghum saccharatum* (Sorgho), dicotyls *Lepidium sativum* (garden cress) and *Sinapis alba* (mustard). The measurements of root and shoot lengths were performed by image analysis using ImageJ® software (NIH, USA).

3. Results and discussion

3.1. Photocatalyst characterization

The characterization of the bulk $g\text{-}C_3N_4$ and gCN_T materials was presented in our previous work [45] (labeled as bulk and T500, respectively), studying its use for the photocatalytic synthesis of benzaldehyde. In the present work, the most relevant characterization for interpretation of the results was selected, and additional data on the gCN_T immobilized on glass rings before and after the photocatalytic reactions, was included. Briefly, the S_{BET} of the bulk $g\text{-}C_3N_4$ material prepared by direct pyrolysis of dicyandiamide is typically low around ~10 $m^2 g^{-1}$, as experimentally determined. However, after thermal post-treatment at 500 °C, the S_{BET} of the resulting material, gCN_T, increased by *ca.* 12 times to 117 $m^2 g^{-1}$. As already described in the literature, oxidation treatment under air atmosphere may lead to unstable structures, due to the low resilience of the hydrogen bonding between strands of polymeric melon by NH or NH_2 groups [41]. The procedure adopted in this work may promote the gradual exfoliation of the bulk material, due to layer-by-layer thermal oxidation and splitting, resulting in a higher S_{BET} [46,47]. For comparison purposes, the S_{BET} determined for TiO_2 was 54 $m^2 g^{-1}$.

The DRUV-Vis spectrum of gCN_T, shows an unresolved broad band fading out between 450 and 500 nm (Fig. 1). It is significantly shifted to the red region leading to a higher light absorption range through the

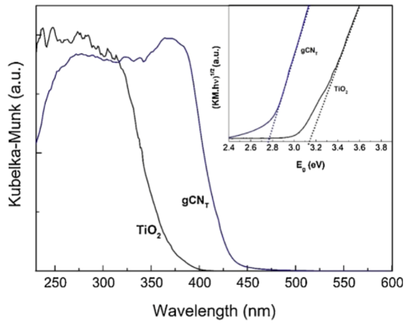


Fig. 1. DRUV-Vis spectra of TiO_2 and gCN_T photocatalysts; inset: plot of Kubelka-Munk units as a function of the light energy.

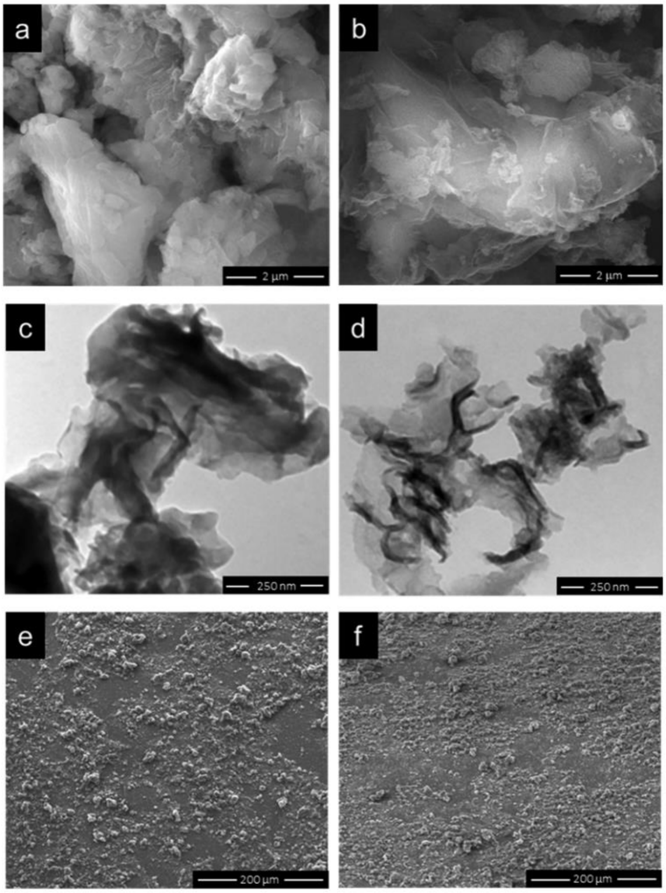


Fig. 2. SEM (a, b) and TEM (c, d) images of bulk g-C_3N_4 (a, c) and gCN_T (b, d) powder materials. SEM of gCN_T immobilized on glass rings before (e) and after (f) usage in the photocatalytic reactions.

visible spectrum, in comparison to TiO_2. The commercial TiO_2 photocatalyst shows the characteristic absorption band sharp edge rising at 400 nm. The bandgap energies (E_g) of both photocatalysts were estimated by the Tauc plot (inset Fig. 1), using the equivalent absorption Kubelka-Munk (*KM*) units of the absorption edges, $(KM.h.\nu)^{1/2}$ versus E_g, where *h* is Plank' constant and ν is the light frequency. The E_g determined for TiO_2 and gCN_T were 3.14 and 2.76 eV, respectively.

The morphology of bulk g-C_3N_4 and gCN_T materials was assessed by SEM and TEM micrographs (Fig. 2), revealing differences in their morphology.

The bulk material is constituted by g-C_3N_4 sheets assembled in dense aggregates (SEM, Fig. 2a), resulting from the stacking of the layers. In the case of gCN_T (SEM, Fig. 2b), thinner plates surrounded by small aggregates of non-exfoliated g-C_3N_4 material were observed, resulting from the progressive oxidation of the hydrogen bonds between the layers, *i.e.* the exfoliation originated by the thermal oxidation treatment, in agreement with the results obtained from S_{BET} measurements. TEM analysis of the materials was also performed (Fig. 2c and 2d) and the results corroborate with those given before, less dense layers being observed in the case of the gCN_T material. A representative SEM micrograph of the fresh gCN_T coated glass rings reveals its regular distribution over the support (Fig. 2e). These gCN_T coated glass rings were analyzed after being employed in the photocatalytic treatment of the UWWTP effluent, under continuous mode and during several hours, the morphology remaining uniform and, thus, confirming their stability under the tested conditions (Fig. 2f).

3.2. Degradation of MPs in batch mode using powder photocatalysts

The photochemical degradation of a set of 9 organic MPs quantified in the UWWTP effluent was carried out in the absence of catalyst by irradiating at 417 nm using LEDs as light source (Fig. 3). Under these

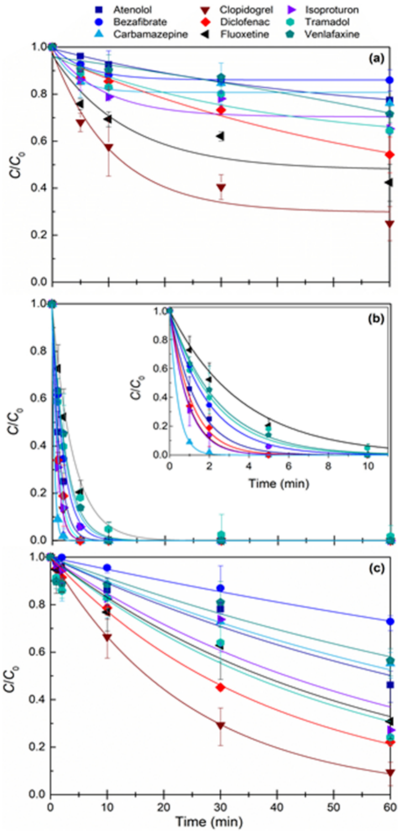


Fig. 3. Normalized concentration of the MPs (C/C_0) identified in the UWWTP effluents, after treatment by photolysis (a), and by heterogeneous photocatalysis using gCN_T (b) and TiO_2 (c). Experiments were performed in batch mode (60 mL) and with four LEDs, using a catalyst load of 1.0 g L^{-1}. LEDs were switched on after a dark period of 30 min to establish the adsorption-desorption equilibrium.

conditions, a surprisingly significant abatement of the MPs (clopidogrel > fluoxetine > diclofenac > isoproturon ≈ tramadol ≈ venlafaxine > carbamazepine ≈ atenolol > bezafibrate) was observed (Fig. 3a). This occurrence could not arise from direct single photon absorption since none of the target compounds will absorb on the emission band of the LED (Fig. S1). However, indirect photolysis may take place by irradiation of the nitrates naturally present in UWWTP effluents (~0.40 mg L^{-1} in this particular case, Table S1): nitrate photochemistry gives raise to reactive oxygen species (ROS), namely singlet oxygen ($^1O_2$), hydroxyl radicals ($HO^{\bullet}$) or alkyl peroxyl radicals ($ROO^{\bullet}$) and hydrated electrons from the ionization of water solvent molecules [48,49]. In addition, photosensitization phenomena may occur by light absorbing species, such as colored organic matter in its triplet state with the ability to break the organic MPs, leading to their indirect photochemical decomposition [48–50]. Less common will be the occurrence of non-linear photon absorption phenomena, such as the simultaneous absorption of two photons [51].

Considering that the complexity of the matrix may affect the degradation kinetics of the MPs [52–54], photolysis experiments were performed using ultrapure water spiked with a solution containing the 9 organic MPs with similar concentrations to those found in the UWWTP effluents, under oxygenated and deoxygenated conditions, and with/without light irradiation. Markedly lower removals were obtained when using ultrapure water as matrix (Fig. S2) instead of the real wastewater (Fig. 3a), and quite similar results were obtained when the solutions were saturated with argon or oxygen, under both dark and light conditions. Thus, photosensitization, indirect photolysis, or other mechanisms involving other matrix species, could take place under visible light driving the degradation of the MPs in the UWWTP effluent.

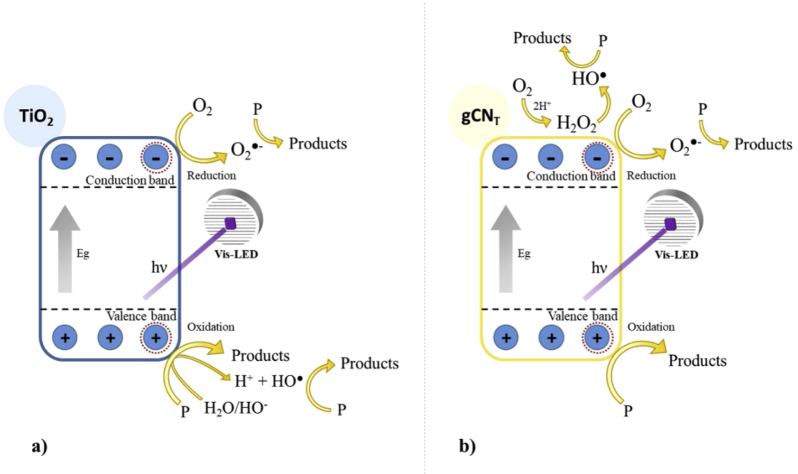
The photocatalytic degradation of the MPs in the UWWTP effluents followed a pseudo-first order rate law (Fig. 3b and c). The apparent first order reaction rate constants (k_{app}) summarized in Table 1 were determined by exponential curve fitting to the experimental data.

The photocatalytic experiments using the powdered gCN_T led to an almost complete removal of all MPs (carbamazepine > isoproturon > clopidogrel > diclofenac > atenolol > bezafibrate > tramadol > venlafaxine > fluoxetine) after 10 min of reaction (*i.e.*, after turning on the LEDs) (Fig. 3b). It is important to refer that the k_{app} obtained for each MP hold one to three orders of magnitude compared with the TiO_2, proving the high efficiency of this catalyst under the reaction conditions used.

Regarding the standard TiO_2 photocatalyst (Fig. 3c), it is observed that the conversion is only slightly better that the photolytic degradation of the MPs. In addition, a different degradation order of the MPs is observed (clopidogrel > diclofenac > tramadol > fluoxetine > isoproturon > atenolol > carbamazepine > venlafaxine > bezafibrate), when comparing with the gCN_T material. The distinct behaviour observed for both catalysts can be expected, especially by using a real matrix containing naturally occurring interferences that may act as promoters or inhibitors (such as organic matter consuming $HO^{\bullet}$ radicals and other oxidizing species; anions acting as $HO^{\bullet}$ scavengers; organic and inorganics species competing for the active reaction sites, etc.). Moreover, the photocatalytic activity of the TiO_2 under the light source used (λ_{max} = 417 nm) can also be explained in terms of the residual overlap between the absorption spectrum of TiO_2 and the emission of the LED (Fig. S3). Obviously, it is not expected that a wide-bandgap optical semiconductor such as TiO_2 (E_g ~ 3.14 eV) would be efficient under 417 nm excitation, since UV-light is required to effectively activate it. Nevertheless, the comparison between the TiO_2 (E_g ~ 3.14 eV) and the gCN_T (E_g ~ 2.76 eV) under visible light was performed based on previous studies using LEDs emitting either in the UV or visible ranges. In those experiments, the performance of both TiO_2 and gCN_T was evaluated on the degradation of diclofenac as model compound (Fig. S4). The results demonstrated that even under UV light, the gCN_T material exhibited better performance compared with TiO_2,

Table 1
Initial concentration (ng L^{-1}) and apparent first order reaction rate constant (k_{app}, min^{-1}) for each MP using different treatment processes.

		k_{app} ($\times 10^{-2}$ min^{-1})	
Compound	Initial concentration (ng L^{-1})	VIS_{LED}/ gCN_T	VIS_{LED}/ TiO_2
Atenolol	12.5 ± 1.2	74.3 ± 2.3	1.2 ± 0.1
Bezafibrate	38.7 ± 6.0	51.9 ± 1.2	0.5 ± 0.0
Carbamazepine	763 ± 18	238 ± 1	1.1 ± 0.2
Clopidogrel	93.2 ± 10.7	108 ± 2	4.1 ± 0.0
Diclofenac	1102 ± 31	90.6 ± 2.6	2.6 ± 0.1
Fluoxetine	21.7 ± 4.5	27.4 ± 4.5	1.9 ± 0.2
Isoproturon	84.6 ± 7.4	110 ± 8	1.7 ± 0.3
Tramadol	3930 ± 244	44.6 ± 3.6	2.0 ± 0.3
Venlafaxine	349 ± 47	41.3 ± 1.2	0.9 ± 0.2



Scheme 2. Schematic representation of a possible photocatalytic mechanism for the degradation of MPs using TiO_2 (a) or gCN_T (b).

showing the high efficiency of gCN_T in both UV and visible ranges.

The efficiency of g-C_3N_4 based photocatalysts to eliminate organic contaminants (mostly dyes) is already known from the literature [19,20,23,24,26,27,29,34,37,39]. In the unique study reported to a spiked effluent from a UWWTP [35], the photocatalytic degradation of four model compounds (phenol, atrazine, sulfamethoxazole and carbamazepine at 100 μM), under visible illumination (xenon lamp; $\lambda > 400$ nm), was attributed to reactive oxygen species and/or contaminant-photocatalyst interfacial interactions, depending on the g-C_3N_4 sample tested. Furthermore, the photocatalytic activity was similar regardless of the realistic matrix tested (water or wastewater), demonstrating the potential of g-C_3N_4 for the treatment of real case effluents.

Scheme 2 shows the possible pathway for gCN_T catalyst compared with the well-known TiO_2 [55], generally based from mechanism studies of model pollutants in aqueous solutions, *i.e.* considering that the others constituents present in the UWWTP effluent (organic matter, nitrates and other inorganics, etc.) do not interfere on the photocatalytic mechanism. However, it is important to remark that the photocatalytic experiments investigating the efficiency of the gCN_T were performed using UWWTP effluent as matrix, which turns the understanding of the mechanisms occurring and governing the degradation pathway at the catalyst surface much more challenging to predict.

Concerning the organic matter, no significant changes on the DOC content of the effluent from the UWWTP were observed before and after the treatment with both photocatalysts. This is not surprisingly due to the complexity of the matrix: there are several orders of magnitude of difference between the concentration of the studied organic MPs (in the range of ng L^{-1} to μg L^{-1}) and the DOC content (in the range of mg L^{-1}).

3.3. Degradation of MPs in continuous mode using gCN_T-coated glass rings

Slurry reactors have as typical disadvantage the need to separate the catalyst from the treated water, in many cases highly energy-consuming processes being required. The immobilization of the photocatalyst on a proper support is seen as a possibility to overcome this drawback in heterogeneous photocatalysis. However, there is a price to pay with immobilization since a decrease in the photocatalytic efficiency must arise from the fact that the reaction mostly occurs at the catalyst-solution interface and a catalyst dispersed in the matrix maximises the contact area [56–58]. The application of continuous mode operating systems, in some cases using immobilized catalysts, was already studied by various authors [57,59–62].

In order to evaluate the feasibility of gCN_T photocatalysis as an option for application in a real case UWWTP, the removal of the target organic MPs of the effluent samples was further studied using the gCN_T immobilized on glass rings, under continuous mode operation. Different residence times (2, 5, 12, 25, 55 min) were tested, as shown in Fig. 4. Control experiments were done conducting the photochemical degradation of the target organic MPs with uncoated glass rings during 55 min of residence time. More significant degrees of removal were found for clopidogrel, fluoxetine and diclofenac, as also observed for photolysis experiments in slurry (*i.e.* batch) mode, reinforcing the idea that other mechanisms besides direct photolysis affected the removal of MPs, namely indirect photolysis, photosensitization and/or photo(hydrolysis).

When the reactor was loaded with gCN_T immobilized on glass rings and, before switching on the LEDs, the UWWTP effluent was passed through the reactor to establish the adsorption-desorption equilibrium, during *ca.* 9 h for the lower flow rate (residence time of 55 min).

Under the photocatalytic process, using gCN_T coated glass rings after approximately 27 h at the highest residence time (55 min), the photocatalyst maintained its activity leading to identical removal efficiencies. Similar results were obtained for the residence times of 25 and 55 min, suggesting that residences times higher than 25 min will not significantly enhance the removal efficiencies of these MPs, probably due to limitations with the effectively irradiated active sites. For lower residence times, these efficiencies decreased, as expected due to the shorter contact between the organic compounds and the semiconductor.

Quite lower removals were obtained in continuous mode operation in comparison with the slurry (batch) system. As an example, nearly complete removal of the MPs was obtained in batch mode after 10 min (Fig. 3b), whereas the following removal efficiencies were obtained in

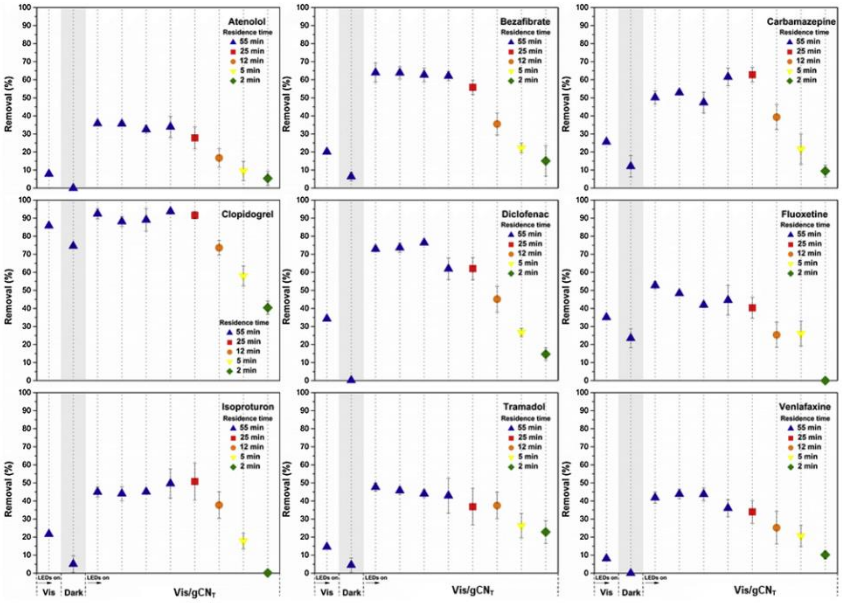


Fig. 4. Removal (%) of the MPs found in UWWTP effluents, after treatment by photolysis, adsorption and photocatalysis using gCN_T immobilized in glass rings under continuous mode operation for different treatment residence times. Experiments were performed in continuous mode (useful volume of 31.5 mL) with four LEDs, packing the reactor with 115 gCN_T-coated glass rings. LEDs were switched on after the adsorption-desorption equilibrium was established (UWWTP effluent was passed through the reactor during ca. 9 h for the lower flow rate (residence time of 55 min)). The samples were withdrawn after the steady state was achieved.

continuous mode for a residence time of 5 min (equivalent treatment time of 10 min in batch mode): atenolol (≈ 10%), bezafibrate (≈ 20%), carbamazepine (≈ 20%), clopidogrel (≈ 60%), diclofenac (≈ 25%), fluoxetine (≈ 20%), isoproturon (≈ 20%), tramadol (≈ 20%) and venlafaxine (≈ 20%). This finding was expected since the catalyst-solution interface decreases when using gCN_T coated glass rings. Again, in what concerns to the amount of organic matter, no significant changes on the DOC content were verified for all the tested residence times, by the same reasons given previously for batch mode. The application of coated-materials requires more treatment time for the effective degradation of pollutants due to the lower area of catalyst per unit of volume, when compared to the use of powder suspensions. However, some practical problems arising from the application of powder photocatalysts are avoided, such as the catalyst separation from the treated water.

The photocatalytic treatment under continuous mode using gCN_T coated glass rings to remove MPs from the effluents of UWWTP is nevertheless possible, but still a challenging technological solution. Further research is needed regarding the optimization of several parameters, such as the length of the column, the amount of catalyst immobilized on the support, the size and number of gCN_T-coated glass rings per unit volume, and the light distribution in the reactor, aiming to increase the process efficiency in continuous mode operation.

3.4. Phytotoxicity measurements

The phytotoxicity was assessed using the PHYTOTOXKIT microbiotest (MicroBioTests Inc.), according to the standard operating protocols for three plant species (*i.e. Sorghum saccharatum, Lepidium sativum* and *Sinapis alba*). The samples from the UWWTP before and after treatment by photocatalysis (using both TiO_2 and gCN_T), did not inhibited the germination of seeds (results not shown).

4. Conclusions

The heterogeneous photocatalysis of MPs in real case effluents of UWWTP was successfully achieved under visible light activation. Among the target MPs found in the UWWTP effluent, the gCN_T photocatalyst showed significantly higher removal rates than the benchmark TiO_2, which can be explained by the suitable E_g and good redox ability of the photogenerated carriers.

Complete removals (to values bellow the limits of quantification) were found in *ca.* 10 min of slurry photocatalytic treatment using gCN_T as catalyst. Moreover, only 5 min were enough to eliminate atenolol, carbamazepine, clopidogrel and diclofenac. As expected, lower efficiencies were observed for the removal of these MPs when the photocatalytic treatment was performed under continuous mode employing gCN_T immobilized on glass rings. Nevertheless, this strategy is important, considering the cost of separation processes and energy requirements for the recovery of the photocatalyst, prior to water reuse or discharge.

The present work showed the potential of gCN_T used in conjunction with visible LEDs to remove several organic MPs present in actual effluents from UWWTP. This can be relevant to the reduction of MPs in UWWTPs, or in other end-of-pipe solutions. It is however clear that parallel to the development of treatment solutions to minimize the impact of MPs on the environment, further optimization is needed.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.apcatb.2019.02.001.

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Appendix D1 – Supplementary information

Metal-free g-C_3N_4 photocatalysis of organic micropollutants in urban wastewater under visible light

Nuno F.F. Moreira, Maria J. Sampaio*, Ana R. Ribeiro, Cláudia G. Silva, Joaquim L. Faria, Adrián M.T. Silva

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Supplementary Material Table of Contents:

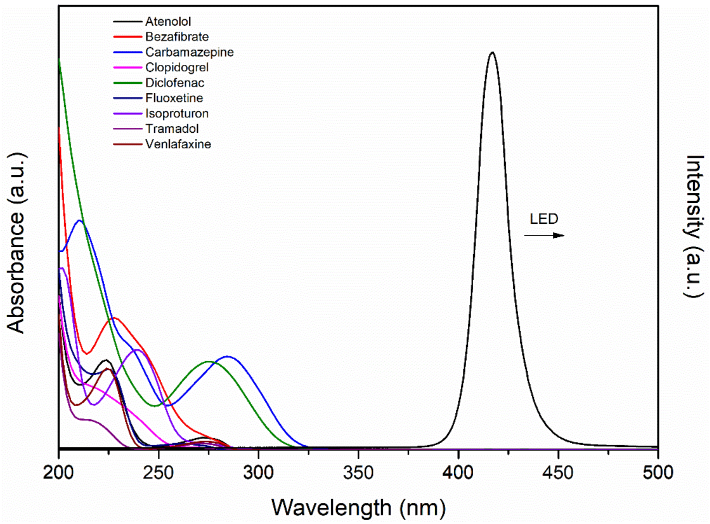

Figure D-S7.1. UV-Vis absorption spectra of the MPs and LED intensity.

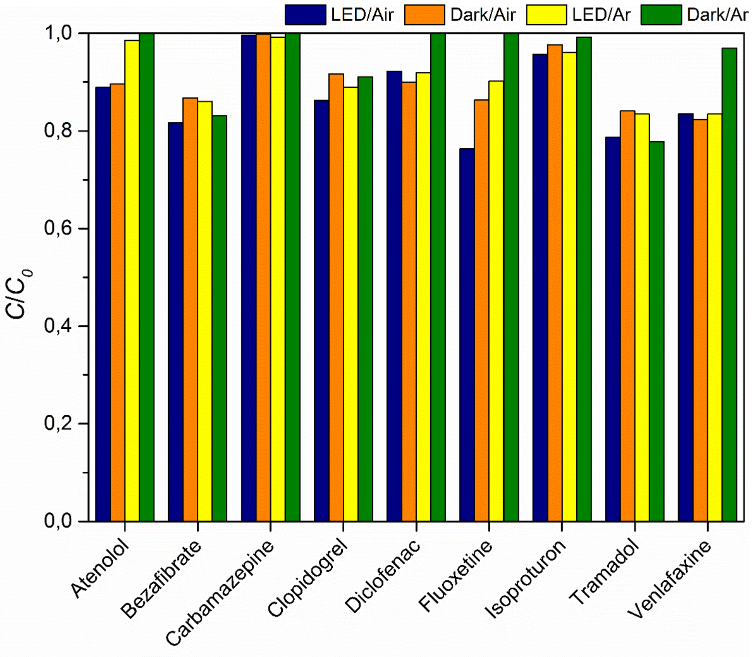

Figure D-S7.2. Normalized concentration of the MPs (spiked in ultrapure water) after 30 min, under LED light irradiation and in the dark under oxygenated (air) and deoxygenated (argon) conditions (standard deviation: < 1.5%).

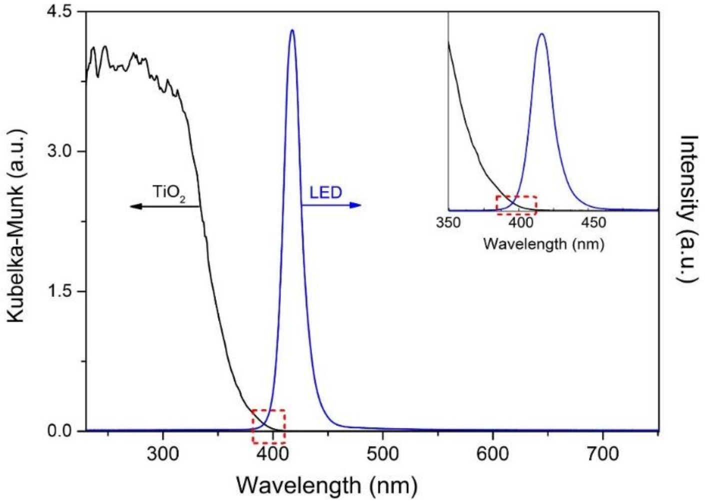

Figure D-S7.3. Diffuse reflectance UV–Vis (DRUV-Vis) spectrum of TiO_2 and LED emission spectrum.

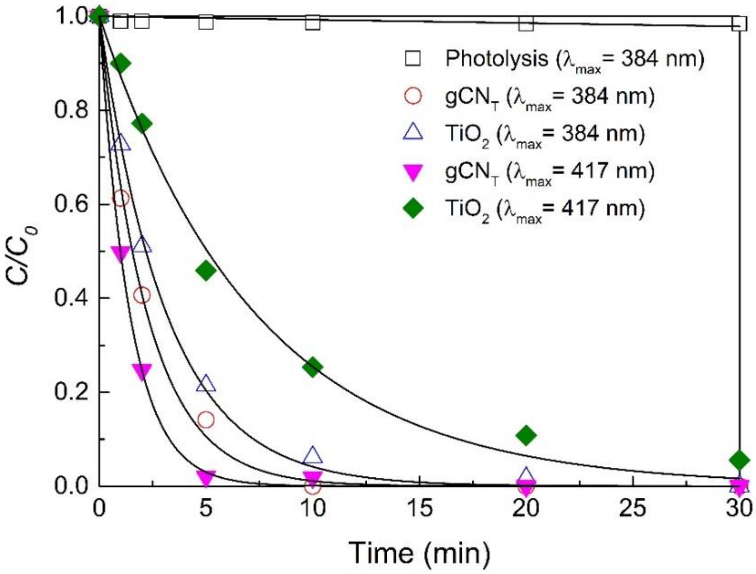

Figure D-S7.4. Normalized concentration of diclofenac (C/C_0) using distilled water as a matrix after treatment by photolysis (a), and by heterogeneous photocatalysis using TiO_2 and gCN_T materials and LEDs (λ_{max} = 384 nm or λ_{max} = 417 nm) as irradiation source. The initial concentration of diclofenac in these experiments was 10 mg L^{-1}.

Table D-S7.1. Physicochemical characterization of the urban wastewater.

Parameter	Value	Parameter	Value	Parameter	Value
DOC (mg L^{-1})	30.0	Na^+ (mg L^{-1})	114.3	BrO_3^- (mg L^{-1})	< 0.05
DIC (mg L^{-1})	77.6	K^+ (mg L^{-1})	22.4	NO_3^- (mg L^{-1})	0.40
Conductivity (μS cm^{-1})	707	NH_4^+ (mg L^{-1})	74.8	NO_2^- (mg L^{-1})	n.d.
pH	7.2	Ca^{2+} (mg L^{-1})	31.3	SO_4^{2-} (mg L^{-1})	45.5
BOD_5 (mg L^{-1})	31	Mg^{2+} (mg L^{-1})	7.65	PO_4^{3-} (mg L^{-1})	9.71
COD (mg L^{-1})	90	Cl^- (mg L^{-1})	695.1		
TSS (mg L^{-1})	35	Br^- (mg L^{-1})	< 0.10		

COD, chemical oxygen demand; BOD_5, 5 day biochemical oxygen demand; DOC, dissolved organic carbon; DIC, dissolved inorganic carbon; TSS, total suspended solids.

Appendix E

Publications & Communications

Appendix E1

Publications in international scientific journals with peer review related to the PhD thesis

acknowledgment to the FCT grant PD/BD/114318/2016 marked with (*)

1. Grehs, B.W.N., Lopes, A.R., **Moreira, N.F.F.**, Fernandes, T., Linton, M.A.O., Silva, A.M.T., Manaia, C.M., Carissimi, E., Nunes, O.C. (2019). Removal of microorganisms and antibiotic resistance genes from treated urban wastewater: A comparison between aluminium sulphate and tannin coagulants, *Water research*, 166, 115056, doi: 10.1016/j.watres.2019.115056 (*).
   (prepared in collaboration under project WaterJPI/0001/2013 STARE: Stopping Antibiotic Resistance Evolution)

2. Iakovides, I.C., Michael-Kordatou, I., **Moreira, N.F.F.**, Ribeiro, A.R., Fernandes, T., Pereira, M.F.R., Nunes, O.C., Manaia, C.M., Silva, A.M.T., Fatta-Kassinos, D. (2019), Continuous ozonation of urban wastewater: Removal of antibiotics, antibiotic-resistant *Escherichia coli* and antibiotic resistance genes and phytotoxicity, *Water Research*, 159, 333-347, doi: 10.1016/j.watres.2019.05.025 (*).
   (prepared in collaboration under project ANSWER: ANtibioticS and mobile resistance elements in WastEwater Reuse applications: risks and innovative solutions, H2020-MSCA-ITN-2015/675530, European Commission)

3. **Moreia, N.F.F.**, Sampaio, M.J., Ribeiro, A.R., Silva, C.G., Faria, J.L., Silva, A.MT. (2019). Metal-free g-C_3N_4 photocatalysis of organic micropollutants in urban wastewater under visible light, *Applied Catalysis B: Environmental*, 248, 184-192, doi: 10.1016/j.apcatb.2019.02.001 (*).
   (corresponds to **Chapter 7**)

4. Biancullo, F., **Moreia, N.F.F.**, Ribeiro, A.R., Manaia, C.M., Faria, J.L., Nunes, O.C., Castro-Silva, S.M., Silva, A.M.T. (2019). Heterogeneous photocatalysis using UVA-LEDs for the removal of antibiotics and antibiotic resistant bacteria from urban wastewater treatment plant effluents, *Chemical Engineering Journal*, 367, 304-313, doi: 10.1016/j.cej.2019.02.012 (*).
   (prepared in collaboration under project ANSWER: ANtibioticS and mobile resistance elements in WastEwater Reuse applications: risks and innovative solutions, H2020-MSCA-ITN-2015/675530, European Commission)

5. Ribeiro, A.R., **Moreira, N.F.F.**, Li Puma, G., Silva, A.M.T. (2019). Impact of water matrix on the removal of micropollutants by advanced oxidation technologies, *Chemical Engineering Journal*, 363, 155-173, doi: 10.1016/j.cej.2019.01.080 (*).
   (partially corresponds to **Chapter 2**, prepared in collaboration under project COST Action ES1403 NEREUS: New and emerging challenges and opportunities in wastewater reuse, COST-European Cooperation in Science and Technology)

6. Chávez, A.M., Ribeiro, A.R., **Moreira, N.F.F.**, Silva, A.M.T., Rey, A., Álvarez, P.M., Beltrán, F.J. (2019). Removal of organic micropollutants from a municipal

wastewater secondary effluent by UVA-LED photocatalytic ozonation, *Catalysts*, 9, 472, doi: 10.3390/catal9050472 (*).
(prepared in collaboration)

7. **Moreira, N. F. F.**, Narciosa-da-Rocha, C., Polo-López, M. I., Pastrana-Martínez, L. M., Faria, J. L., Manaia, C. M., Fernández-Ibáñez, P., Nunes, O. C., Silva, A. M. T. (2018). Solar treatment (H_2O_2, TiO_2-P25 and GO-TiO_2 photocatalysis, photo-Fenton) of organic micropollutants, human pathogen indicators, antibiotic resistant bacteria and related genes in urban wastewater, *Water Research*, 135, 195-206, doi: 10.1016/j.watres.2018.01.064 (*).
(corresponds to **Chapter 6**)

8. Jallouli, N., Pastrana-Martínez, L. M., Ribeiro, A. R., **Moreira, N. F. F.**, Faria, J. L., Hentati, O., Silva, A. M. T., Ksibi, M. (2018). Heterogeneous photocatalytic degradation of ibuprofen in ultrapure water, municipal and pharmaceutical wastewaters using a UV-LEDs/TiO_2 system, *Chemical Engineering Journal*, doi: 10.1016/j.cej.2017.10.045 (*).
(prepared in collaboration)

9. Lopes, A.R., Sousa, V.M., Estevinho, B.N., Leite, J.P, **Moreira, N.F.F.**, Gales, L., Rocha, F., Nunes, O.C. (2016). Production of microparticles of molinate degrading biocatalysts using the spray drying technique, *Chemosphere*, 161, 61-68, doi: 10.1016/j.chemosphere.2016.07.006.
(prepared in collaboration)

10. Barbosa, M., **Moreira, N.F.F.**, Ribeiro, A.R., Pereira, M.F.R., Silva, A.M.T. (2016). Occurrence and removal of organic micropollutants: an overview of the watch list of EU Decision 2015/495, *Water Research*, 94, 257-279, doi: 10.1016/j.watres.2016.02.047 (*).
(prepared in collaboration)

11. **Moreira, N.F.F.**, Sousa, M.J., Macedo, G., Ribeiro, A.R., Barreiros, L., Pedrosa, M., Faria, J.L., Pereira, M.F.R., Castro-Silva, S., Segundo, M.A., Manaia, C.M., Nunes, O.C., Silva, A.M.T. (2016). Photocatalytic ozonation of urban wastewater and surface water using immobilized TiO_2 with LEDs: micropollutants, antibiotic resistance genes and estrogenic activity, *Water Research*, 94, 10-22, doi: 10.1016/j.watres.2016.02.003 (*).
(corresponds to **Chapter 5**)

12. **Moreira, N.F.F.**, Orge, C.A., Ribeiro, A.R., Faria, J.L., Nunes, O.C., Pereira, M.F.R., Silva, A.M.T. (2015). Fast mineralization and detoxification of amoxicillin and diclofenac by photocatalytic ozonation and application to an urban wastewater, *Water Research*, 87, 87-96, doi: 10.1016/j.watres.2015.08.059
(resulting from my Master thesis and prepared in collaboration under project NEPCAT: New EquiPment involving integrated CATalytic processes for treatment of organic pollutants and disinfection of water, NORTE-07-0202-FEDER-038900, corresponds to **Chapter 4**)

13. Ribeiro, A.R., Pedrosa, M., **Moreira, N.F.F.**, Pereira, M.F.R., Silva, A.M.T. (2015). Environmental friendly method for urban wastewater monitoring of micropollutants defined in the Directive 2013/39/EU and Decision 2015/495/EU, *Journal of Chromatography A*, 1418, 140-149, doi: 10.1016/j.chroma.2015.09.057.

(prepared in collaboration under project NEPCAT: New EquiPment involving integrated CATalytic processes for treatment of organic pollutants and disinfection of water, NORTE-07-0202-FEDER-038900)

Appendix E2

Oral communications in scientific meetings

acknowledgment to the FCT grant PD/BD/114318/2016 marked with (*)

1. Iakovides, I.C., **Moreira, N.F.F.**, Fernandes, T., Ribeiro, A.R., Ioannou-Ttofa, L., Michael-Kordatou, I., Pereira, M.F.R. , Manaia, C.M., Nunes, O.C. , Silva, A.M.T., Fatta-Kassinos, D. "Evaluation of the efficiency of the ozonation process in continuous mode for the removal of phytotoxicity, antibiotics and antibiotic resistance from urban wastewater", XENOWAC II, Limassol, Cyprus, October 10-12, 2018 (*).

2. Biancullo, F., **Moreira, N.F.F.**, Ribeiro, A. R., Castro-Silva, S., Nunes, O. C., Faria, J. L., Silva, A.M.T. "Antibiotics and Antibiotic Resistant Bacteria Removal in Urban Wastewater by Heterogeneous Photocatalysis using UVA-LEDs", XENOWAC II, Limassol, Cyprus, October 10-12, 2018.

3. **Moreira, N.F.F.**, Narciosa-da-Rocha, C., Polo-López, M. I., Pastrana-Martínez, L. M., Faria, J. L., Manaia, C. M., Fernández-Ibáñez, P., Nunes, O. C., Silva, A. M. T. "Microbiological quality of treated water by solar-driven oxidation processes", Microbiotec'17, Porto, Portugal, December 7-9, 2017 (*).

4. Jallouli, N., Pastrana-Martínez, L.M., Ribeiro, A.R., **Moreira, N.F.F.**, Faria, J.L., Hentati, O., Silva, A.M.T., Ksibi, M., "Photocatalytic degradation of ibuprofen in pharmaceutical industry wastewater using a TiO_2/UV-LED system", 1st Euro-Mediterranean Conference for Environmental Integration (EMCEI), Sousse, Tunisia, 22-25 November, 2017.

5. Ribeiro, A.R., Gorito, A.M., **Moreira, N.F.F.**, Pereira, M.F.R., Almeida, C.M.R., Silva, A.M.T. "Constructed Wetlands and Advanced Oxidation Processes to Remove Organic Micropollutants" 3rd Iberoamerican Conference On Advanced Oxidation Technologies (III CIPOA) and 2nd Colombian Conference on Advanced Oxidation Processes (II CCPAOX), Medellin (Guatapé), Colombia, 14-17 November 2017 (*).

6. **Moreira, N.F.F.**, Narciosa-da-Rocha, C., Polo-López, M. I., Pastrana-Martínez, L. M., Faria, J. L., Manaia, C. M., Fernández-Ibáñez, P., Nunes, O. C., Silva, A. M. T. "Chemical and biological response of H_2O_2-assisted heterogeneous photocatalytic systems used on effluents from urban wastewater treatment plants", Europacat 2017, Florence, Italy, August 27-31, 2017 (*).

7. **Moreira, N.F.F.**, Narciosa-da-Rocha, C., Polo-López, M. I., Pastrana-Martínez, L. M., Faria, J. L., Manaia, C. M., Fernández-Ibáñez, P., Nunes, O. C., Silva, A. M. T. "Solar Advanced Oxidation Processes for the Treatment of Urban Wastewater: Chemical and Biological Response", 2nd Summer School on Environmental Applications of Advanced Oxidation Processes and Training School on Advanced Treatment Technologies and Contaminants of Emerging Concern, Porto, Portugal, July 10-14, 2017 (*).

8. **Moreira, N.F.F.**, Narciosa-da-Rocha, C., Polo-López, M. I., Pastrana-Martínez, L. M., Faria, J. L., Manaia, C. M., Fernández-Ibáñez, P., Nunes, O. C., Silva, A. M. T. “Pilot-scale H_2O_2-assisted heterogeneous photocatalytic treatment of effluents from urban wastewater treatment plants”, 2nd Doctoral Congress of Engineering, Porto, Portugal, June 8-9, 2017 (*).

9. **Moreira, N. F. F.**, Sousa, J. M., Macedo G., Ribeiro, A. R., Barreiros, L., Pedrosa, M., Faria, J. L., Pereira, M. F. R., Castro-Silva, S., Segundo, M. A., Manaia, C. M., Nunes, O. C., Silva, A. M. T. “Micropollutants, Potential Pathogens and Antibiotic Resistance Genes Inactivation by Photocatalytic Ozonation Using LEDs”, SPEA9, Strasbourg, France, June 13-17, 2016 (*).

10. Ribeiro, A.R., Pedrosa, M., **Moreira, N.F.F.**, Pereira, M.F.R., Silva, A.M.T., “Eco-friendly SPE-UHPLC-MS/MS method for wastewater monitoring of micropollutants”, XVI Latin-American Congress on Chromatography (XVI COLACRO) & 9th National Meeting on Chromatography (9ENC), p. 27, Faculdade de Ciências da Universidade de Lisboa, Portugal, January 5-9, 2016.

11. **Moreira, N.F.F.**, Orge, C.A., Faria, J.L., Nunes, O.C., Pereira, M.F.R., Silva, A.M.T. “Integrated catalytic processes for the treatment of organic pollutants and inactivation of microorganisms”, XX Encontro Luso-Galego de Química, Porto, Portugal, November 26-28, 2014.

Appendix E3

Poster communications in scientific meetings

acknowledgment to the FCT grant PD/BD/114318/2016 marked with (*)

1. **Moreira, N.F.F.**, Viana, A.T., Graça, C.A.L., Ribeiro, A.R., Pereira, M.F.R., Silva, A.M.T., Nunes, O.C. "Are ozonation by-products the main regrowth drivers after wastewater treatment?", BioRemid2019, Porto, Portugal, October 24-25, 2019 (*).

2. Ribeiro, A.R., **Moreira, N.F.F.**, Li Puma, G., Silva, A.M.T. "Impact of water matrix on the effectiveness of advanced oxidation technologies", 6th European Conference on ENVIRONMENTAL APPLICATIONS OF ADVANCED OXIDATION PROCESSES, Portoroz-Portorose, Slovenia, June 26-30, 2019 (*).

3. Ribeiro, A.R., **Moreira, N.F.F.**, Barbosa, M. O., Pereira, M.F.R., Li Puma, G., Silva, A. M. T. "Removal of micropollutants by ozonation in wastewater matrices", XENOWAC II, Limassol, Cyprus, October 10-12, 2018 (*).

4. **Moreira, N.F.F.**, Narciosa-da-Rocha, C., Polo-López, M. I., Pastrana-Martínez, L. M., Faria, J. L., Manaia, C. M., Fernández-Ibáñez, P., Nunes, O. C., Silva, A. M. T. "Urban wastewater treatment by solar advanced oxidation processes: chemical and biological response", SPEA10, Almeria, Spain, June 4-8, 2018 (*).

5. Biancullo, F., **Moreira, N.F.F.**, Ribeiro, A.R., Faria, J. L., Castro-Silva, S., Nunes, O. C., Silva, A.M.T. "UVA-LEDs Heterogeneous Photocatalysis for the Removal of Antibiotics and Antibiotic Resistant Bacteria in Municipal Wastewater", SPEA10, Almeria, Spain, June 4-8, 2018 (*).

6. Biancullo, F., **Moreira, N.F.F.**, Ribeiro, A.R., Faria, J. L., Castro-Silva, S., Silva, A.M.T. "Removal of Organic Micropollutants in Urban Wastewater by using UV-LEDs Heterogeneous Photocatalysis" 18th European Meeting on Environmental Chemistry (EMEC), Porto, Portugal, 26-29 November 2017 (*).

7. Gorito, A. M., **Moreira, N.F.F.**, Ribeiro, A. R., Pereira, M. F. R., Almeida C. M. R., Silva, A.M.T. "Coupling Constructed Wetlands and Advanced Oxidation Processes to Remove Organic Micropollutants", 2nd Summer School on Environmental Applications of Advanced Oxidation Processes and Training School on Advanced Treatment Technologies and Contaminants of Emerging Concern, Porto, Portugal, July 10-14, 2017(*).

Appendix F

Appendix F1 *- Fast mineralization and detoxification of amoxicillin and diclofenac by photocatalytic ozonation and application to an urban wastewater, Water Research* 87 (2015) 87-96, doi: 10.1016/j.watres.2015.08.059

(Authors: Nuno F. F. Moreira, Carla A. Orge. Ana R. Ribeiro, Joaquim L. Faria, Olga C. Nunes, M. Fernando R. Pereira, Adrián M. T. Silva)

Appendix F2 - *Photocatalytic ozonation of urban wastewater and surface water using immobilized* TiO_2 *with LEDs: Micropollutants, antibiotic resistance genes and estrogenic activity, Water Research* 94 (2016) 10-22, doi: 10.1016/j.watres.2016.02.003

(Authors: Nuno F. F. Moreira, José M. Sousa, Gonçalo Macedo, Ana R. Ribeiro, Luisa Barreiros, Marta Pedrosa, Joaquim L. Faria, M. Fernando R. Pereira, Sérgio Castro-Silva, Marcela A. Segundo, Célia M. Manaia, Olga C. Nunes, Adrián M.T. Silva)

Appendix F3 - *Solar treatment (*H_2O_2*,* TiO_2*-P25 and GO-*TiO_2 *photocatalysis, photo-Fenton) of organic micropollutants, human pathogen indicators, antibiotic resistant bacteria and related genes in urban wastewater, Water Research* 135 (2018) 195-206, doi: 10.1016/j.watres.2018.01.064

(Authors: Nuno F. F. Moreira, Carlos Narciso-da-Rocha, M. Inmaculada Polo-López, Luisa M. Pastrana-Martínez, Joaquim L. Faria, Célia M. Manaia, Pilar Fernández-Ibáñez, Olga C. Nunes, Adrián M. T. Silva)

Appendix F4 - *Metal-free g-*C_3N_4 *photocatalysis of organic micropollutants in urban wastewater under visible light, Applied Catalysis B: Environmental* 248 (2019) 184-192, doi: 10.1016/j.apcatb.2019.02.001

(Authors: Nuno F. F. Moreira, Maria J. Sampaio, Ana R. Ribeiro, Cláudia G. Silva, Joaquim L. Faria, Adrián M. T. Silva)

Appendix F5 - *Impact of water matrix on the removal of micropollutants by advanced oxidation technologies, Chemical Engineering Journal* 363 (2019) 155-173, doi: 10.1016/j.cej.2019.01.080

(Authors: Ana R. Ribeiro, Nuno F. F. Moreira, Gianluca Li Puma, Adrián M. T. Silva)

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11/05/2020

To Whom it May Concern

Carla Alexandra Orge Fonseca, PhD, Associate Laboratory LSRE-LCM, Faculty of Engineering – University of Porto (FEUP), hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled "Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes", as a result of his research activity:

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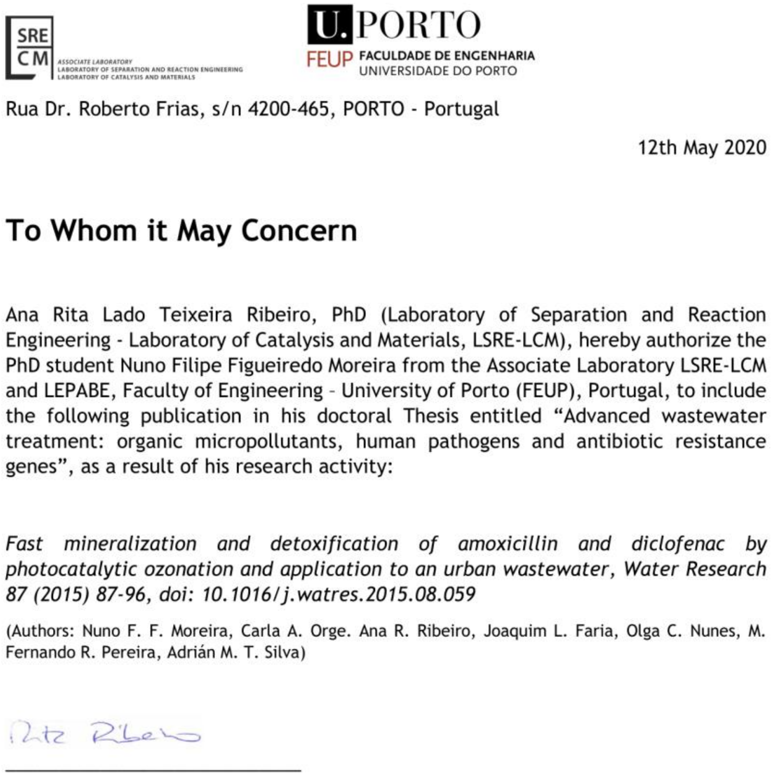
(Authors: Nuno F. F. Moreira, Carla A. Orge. Ana R. Ribeiro, Joaquim L. Faria, Olga C. Nunes, M. Fernando R. Pereira, Adrián M. T. Silva)

Carla Alexandra Orge Fonseca



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12th May 2020

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Ana Rita Lado Teixeira Ribeiro, PhD (Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials, LSRE-LCM), hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled “Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes”, as a result of his research activity:

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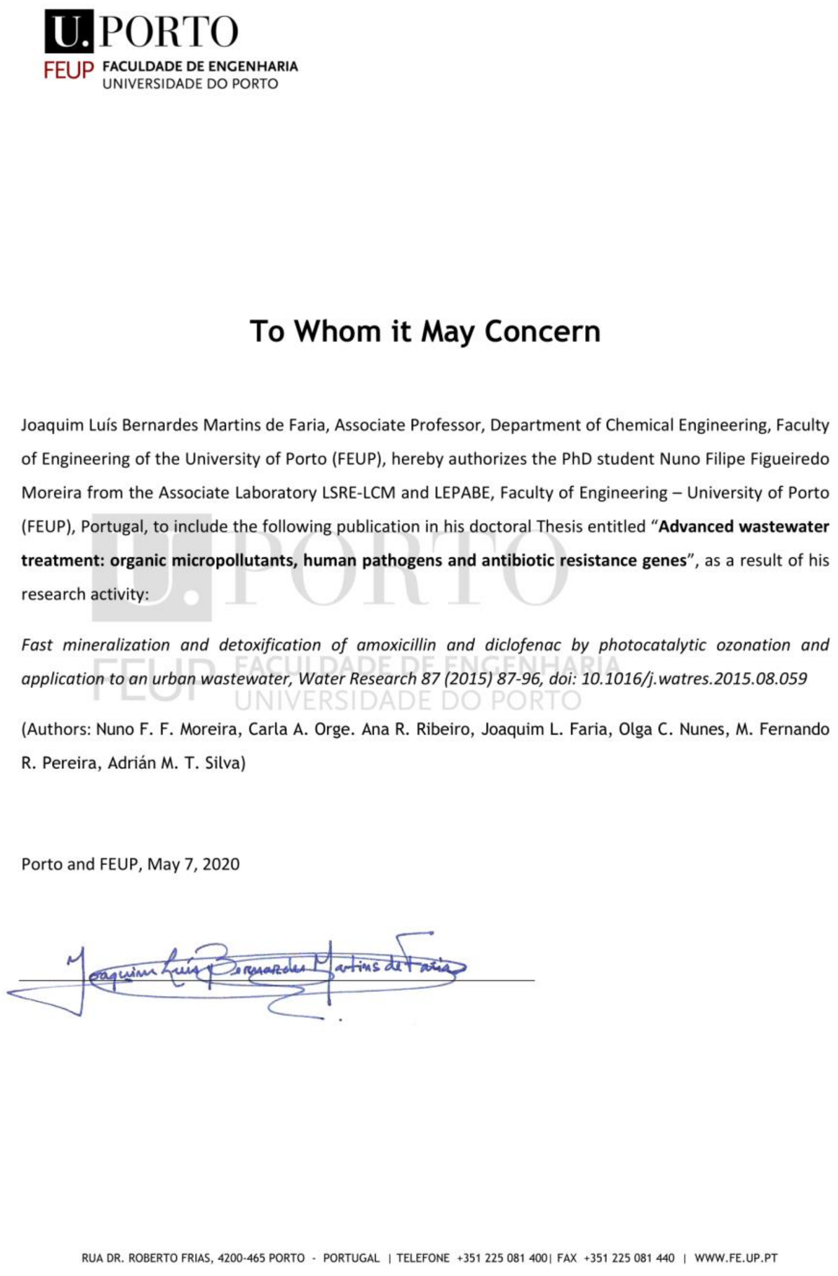
(Authors: Nuno F. F. Moreira, Carla A. Orge. Ana R. Ribeiro, Joaquim L. Faria, Olga C. Nunes, M. Fernando R. Pereira, Adrián M. T. Silva)

Rita Ribeiro



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To Whom it May Concern

Joaquim Luís Bernardes Martins de Faria, Associate Professor, Department of Chemical Engineering, Faculty of Engineering of the University of Porto (FEUP), hereby authorizes the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled "**Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes**", as a result of his research activity:

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(Authors: Nuno F. F. Moreira, Carla A. Orge. Ana R. Ribeiro, Joaquim L. Faria, Olga C. Nunes, M. Fernando R. Pereira, Adrián M. T. Silva)

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Porto and FEUP, May 7, 2020

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To Whom it May Concern

I José Mário de Sousa, MSc in Bioengineering, hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled "Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes", as a result of his research activity:

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(Authors: Nuno F. F. Moreira, José M. Sousa, Gonçalo Macedo, Ana R. Ribeiro, Luisa Barreiros, Marta Pedrosa, Joaquim L. Faria, M. Fernando R. Pereira, Sérgio Castro-Silva, Marcela A. Segundo, Célia M. Manaia, Olga C. Nunes, Adrián M.T. Silva)

José Mário de Sousa

Sousa

11th May 2020

To Whom it May Concern

Gonçalo Nuno Barroca de Macedo, MSc., hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled “Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes”, as a result of his research activity:

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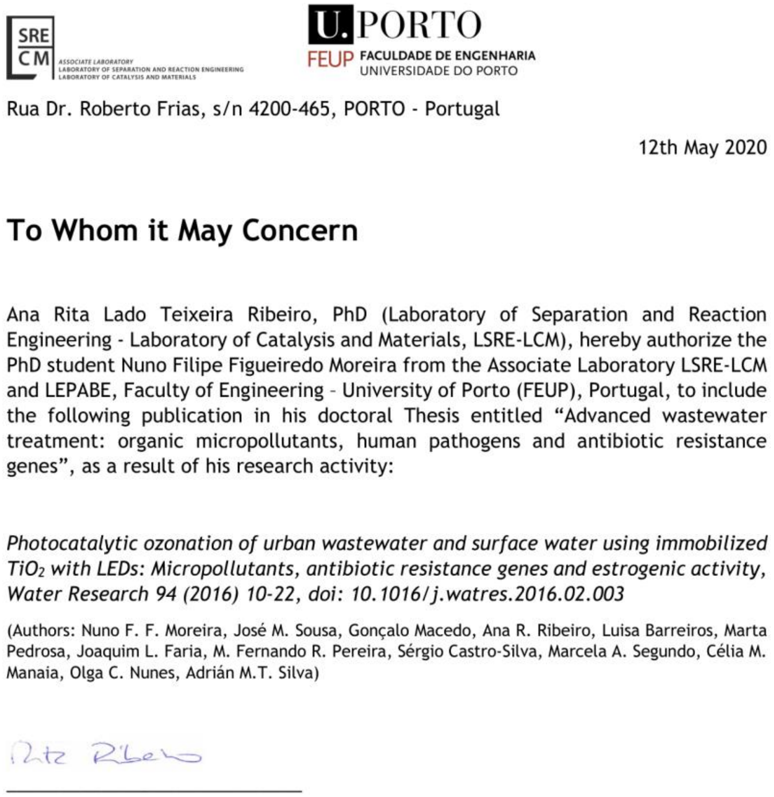
Gonçalo Macedo



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To Whom it May Concern

Ana Rita Lado Teixeira Ribeiro, PhD (Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials, LSRE-LCM), hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled “Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes”, as a result of his research activity:

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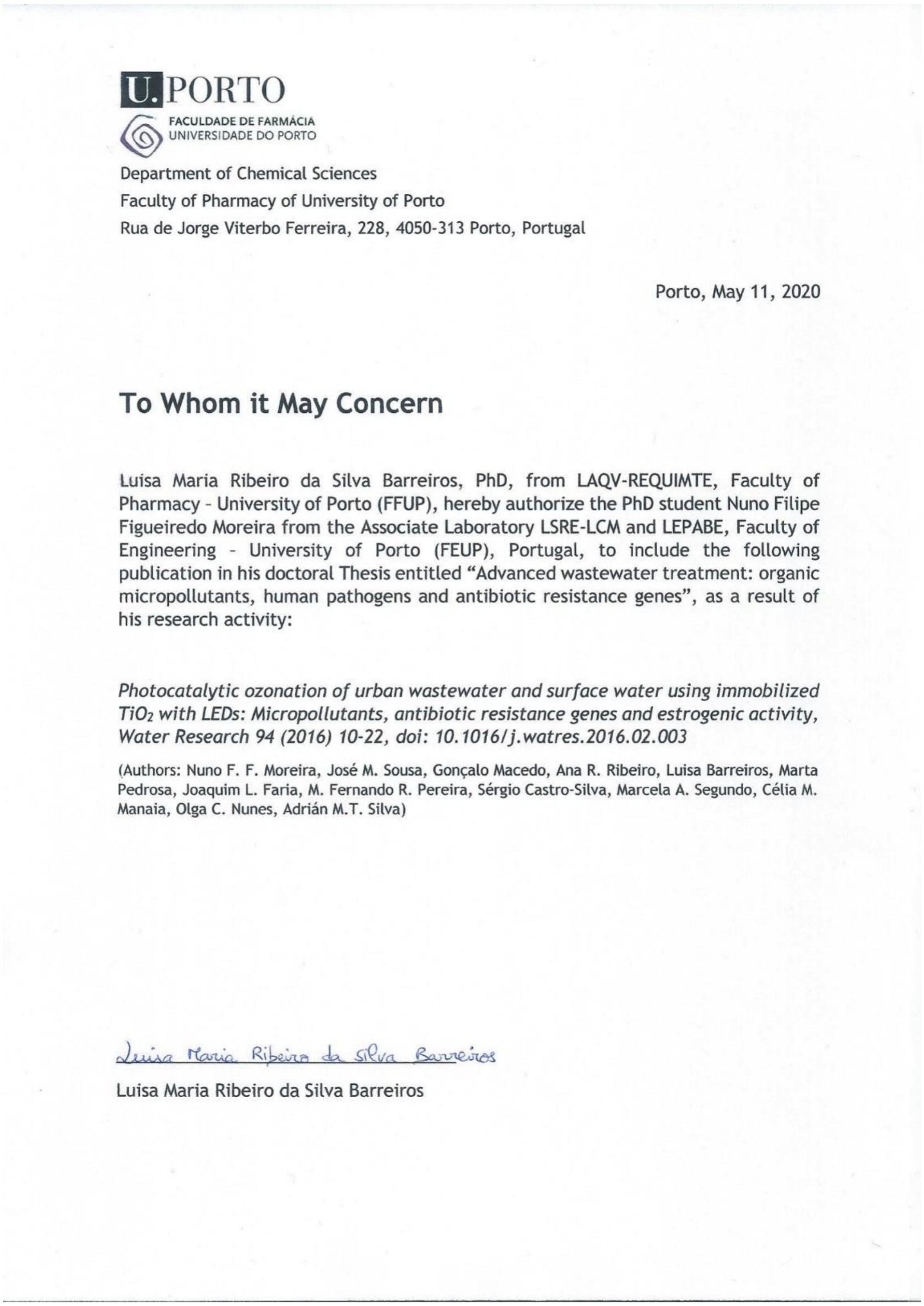
Rita Ribeiro



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Porto, May 11, 2020

To Whom it May Concern

Luisa Maria Ribeiro da Silva Barreiros, PhD, from LAQV-REQUIMTE, Faculty of Pharmacy - University of Porto (FFUP), hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering - University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled “Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes”, as a result of his research activity:

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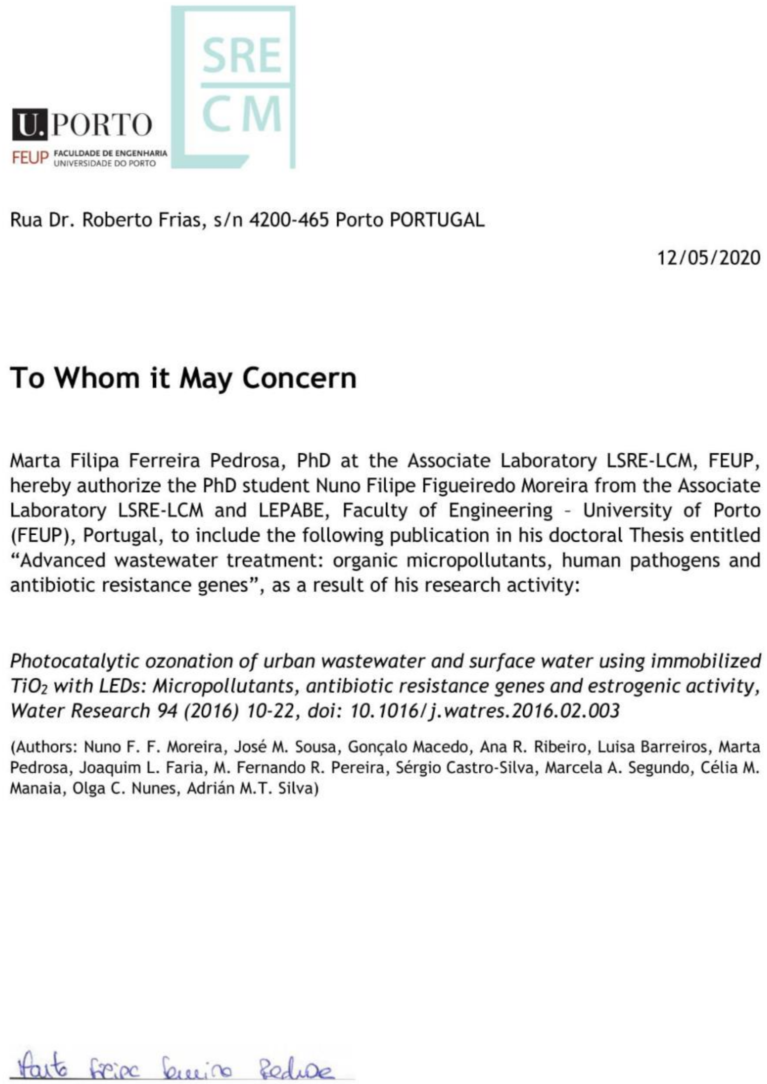
Luisa Maria Ribeiro da Silva Barreiros

Luisa Maria Ribeiro da Silva Barreiros



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12/05/2020

To Whom it May Concern

Marta Filipa Ferreira Pedrosa, PhD at the Associate Laboratory LSRE-LCM, FEUP, hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled "Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes", as a result of his research activity:

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(Authors: Nuno F. F. Moreira, José M. Sousa, Gonçalo Macedo, Ana R. Ribeiro, Luisa Barreiros, Marta Pedrosa, Joaquim L. Faria, M. Fernando R. Pereira, Sérgio Castro-Silva, Marcela A. Segundo, Célia M. Manaia, Olga C. Nunes, Adrián M.T. Silva)

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Joaquim Luís Bernardes Martins de Faria, Associate Professor, Department of Chemical Engineering, Faculty of Engineering of the University of Porto (FEUP), hereby authorizes the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled "**Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes**", as a result of his research activity:

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(Authors: Nuno F. F. Moreira, José M. Sousa, Gonçalo Macedo, Ana R. Ribeiro, Luisa Barreiros, Marta Pedrosa, Joaquim L. Faria, M. Fernando R. Pereira, Sérgio Castro-Silva, Marcela A. Segundo, Célia M. Manaia, Olga C. Nunes, Adrián M.T. Silva)

Porto and FEUP, May 7, 2020

Joaquim Luís Bernardes Martins de Faria

To Whom it May Concern

Sérgio Manuel Castro Silva, Adventech, Lda, hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled "Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes", as a result of his research activity:

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São João da Madeira, Portugal
11 May 2020

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Porto, May 13th, 2020

To Whom it May Concern

Marcela Alves Segundo, *Professor Auxiliar*, Faculty of Pharmacy – University of Porto (FFUP), hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled "Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes", as a result of his research activity:

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Célia Manaia (Associate Professor at ESB/UCP) hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled “Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes”, as a result of his research activity:

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Porto, 18th May 2020

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Professora Associada
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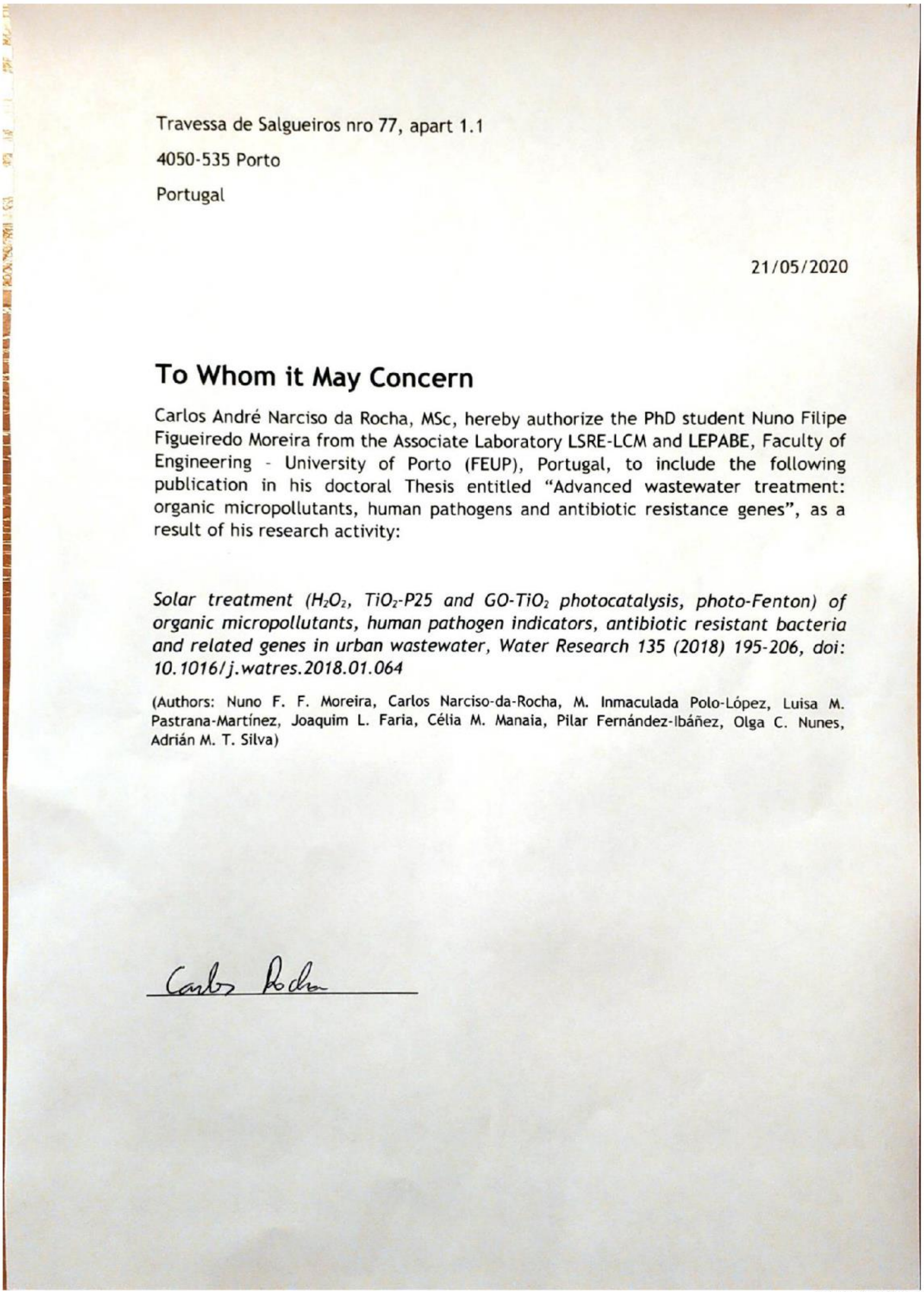

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21/05/2020

To Whom it May Concern

Carlos André Narciso da Rocha, MSc, hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering - University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled “Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes”, as a result of his research activity:

Solar treatment (H_2O_2, TiO_2-P25 and GO-TiO_2 photocatalysis, photo-Fenton) of organic micropollutants, human pathogen indicators, antibiotic resistant bacteria and related genes in urban wastewater, Water Research 135 (2018) 195-206, doi: 10.1016/j.watres.2018.01.064

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Carlos Rocha

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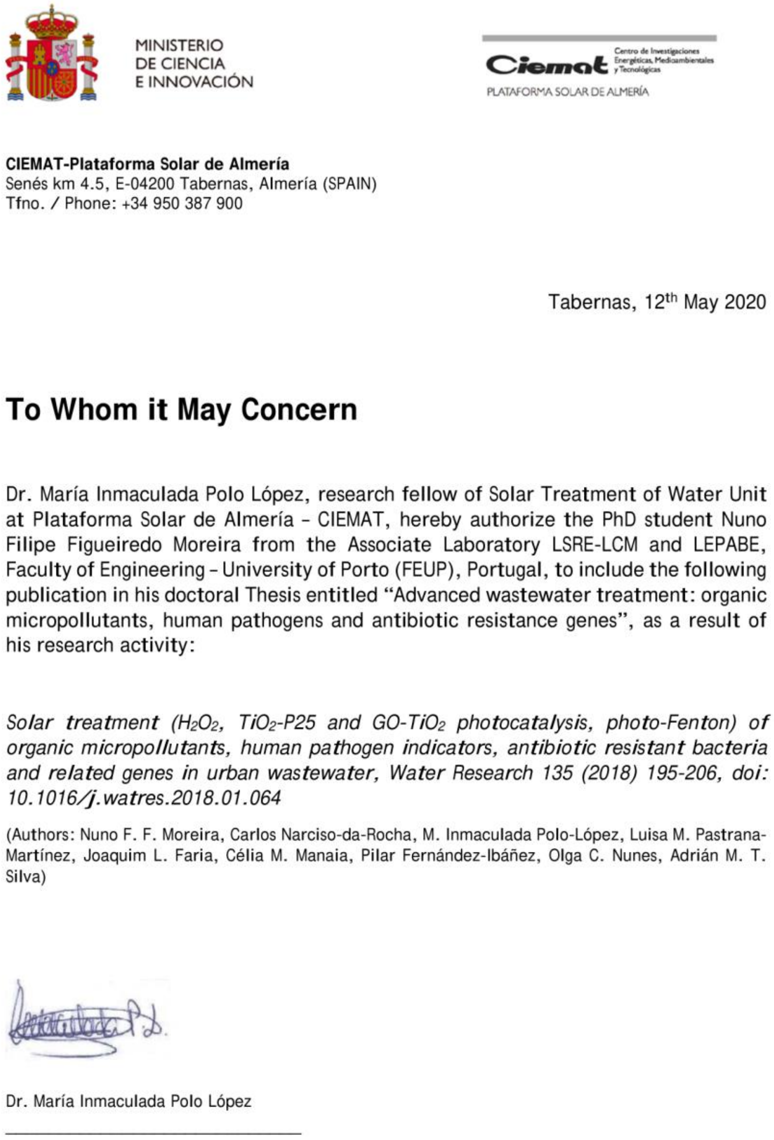

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Tabernas, 12th May 2020

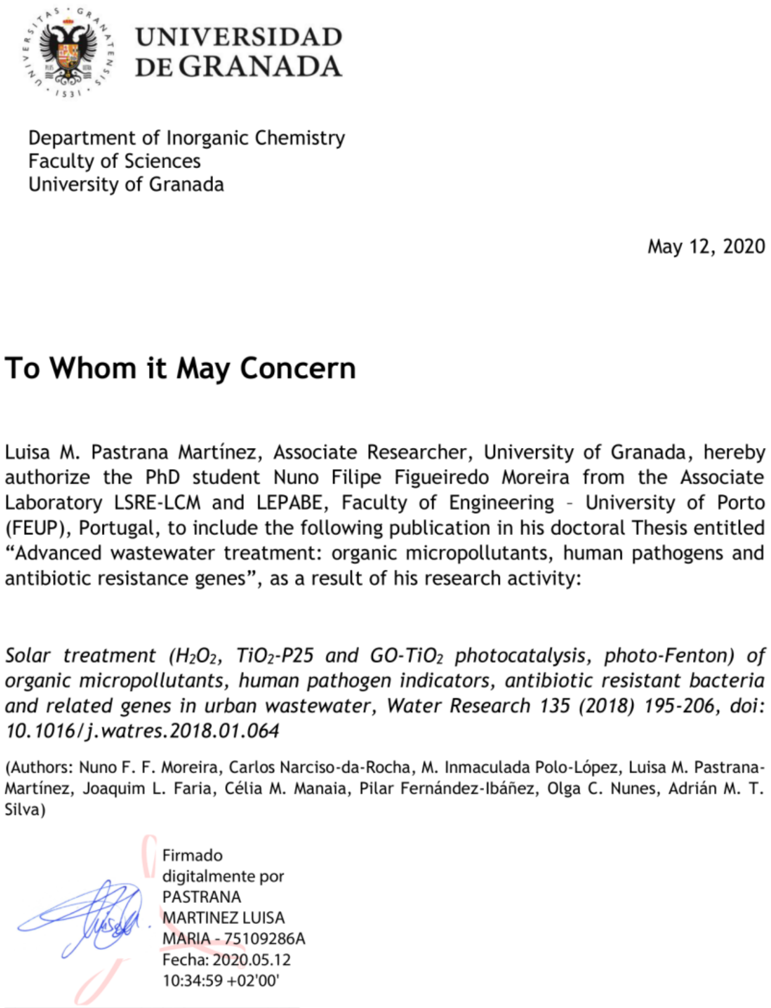
To Whom it May Concern

Dr. María Inmaculada Polo López, research fellow of Solar Treatment of Water Unit at Plataforma Solar de Almería – CIEMAT, hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled "Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes", as a result of his research activity:

Solar treatment (H_2O_2, TiO_2-P25 and GO-TiO_2 photocatalysis, photo-Fenton) of organic micropollutants, human pathogen indicators, antibiotic resistant bacteria and related genes in urban wastewater, Water Research 135 (2018) 195-206, doi: 10.1016/j.watres.2018.01.064

(Authors: Nuno F. F. Moreira, Carlos Narciso-da-Rocha, M. Inmaculada Polo-López, Luisa M. Pastrana-Martínez, Joaquim L. Faria, Célia M. Manaia, Pilar Fernández-Ibáñez, Olga C. Nunes, Adrián M. T. Silva)

Dr. María Inmaculada Polo López



UNIVERSIDAD
DE GRANADA

Department of Inorganic Chemistry
Faculty of Sciences
University of Granada

May 12, 2020

To Whom it May Concern

Luisa M. Pastrana Martínez, Associate Researcher, University of Granada, hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled “Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes”, as a result of his research activity:

Solar treatment (H_2O_2, TiO_2-P25 and GO-TiO_2 photocatalysis, photo-Fenton) of organic micropollutants, human pathogen indicators, antibiotic resistant bacteria and related genes in urban wastewater, Water Research 135 (2018) 195-206, doi: 10.1016/j.watres.2018.01.064

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To Whom it May Concern

Joaquim Luís Bernardes Martins de Faria, Associate Professor, Department of Chemical Engineering, Faculty of Engineering of the University of Porto (FEUP), hereby authorizes the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled "**Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes**", as a result of his research activity:

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Porto and FEUP, May 7, 2020

Joaquim Luís Bernardes Martins de Faria

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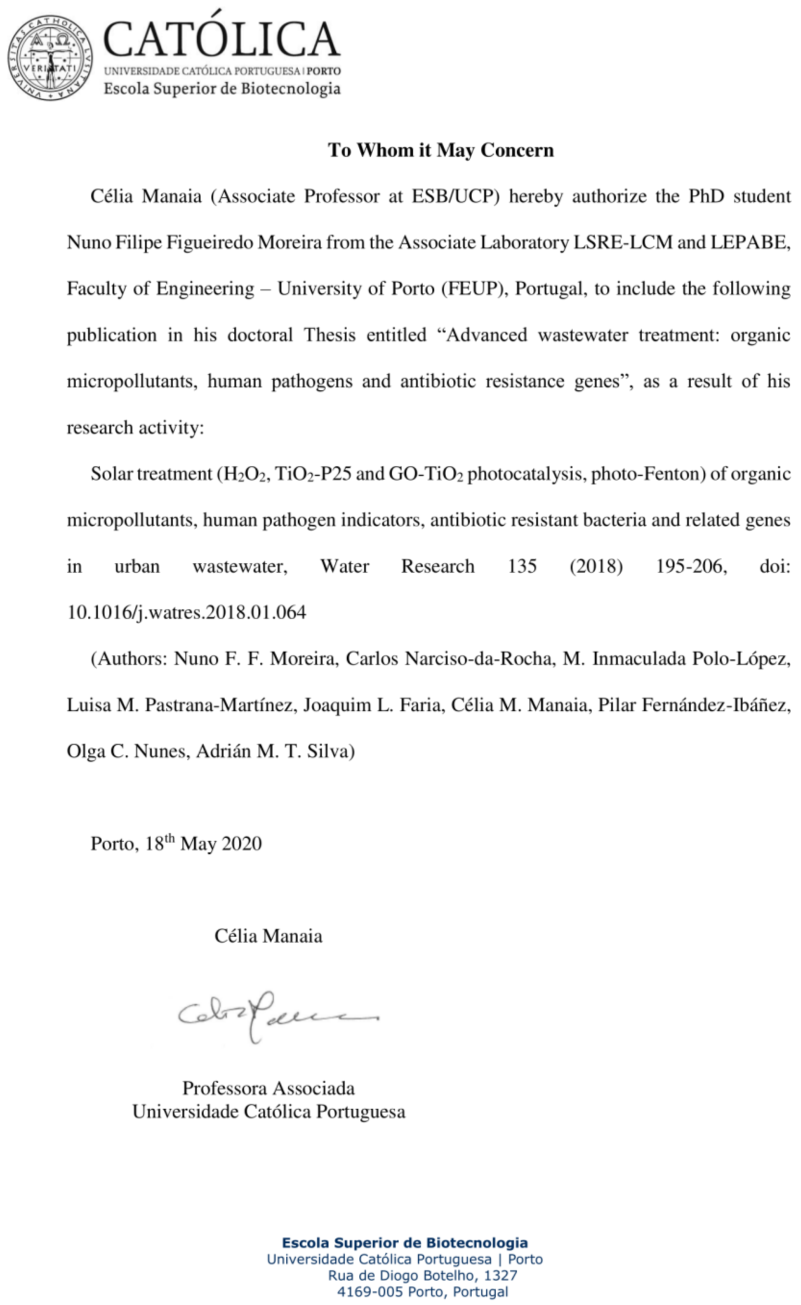

CATÓLICA
UNIVERSIDADE CATÓLICA PORTUGUESA | PORTO
Escola Superior de Biotecnologia

To Whom it May Concern

Célia Manaia (Associate Professor at ESB/UCP) hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled “Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes”, as a result of his research activity:

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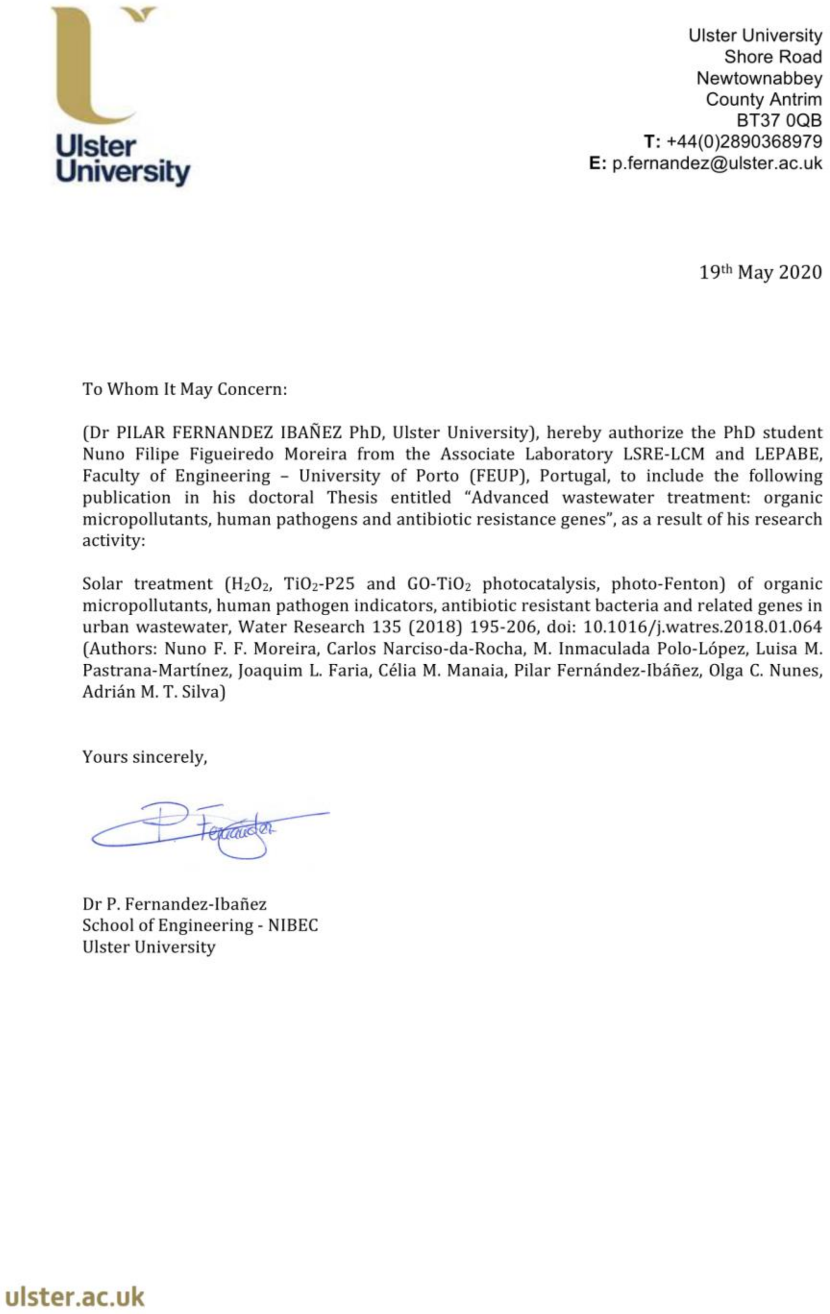
Porto, 18th May 2020

Célia Manaia

Professora Associada
Universidade Católica Portuguesa

Escola Superior de Biotecnologia
Universidade Católica Portuguesa | Porto
Rua de Diogo Botelho, 1327
4169-005 Porto, Portugal



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E: p.fernandez@ulster.ac.uk

19th May 2020

To Whom It May Concern:

(Dr PILAR FERNANDEZ IBAÑEZ PhD, Ulster University), hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled "Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes", as a result of his research activity:

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Yours sincerely,

Dr P. Fernandez-Ibañez
School of Engineering - NIBEC
Ulster University

ulster.ac.uk

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UNIVERSIDADE DO PORTO

Rua Dr. Roberto Frias s/n, Porto, 4200-465,
Portugal

11th of May, 2020

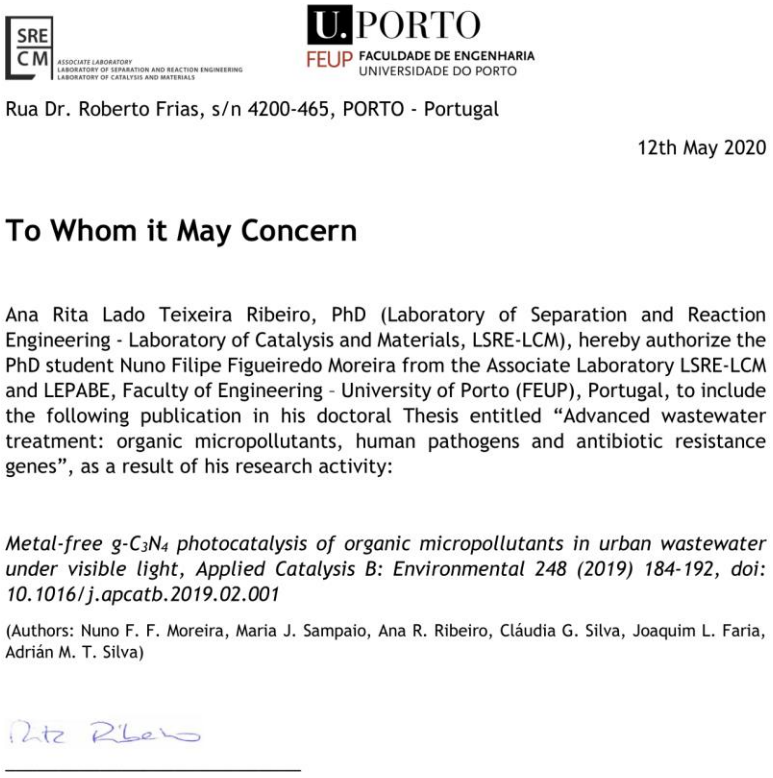
To Whom it May Concern

Maria José Fernandes Sampaio, PhD, at Laboratory of Separation and Reaction Engineering – Laboratory of Catalysis and Materials (LSRE-LCM), Departamento de Engenharia Química, Faculdade de Engenharia, Universidade do Porto, hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled "Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes", as a result of his research activity:

Metal-free g-C_3N_4 *photocatalysis of organic micropollutants in urban wastewater under visible light, Applied Catalysis B: Environmental 248 (2019) 184-192, doi: 10.1016/j.apcatb.2019.02.001*

(Authors: Nuno F. F. Moreira, Maria J. Sampaio, Ana R. Ribeiro, Cláudia G. Silva, Joaquim L. Faria, Adrián M. T. Silva)

Maria José Fernandes Sampaio



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LABORATORY OF CATALYSIS AND MATERIALS

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UNIVERSIDADE DO PORTO

Rua Dr. Roberto Frias, s/n 4200-465, PORTO - Portugal

12th May 2020

To Whom it May Concern

Ana Rita Lado Teixeira Ribeiro, PhD (Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials, LSRE-LCM), hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled “Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes”, as a result of his research activity:

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(Authors: Nuno F. F. Moreira, Maria J. Sampaio, Ana R. Ribeiro, Cláudia G. Silva, Joaquim L. Faria, Adrián M. T. Silva)

Rita Ribeiro

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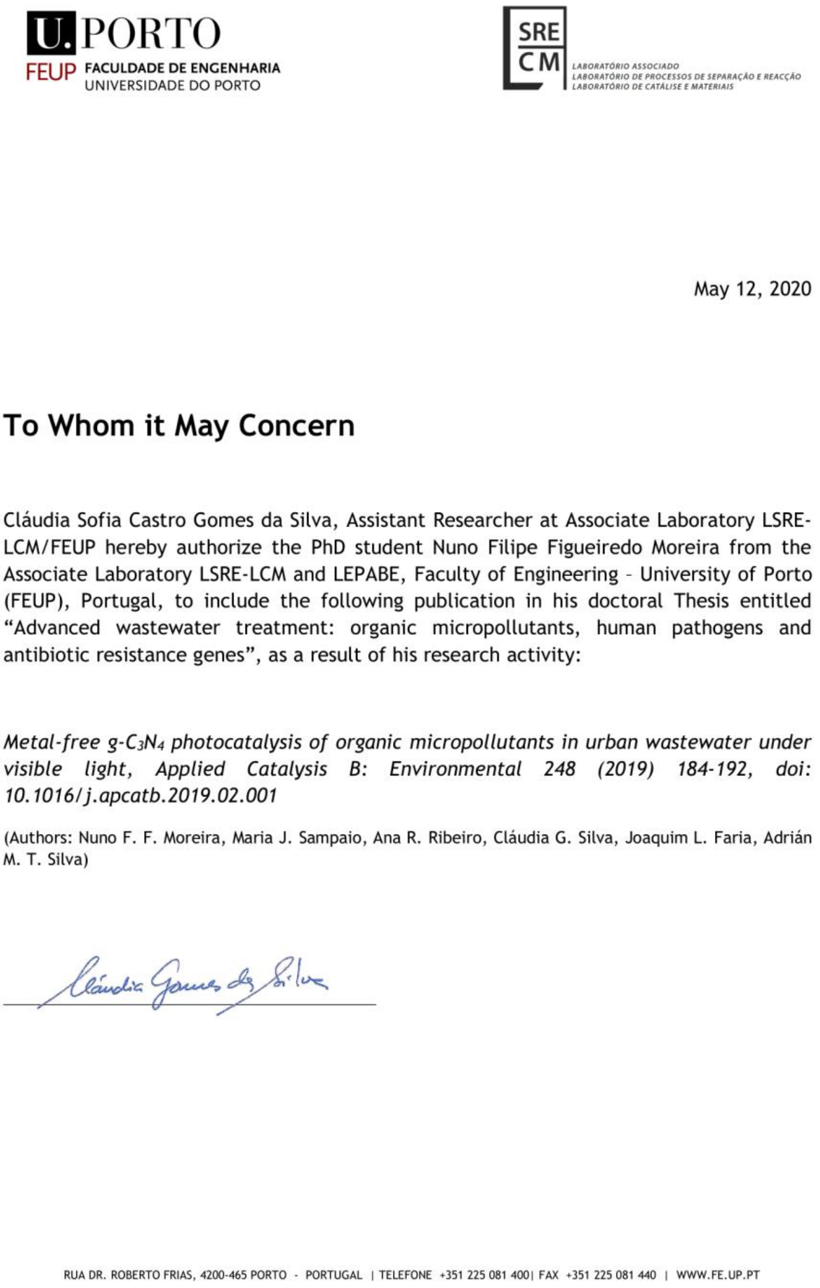

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LABORATÓRIO DE PROCESSOS DE SEPARAÇÃO E REACÇÃO
LABORATÓRIO DE CATÁLISE E MATERIAIS

May 12, 2020

To Whom it May Concern

Cláudia Sofia Castro Gomes da Silva, Assistant Researcher at Associate Laboratory LSRE-LCM/FEUP hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled "Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes", as a result of his research activity:

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(Authors: Nuno F. F. Moreira, Maria J. Sampaio, Ana R. Ribeiro, Cláudia G. Silva, Joaquim L. Faria, Adrián M. T. Silva)

Cláudia Gomes da Silva

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To Whom it May Concern

Joaquim Luís Bernardes Martins de Faria, Associate Professor, Department of Chemical Engineering, Faculty of Engineering of the University of Porto (FEUP), hereby authorizes the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled "**Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes**", as a result of his research activity:

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(Authors: Nuno F. F. Moreira, Maria J. Sampaio, Ana R. Ribeiro, Cláudia G. Silva, Joaquim L. Faria, Adrián M. T. Silva)

Porto and FEUP, May 7, 2020

Joaquim Luís Bernardes Martins de Faria

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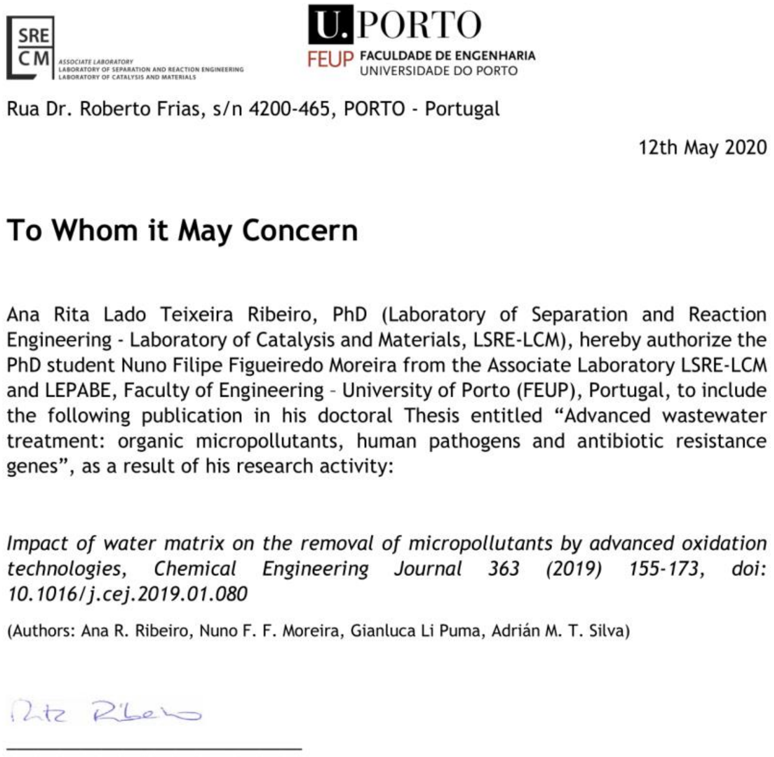

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Rua Dr. Roberto Frias, s/n 4200-465, PORTO - Portugal

12th May 2020

To Whom it May Concern

Ana Rita Lado Teixeira Ribeiro, PhD (Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials, LSRE-LCM), hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled “Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes”, as a result of his research activity:

Impact of water matrix on the removal of micropollutants by advanced oxidation technologies, Chemical Engineering Journal 363 (2019) 155-173, doi: 10.1016/j.cej.2019.01.080

(Authors: Ana R. Ribeiro, Nuno F. F. Moreira, Gianluca Li Puma, Adrián M. T. Silva)

Rita Ribeiro

Loughborough
University

Loughborough LE11 3TU, United Kingdom

12th May 2020

To Whom it May Concern

Gianluca Li Puma, PhD (Loughborough University), hereby authorize the PhD student Nuno Filipe Figueiredo Moreira from the Associate Laboratory LSRE-LCM and LEPABE, Faculty of Engineering – University of Porto (FEUP), Portugal, to include the following publication in his doctoral Thesis entitled “Advanced wastewater treatment: organic micropollutants, human pathogens and antibiotic resistance genes”, as a result of his research activity:

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(Authors: Ana R. Ribeiro, Nuno F. F. Moreira, Gianluca Li Puma, Adrián M. T. Silva)

Gianluca Li Puma, PhD
Professor of Chemical and Environmental Engineering
Environmental Nanocatalysis & Photoreaction Engineering
Department of Chemical Engineering
Loughborough University, United Kingdom