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ANALYSIS OF PHARMACEUTICAL RESIDUES IN WASTEWATERS, SURFACE AND DRINKING WATERS - STUDY OF THE REMOVAL EFFICIENCY THROUGH CONVENTIONAL AND ADVANCED TREATMENT PROCESSES

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LSRE – Laboratory of Separation and Reaction Engineering, Associate Laboratory LSRE/LCM - Department of Chemical Engineering, Faculty of Engineering of the University of Porto, Porto, Portugal

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"What we are doing is nothing but a drop in the ocean.
But if we didn't do it, the ocean would be less."
Mother Teresa of Calcutta

Abstract

The Water Framework Directive (WFD) was created and adopted as an operational tool for quality recovery, protection and assurance of the sustainable use of all water bodies in the European Union (EU). Recently, special focus has been paid to the so-called "emerging pollutants" (EPs), many of which are ubiquitously distributed in the aquatic compartment. Among them, pharmaceutically active substances have generated rising awareness owing to their huge variety and consumption, recognised (eco)toxicity, as well as their unpredictable environmental impact, even at low concentration levels.

Pharmaceutical compounds reach the aquatic environment mainly through effluent discharges in wastewater treatment plants (WWTPs). Given their high consumption and the increasing amount of identified metabolites and transformation products (TPs), complex mixtures or "cocktails" of these pollutants are expected to be found in the aquatic compartment. Therefore, the first stage of this work consisted on the development and validation of an offline solid-phase extraction with simultaneous cleanup capability, followed by liquid chromatography–(electrospray ionisation)–ion trap mass spectrometry, enabling the concurrent determination of 23 pharmaceuticals of diverse chemical nature, among the most consumed in Portugal, in wastewater samples. Several cleanup strategies were assayed, exploiting the physical and chemical properties of the analytes vs. interferences. After testing all combinations of adsorbents and elution solvents, the best results were achieved with the mixed-anion exchange Oasis MAX cartridges. They provided recovery rates generally higher than 60%. The precision of the method ranged from 2% to 18% and 4% to 19% (except for diclofenac (22%) and simvastatin (26%)) for intra- and inter-day analyses, respectively. Method detection limits varied between 1 and 20 ng L^{-1} (excluding ibuprofen).

The developed analytical method was further applied to evaluate the impact caused by the discharge of a municipal WWTP on Febros river, a small tributary of Douro river, located in the northern region of Portugal, regarding pharmaceutical content only. Febros WWTP implication on the pharmaceutical input to Febros river was evidenced by the higher pharmaceutical load in samples collected immediately downstream the WWTP discharge point, when compared to upstream sampling sites. Moreover, the performance of the method was assessed through the participation in two European level inter-laboratory exercises (ILEs), promoted by EU-JRC and PHARMAS FP7. Most of our results fell in a satisfactory z-score range, which demonstrated the reliability of the developed procedure for monitoring pharmaceuticals in water samples.

Since the conventional treatment processes employed in most WWTPs seem to be inefficient for pharmaceuticals' complete removal, a tremendous amount of research has been recalled in the field of robust new water treatment technologies, leading to the complete mineralization of organic pollutants at lower cost and with less energy. In this field, advanced oxidation processes (AOPs) have been regarded as promising tools.

Thus, our following work was mainly focused on advanced photocatalytic treatment processes, primarily tested on lorazepam (LZP), a recalcitrant drug, frequently quantified in WWTPs' effluents and surface waters. In a preliminary study, LZP photolytic and photocatalytic degradation kinetics were comparatively evaluated using three experimental systems: two lab-scale photochemical reactors, one provided with an UV medium pressure mercury lamp (*LsAUVP*) and the other with a blacklight blue lamp (*LsBLBUV*), and a solar pilot plant with compound parabolic collectors (*SPP-CPCs*). Results showed that the best degradation performance was achieved using the *SPP-CPCs* system, by photocatalysis with a TiO_2 concentration of 200 mg L^{-1} (pseudo-first order degradation kinetic constant k=1.49±0.03 L kJ^{-1}). Subsequently, LZP phototransformation pathways were assessed using ultra-high performance liquid chromatography–quadrupole-time-of-flight–mass spectrometry (UHPLC/QToF-MS). Six major lorazepam by-products (LBPs) were identified and elucidated, with nominal $[M+H]^+$ masses of 337, 303, 319, 275, 291 and 293 Da.

Afterwards, the optimized photocatalytic treatment method was further applied to the treatment of a real municipal WWTP effluent, containing 22 pharmaceutical compounds in moderate concentrations (maximum of 680 ng L^{-1}, except for diclofenac ~24 μg L^{-1} and hydrochlorothiazide ~3 μg L^{-1}). A pseudo-first order kinetic model was able to successfully predict all pharmaceuticals' degradation kinetics. The overall treatment was considered efficient, with a complete removal of the majority of these micropollutants, except for ciprofloxacin (35%), ketoprofen (61%) and bisoprolol (77%). Finally, *V. fischeri* acute toxicity test showed that the effluent itself presented no significant toxicity and that the intermediate oxidation compounds, possibly formed during phototreatment, did not reflect any significant increase of toxicity. Therefore, photocatalysis with TiO_2 was proposed as a viable, green, low cost decontamination method of EPs in domestic wastewaters.

Keywords: emerging pollutants; pharmaceutical compounds; liquid chromatography-mass spectrometry; wastewater treatment plants; advanced oxidation processes

Resumo

A Diretiva-Quadro da Água (DQA) constitui uma ferramenta operacional para a recuperação da qualidade de todos os cursos de água na União Europeia (UE), sua proteção e garantia do uso sustentável. Recentemente tem sido dado especial ênfase aos designados "poluentes emergentes" (PEs), muitos dos quais se encontram distribuídos de forma ubíqua no meio aquático. De entre eles, as substâncias farmacêuticas geram crescente preocupação, dada a sua grande variedade e consumo, reconhecida (eco)toxicidade, além do seu impacto ambiental imprevisível, mesmo em baixos teores.

Os compostos farmacêuticos atingem o meio aquático principalmente através da descarga de efluentes em estações de tratamento de águas residuais (ETARs). Juntamente com estas substâncias tem sido identificado um crescente número de metabolitos e produtos de transformação (PTs), sendo esperadas misturas complexas ou "*cocktails*" destes poluentes no ambiente. Assim, numa primeira etapa deste trabalho procedeu-se ao desenvolvimento e validação de um método de extração em fase sólida *offline* com *cleanup* simultâneo, seguida de cromatografia líquida-(ionização em *electrospray*)-espectrometria de massa (*ion trap*), permitindo a determinação concomitante de 23 fármacos, de entre os mais consumidos em Portugal, em águas residuais. Testaram-se diferentes estratégias de *cleanup*, explorando as propriedades físico-químicas dos analitos vs. interferências. Após o ensaio de várias combinações de adsorventes e solventes de eluição, os melhores resultados foram obtidos com os cartuchos de troca iónica mistos Oasis MAX (*mixed-anion exchange*). Estes cartuchos permitiram a obtenção de taxas de recuperação, em geral, superiores a 60%. A precisão do método variou entre os 2-18% e os 4-19% (exceto para a sinvastatina (26%) e o diclofenac (22%)), expressa em repetibilidade e precisão intermédia, respectivamente. Os limites de detecção variaram entre 1-20 ng L^{-1} (exceto o ibuprofeno).

O método analítico desenvolvido foi posteriormente aplicado na avaliação do impacto causado pela descarga de uma ETAR municipal no rio Febros, um afluente do rio Douro. A contribuição da ETAR de Febros para a contaminação daquele rio com compostos farmacêuticos foi evidenciada pela elevada carga destes compostos nas amostras colhidas imediatamente a jusante do ponto de descarga da ETAR, quando comparadas com os pontos a montante. A *performance* do método foi também avaliada pela participação em dois ensaios interlaboratoriais (EILs), promovidos pelo *EU-JRC* e pelo projeto *PHARMAS FP7*. A maioria dos resultados obtidos foi classificada com *z-score* satisfatório demonstrando assim a fiabilidade do método desenvolvido para a monitorização de fármacos em amostras de água.

Dado que os processos de tratamento convencionais utilizados na maioria das ETARs se têm revelado ineficazes na remoção completa dos fármacos, tem-se assistido a um aumento exponencial da investigação em novas tecnologias de tratamento de águas, capazes de promover a completa mineralização dos poluentes orgânicos, com baixo custo e reduzido consumo energético. Neste campo, os processos avançados de oxidação (PAOs) têm-se revelado ferramentas bastante promissoras.

Assim, a etapa seguinte deste trabalho centrou-se nos processos de tratamento fotocatalítico avançados, usando o lorazepam (LZP) como modelo, um fármaco recalcitrante presente em efluentes de ETARs e águas superficiais. Compararam-se as cinéticas de degradação fotolítica e fotocatalítica do LZP, utilizando três sistemas experimentas: dois reatores fotoquímicos à escala laboratorial, um constituído por uma lâmpada UV de mercúrio de média pressão (*LsAUVP*) e outro por uma lâmpada de luz negra (*LsBLBUV*), e ainda uma instalação solar piloto com coletores parabólicos compostos (*SPP-CPCs*). Os resultados obtidos mostraram que o maior rendimento de degradação se obteve com o sistema *SPP-CPCs*, por fotocatálise com TiO_2, na concentração de 200 mg L^{-1} (cinética de pseudo-primeira ordem, k=1.49±0.03 L kJ^{-1}). De seguida, estudaram-se os mecanismos de fototransformação do LZP, utilizando a técnica de cromatografia líquida de ultra *performance*–espectrometria de massa por "tempo-de-vôo" (time-of-flight) (UHPLC/QToFMS). Identificaram-se seis produtos de degradação do LZP com massas nominais $[M+H]^+$ de 337, 303, 319, 275, 291 e 293 Da.

Por último, o método de tratamento fotocatalítico otimizado foi posteriormente aplicado no tratamento de efluente de uma ETAR municipal, contaminado com 22 compostos farmacêuticos em concentrações moderadas (máximo de 680 ng L^{-1}, exceto diclofenac ~24 μg L^{-1} e hidroclorotiazida ~24 μg L^{-1}). A degradação dos fármacos seguiu uma cinética de pseudo-primeira ordem. Em geral, o tratamento foi considerado bastante eficiente, conseguindo-se uma remoção completa destes micropoluentes, com a exceção da ciprofloxacina (35%), do cetoprofeno (61%) e do bisoprolol (77%). Finalmente, foram realizados testes de toxicidade aguda ao *V. fischeri*, tendo-se revelado a ausência de toxicidade significativa no efluente inicial. Verificou-se ainda que os compostos intermediários potencialmente formados durante o fototratamento não contribuíram para o aumento da toxicidade. Assim sendo, o processo fotocatalítico com TiO_2 foi proposto como um processo viável, amigo do ambiente e de baixo custo para a descontaminação de águas residuais com poluentes emergentes.

Palavras-chave: poluentes emergentes; compostos farmacêuticos; cromatografia líquida–espectrometria de massa; estações de tratamento de águas residuais; processos avançados de oxidação

Index

Figure Index

Table Index

Publications, Oral and Poster Communications

• List of Publications

Paper 1 *(In English language)*

Sousa, M.A., Gonçalves, C., Cunha, E., Hajšlová, J. and Alpendurada, M.F., *Cleanup strategies and advantages in the determination of several therapeutic classes of pharmaceuticals in wastewater samples by SPE–LC–MS/MS*. Analytical and Bioanalytical Chemistry, 2011. 399: 807-822.

Paper 2 *(In Portuguese language)*

Sousa, M.A., Gonçalves, C., Vilar, V.J.P., Boaventura, R.A.R. e Alpendurada, M.F., *Efeitos dos efluentes domésticos tratados e rios tributários no pool de resíduos farmacêuticos do Rio Douro - Processos de Mitigação [Effects of treated domestic effluents and tributaries on the contamination of Douro river with pharmaceutical compounds – Mitigation processes]*. Submitted for publication in *Recursos Hídricos* (APRH - *Associação Portuguesa de Recursos Hídricos*), June 2011.

Paper 3 *(In English language)*

Sousa, M.A., Gonçalves, C., Pereira, J.H.O.S., Vilar, V.J.P., Boaventura, R.A.R. and Alpendurada, M.F., *Photolytic and TiO_2-Assisted Photocatalytic Oxidation of the Anxiolytic Drug Lorazepam (Lorenin® pills) under Artificial UV Light and Natural Sunlight: A Comparative and Comprehensive Study*. Solar Energy, 2013. 87: 219-228.

Paper 4 *(In English language)*

Sousa, M.A., Gonçalves, C., Vilar, V.J.P., Boaventura, R.A.R. and Alpendurada, M.F., *Suspended TiO_2-Assisted Photocatalytic Degradation of Emerging Contaminants in a Municipal WWTP Effluent using a Solar Pilot Plant with CPCs*. Chemical Engineering Journal, 2012. 198-199: 301-309.

Paper 5 *(In English language)*

Sousa, M.A., Lacina, O., Hrádková, P., Pulkrabová, J., Vilar, V.J.P., Gonçalves, C., Boaventura, R.A.R., Hajšlová, J. and Alpendurada, M.F., *Lorazepam Photofate under Photolysis and TiO_2-assisted Photocatalysis: By-products' Identification and Evolution Profiles during Phototreatment of a Contaminated WWTP Effluent*. Submitted for publication in Environmental Science and Technology, September 25, 2012.

Associated Papers

(Annex II)

Gonçalves, C., Sousa, M.A. and Alpendurada, M.F., *Analysis of acidic, basic and neutral pharmaceuticals in river waters: clean-up by 1º,2º amino anion exchange and enrichment using an hydrophilic adsorbent*. International Journal of Environmental Analytical Chemistry, 2013. 93: 1-22.

(Annex IV)

Moreira, F.C., Vilar, V.J.P., Ferreira, A.C.C., Santos, F.R.A., Dezotti, M., Sousa, M.A., Gonçalves, C., Boaventura, R.A.R. and Alpendurada, M.F., *Treatment of a pesticide-containing wastewater using combined biological and solar-driven AOPs at pilot scale*. Chemical Engineering Journal, 2012. 209: 429-441.

(Not enclosed)

Vilar, V.J.P., Moreira, F.C., Ferreira, A.C.C., Sousa, M.A., Gonçalves, C., Alpendurada, M.F. and Boaventura, R.A.R., *Biodegradability enhancement of a pesticide-containing bio-treated wastewater using a solar photo-Fenton treatment step followed by a biological oxidation process*. Water Research, 2012. 46: 4599-4613.

Book Chapters

Gonçalves, C., Sousa, M.A. and Alpendurada, M.F., *Occurrence of Transformation Products of Emerging Contaminants in Water Resources*. In: Transformation Products of Emerging Contaminants in the Environment: Analysis, Processes, Occurrence, Effects and Risks. Book to be edited by Dimitra A. Lambropoulou and Leo M.L. Nollet. John Wiler & Sons Inc. (2013).

- **Oral Communications**

[In English language]

[5] Sousa, M.A., Lacina, O., Hrádková, P., Pulkrabová, J., Gonçalves, C., Vilar, V.J.P., Boaventura, R.A.R., Hajšlová, J. and Alpendurada, M.F., *Tracing the Formation of Emerging Pollutants' By-products over the Phototreatment of a Contaminated Municipal WWTP Effluent*. SPEA7 – 7th European Meeting on Solar Chemistry and Photocatalysis: Environmental Applications, 17-19th of June, 2012, Porto, Portugal.

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[2] Gonçalves, C., Sousa, M.A., Guimarães, A. and Alpendurada, M.F*., Assessment of priority and emerging pollutants in two distinct rivers in the north of Portugal: support to the WFD and RBMPs*. Pesticides2010 – 6th European Conference on Pesticides and Related Organic Micropollutants in the Environment and 12th Symposium on Chemistry and Fate of Modern Pesticides, 7-10th of September, 2010, Matera, Italy.

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[5] Sousa, M.A., Gonçalves, C., Vilar, V.J.P., Boaventura, R.A.R. e Alpendurada, M.F., *Processos Avançados de Oxidação na Eliminação de Fármacos em Águas Residuais*. Workshop IAREN - Poluentes Emergentes no Meio Aquático: Análise, Níveis de Contaminação e Preocupações Ambientais, 10th of February, 2012, Alfândega do Porto, Porto, Portugal.

[4] Gonçalves, C., Sousa, M.A., Machado, S., Machado, A., Guimarães, A. e Alpendurada, M.F., *Monitorização de poluentes emergentes nos rios Leça e Douro*. Workshop IAREN - Poluentes Emergentes no Meio Aquático: Análise, Níveis de Contaminação e Preocupações Ambientais, 10th of February, 2012, Alfândega do Porto, Porto, Portugal.

[3] Sousa, M.A., Gonçalves, C., Vilar, V., Boaventura, R. e Alpendurada, M.F., *Contributo de efluentes domésticos tratados e rios tributários para o pool de resíduos farmacêuticos no Rio Douro – Processos de Mitigação*. 3º Seminário sobre Gestão de Bacias Hidrográficas - Os Estuários, APRH, 2nd-3rd of June, 2011, IP Viana do Castelo, Portugal.

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- **Poster Communications**

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List of Acronyms and Abbreviations

ACTC	Anhydrochlortetracycline
ALP	Alprazolam
AOPs	Advanced oxidation processes
APEOs	Alkylphenol ethoxylates
ATC	Anhydrotetracycline
BGE	Band gap energy
BOD	Biochemical oxygen demand
BP	By-product
BRFs	Brominated flame retardants
BZP	Benzodiazepine
CB	Conduction band
CIP	Ciprofloxacin
CNS	Central nervous system
DLLME	Dispersive liquid-liquid microextraction
DO	Dissolved oxygen
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
DWD	Drinking Water Directive
DZP	Diazepam
E1	Estrone
E2	17β-estradiol
EACTC	Epianhydrochlortetracycline
EC	European Commission
EC50	Half-maximal effective concentration
EDCs	Endocrine disrupting compounds
EE2	17β-ethinylestradiol
EMEA	European Medicines Agency
EOTC	4-Epioxytetracycline
EPs	Emerging pollutants
EQS	Environmental quality standards
ERY	Erythromycin
ESA	Ethane sulfonic acid
ESB	Environmental Specimen Banks
ESI	Electrospray ionization

ETC	4-Epitetracycline
EU	European Union
FDA	Food and Drug Administration
FEDESA	European Federation of Animal Health
GCB	Graphitised carbon black
GC-MS	Gas chromatography-mass spectrometry
HLB	Hydrophilic-lipophilic-balanced
HPLC-MS	High performance liquid chromatography-mass spectrometry
HRT	Hydraulic retention time
HS	Humic substances
HTLC	High-temperature liquid chromatography
IAC	Immuno-affinity chromatography
ICTC	Isochlortetracycline
ILE	Inter-laboratory exercise
INFARMED	Portuguese National Authority of Medicines and Health Products, IP
JRC	Joint Research Centre
LC	Liquid chromatography
LC50	Half-maximal lethal concentration
LC-MS	Liquid chromatography-mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOEC	Lowest observed effect concentration
LOQ	Limit of quantification
LPME	Liquid-phase microextraction
LsBLBUV	Lab-scale blacklight blue-UV system
LsTQUV	Lab-scale medium pressure mercury lamp Heraeus TQ 150W
LZP	Lorazepam
MAX	Mixed-mode anion exchange sorbent
MCX	Mixed-mode cation exchange sorbent
MDA	4-Methylenedioxyamphetamine
MDEA	3,4-Methylenedioxyethamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
MESCO	Membrane-enclosed sorptive coating
MIP	Molecularly imprinted polymer
MISPE	Molecularly imprinted solid-phase extraction
MS	Mass spectrometry

NMR	Nuclear magnetic resonance
NOEC	No-observed effect concentration
NPEOs	Nonylphenol polyethoxylates
NRZ	Nordazepam
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Oxanilic acid
OFL	Ofloxacin
OTC	Over-the-counter
OXA	Oxazepam
PAHs	Polycyclic aromatic hydrocarbons
PBDEs	Polybrominated diphenyl ethers
PCBs	Polychlorinated biphenyls
PDB	Passive diffusion bag
PEC	Predicted environmental concentration
PFAs	Poly- and perfluoroalkyl substances
PFOS	Perfluorooctanosulfonates
PFCA	Perfluoroalkylcarboxylates
PISCES	Passive *in situ* concentration-extraction sampler
PNEC	Predicted no-effect concentration
POCIS	Polar organic integrative sampler
PoD	Points of departure
PS-DVB	Poly(styrene-divinylbenzene)
PTC	parabolic-trough concentrator
QAPP	Quality assurance project plan (previously known as QA/QC)
QA/QC	Quality assurance/quality control
QTRAP	Hybrid triple-quadrupole linear ion trap mass spectrometer
QUV	Accumulated UV energy
RAMs	Restricted access materials
REACH	Registration, evaluation and authorisation of chemicals
SC	Semiconductor
SM-LLME	Stir membrane liquid-liquid microextraction
SMX	Sulfamethoxazole
SMZ	Sulfamethazine
SPATT	Solid-phase adsorption toxin tracking
SPE	Solid-phase extraction
SPMD	Semi-permeable membrane device

SPME	Solid-phase microextraction
SPP-CPCs	Solar pilot plant with compound parabolic collectors
SPY	Sulfapyridine
STPs	Sewage treatment plants
TC	Tetracyclines
THF	Tetrahydrofuran
TMP	Trimethoprim
TMZ	Temazepam
TOF-MS	Time-of-flight mass spectrometry
TP	Transformation product
UHPLC	Ultra high-performance liquid chromatography
USA	United States of America
UV	Ultraviolet
UWWD	Urban Waste Water Directive
VB	Valence band
VOCs	Volatile organic compounds
WAX	Weak-anion exchange
WFD	Water Framework Directive
WWTPs	Wastewater treatment plants

Chapter 1
General Introduction

1.1. Water Quality Policy and Legislation

Nowadays, one of the most relevant topics in the environmental field is water quality. It has been evident for a considerable time now the increasing demand by citizens and environmental organizations for cleaner rivers, lakes, groundwater and coastal beaches. This demand was precisely one of the main reasons why the European Commission (EC) has made water protection one of its top work priorities. Whilst some actions taken in the past by the European Union (EU), such as the Drinking Water Directive (DWD) and the Urban Waste Water Directive (UWWD), can properly be considered milestones, a renewed European Water Policy was mandatory to address the increasing awareness of citizens and other involved parties. Consequently, and as the outcome of a consultation process involving all interested parties, the Commission presented a Proposal for a Water Framework Directive, with the following key aims (1):

i) expanding the scope of water protection to all waters, surface waters and groundwater;
ii) achieving "good status" for all waters by a set deadline;
iii) water management based on river basins;
iv) "combined approach" of emission limit values and quality standards;
v) getting the prices right;
vi) getting the citizens involved more closely;
vii) streamlining legislation.

Hence, the Water Framework Directive (WFD, Directive 2000/60/EC) was created and adopted as an operational tool, setting the objectives for water protection. It presented a huge breakthrough in the EU water policy, aiming to achieve a good ecological and chemical status for all surface waters at the latest 15 years from the date of entry into force, i.e., 22 December 2000. Furthermore, the WFD was later amended by Decision no. 2455/2001/EC, that established a list of 33 priority substances given their significant risk

to the aquatic environment, and even more recently by Directive 2008/105/EC, which laid down environmental quality standards (EQS) for priority substances and other certain pollutants, as provided for in Article 16 of the WFD (1, 2).

In order to accomplish the co-ordination of the objectives and achieve a good status for all waters by the set deadline, it became necessary to define the best model for a single system of water management. As a result, the management by river basin – the natural geographical and hydrological unit – was adopted, instead of according to administrative or political boundaries. Meanwhile, though several Member States are already undertaking "the river basin approach", some national and international river basin districts are still not established (Fig. 1.1). Furthermore, all river basin districts must be updated every six years, in order to provide the context for all co-ordination requirements aforementioned (1).

1.2. Emerging Environmental Pollutants

Over the last few decades, the focus of environmental research has been extended from the conventional "priority pollutants", such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins and pesticides, to the so called "emerging pollutants" (EPs), many of which are ubiquitously distributed in the aquatic compartment. EPs are not necessarily new chemicals; they also include substances that have long been present in the environment but whose presence and significance are only now being elucidated. Therefore, the rising interest on such organic micropollutants is not only concomitant with their increased diversity, widespread and growing use, but also with the development of new highly sensitive analytical tools, namely liquid chromatography (LC) coupled with mass spectrometry (MS). Such analytical hyphenated technique led to a "revolution" in environmental analysis, enabling the identification and quantification of polar organic pollutants down to relatively low concentrations, in all kinds of waters (wastewater, surface water, ground water, drinking water) and in solid matrices (sewage sludge, manure, sediment) (3-5).

However, emerging pollutants correspond in most cases to unregulated contaminants and are generally not included in the monitoring campaigns enforced by EU legislation. Consequently, there is the need to build knowledge on their occurrence, levels and fate in the environment, as well as to elucidate their long-term risks, ecotoxicity and human

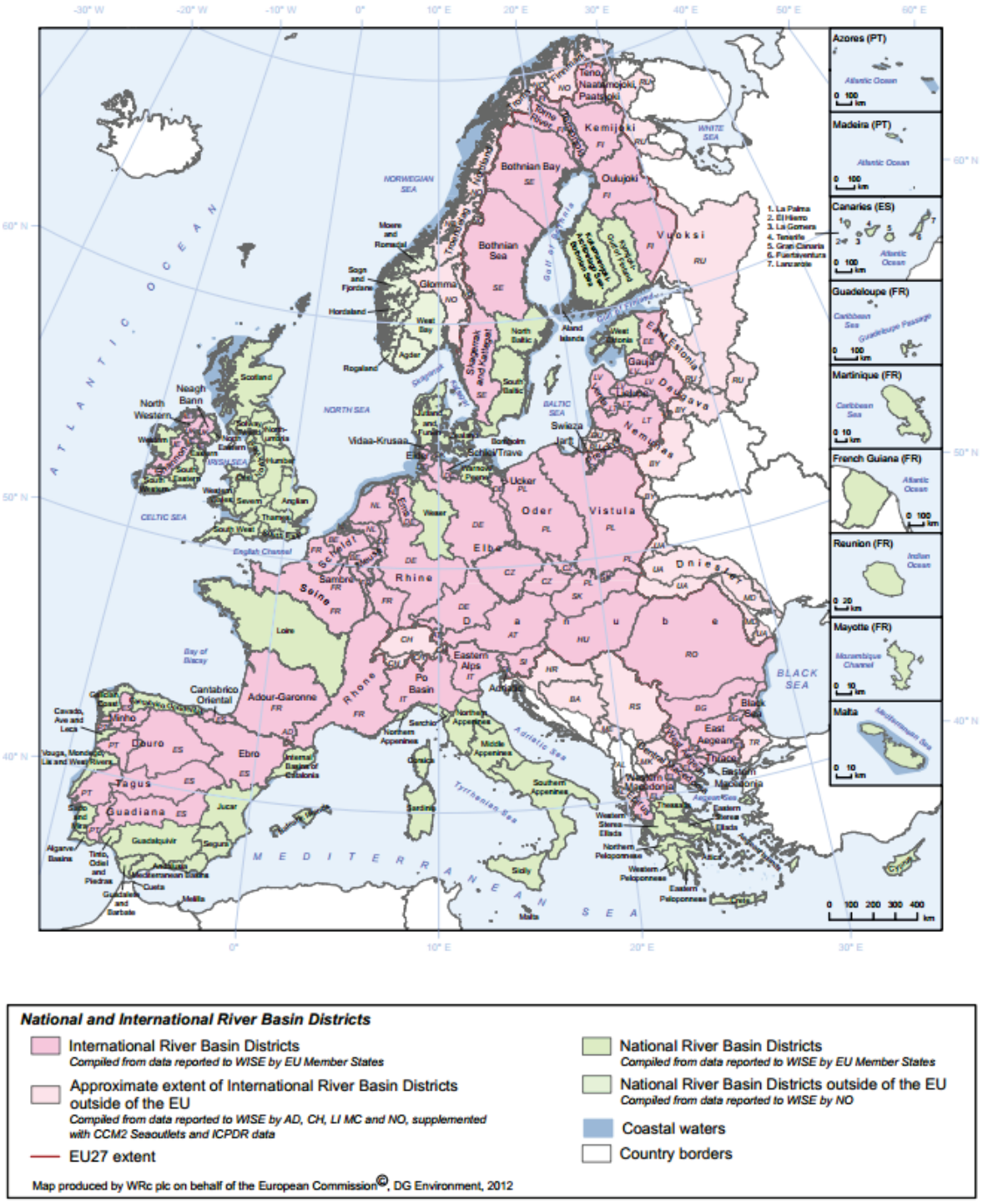

Fig. 1.1. Map presenting the national and international river basin districts, as designated by Member States (1).

health impact, which might be used as evidence for future regulation (2, 6). Emerging pollutants are considered "pseudo-persistent" compounds, since their transformation/removal rates can be compensated by their continuous introduction into the environment. Moreover, their recalcitrant character, together with their polarity, favour their spread along the aquatic compartment (7).

The NORMAN Network, established since 2009 as a permanent self-sustaining network of reference laboratories, research centres and related organizations for the monitoring and biomonitoring of emerging environmental substances, started its activities in September 2005, with the financial support of the European Commission (NORMAN project – 6th Framework Programme – Priority 6.3). Currently, it proposes a preliminary list of emerging substances, built upon the results collected by Member States, after several European surveys. Herein, different classes of compounds are included, from algal toxins to anticorrosives, antifoaming agents, antifouling compounds, antioxidants, biocides, bio-terrorism/sabotage agents, complexing agents, detergents, disinfection by-products (drinking water), drugs of abuse, flame retardants, food additives, fragrances, gasoline additives, industrial chemicals, nanoparticles, perfluoroalkylated substances and their transformation products, personal care products, pesticides, pharmaceuticals, plasticizers, trace metals and their compounds, wood preservatives, among others (for individual substances, please refer to Annex I) (8).

Furthermore, the NORMAN Association has also created an interactive database – EMPODAT, designed to assemble the geo-referenced monitoring and bio-monitoring data collected by its members on emerging substances in air, water and soil. Each contact point across the Member States is requested to provide information concerning data sources, sample matrices and analytical methods employed to obtaining the results. In conclusion, the EMPODAT database allows access to the latest information on emerging pollutants, with an overview of benchmark values on the occurrence of emerging substances across Europe, as well as the identification of gaps in data relating to time, geographical areas and/or environmental matrices.

Among all emerging substances in water, special attention has been given to pharmaceutical products and residues. This rising awareness is attributed to their growing variety and consumption, recognised (eco)toxicity, as well as their unpredictable environmental impact, even at low concentration levels (9, 10).

1.2.1. Pharmaceutical Compounds

1.2.1.1. Definition and Classification by Therapeutic Group

The European Medicines Agency (EMEA) is responsible for establishing the rules governing human and veterinary medicinal products in the EU. According to Directive 2001/83/EC, of November 6, on the Community code relating to medicinal products for human use, later amended by Directive 2004/27/EC, of March 31, a *medicinal product* is (11):

i) any substance or combination of substances presented as having properties for treating or preventing disease in human beings; or
ii) any substance or combination of substances which may be used in or administered to human beings either with a view to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis.

In Portugal, there is an annual edition of the Therapeutic Handbook, mainly addressed to medical doctors, pharmacists and other health care professionals, which sets the guidelines for the therapeutic use of drugs and constitutes an essential tool for the rational use of medicines, providing guidance during prescription. It contains the monographs of all medicines marketed in Portugal and available in pharmacies, as well as some that, although available only in hospitals, are of particular interest for patients continuing the treatment process at the clinic. In the Therapeutic Handbook, medicines are grouped by pharmaceutically active substance, according to the respective therapeutic group. The first 10 therapeutic groups and respective sub-groups included in this Handbook are displayed in Table 1.1, since they include the pharmaceutical compounds most widely studied as environmental pollutants (12).

According to NORMAN, Table 1.2 shows a list of pharmaceutical pollutants which are object of study by EU Member States, as well as all over the world. Moreover, at the NORMAN Association website, further information including the systematic name (IUPAC), CAS number and therapeutic sub-class of every pharmaceutical compound can be found (8).

Table 1.1. Therapeutic groups according to the Portuguese Therapeutic Handbook (12)

THERAPEUTIC HANDBOOK

Therapeutic Groups *Edition 2011*

GROUP 1 - Anti-infective Medicines
1.1. Antibacterials
1.2. Antifungals
1.3. Antivirals
1.4. Antiparasitics

GROUP 2 - Central Nervous System (CNS)
2.1. General Anesthetics
2.2. Local Anesthetics
2.3. Muscle Relaxing Agents
2.4. Antimyasthenia Agents
2.5. Antiparkinsonian Agents
2.6. Antiepileptics and Anticonvulsivants
2.7. Antiemetics and Antivertigo Agents
2.8. Unspecific Stimulants of CNS
2.9. Psychopharmaceuticals
2.10. Analgesics and Antipyretics
2.11. Medicines for Headache
2.12. Narcotics
2.13. Others

GROUP 3 - Cardiovascular System
3.1. Cardiotonics
3.2. Antiarrhythmics
3.3. Sympathomimetics
3.4. Antihypertensives
3.5. Vasodilators
3.6. Venotropics
3.7. Lipid Regulators

GROUP 4 - Blood
4.1. Antianemic Agents
4.2. Hematopoietic Growth Factors
4.3. Anticoagulant and Antithrombotic Agents
4.4. Antihemorrhagic Agents

GROUP 5 - Respiratory System
5.1. Antiasthmatics and Bronchodilators
5.2. Antitussives and Expectorants
5.3. Pulmonary Tensoactive Agents (Surfactants)

GROUP 6 - Digestive System
6.1. Medicines acting on Mouth and Orofaringe
6.2. Antacids and Anti-ulcer Agents
6.3. Modifiers of Gastrointestinal Motility
6.4. Antispasmodics
6.5. Enzyme Inhibitors
6.6. Enzyme Suplements and Analogs
6.7. Antihemorrhoid Agents
6.8. Intestinal Anti-inflammatory Agents
6.9. Medicines acting on Liver and Biliary

GROUP 7 - Genitourinary System
7.1. Vaginal Medicines
7.2. Medicines acting on Uterus
7.3. Urinary Anti-infective and Antiseptic Agents
7.4. Others

GROUP 8 - Hormones and Medicines for Endocrine Diseases
8.1. Hypothalamus and Pituitary Hormones, their Analogs and Antagonists
8.2. Corticosteroids
8.3. Thyroid Hormones and Antagonists
8.4. Insulins, Oral Antidiabetics and Glucagon
8.5. Sex Hormones
8.6. Ovulation Stimulants and Gonadotropins
8.7. Anti-hormones

GROUP 9 - Locomotor System
9.1. Non-Steroidal Anti-inflammatory Drugs
9.2. Rheumatism Modifiers
9.3. Medicines used for Arthritis
9.4. Medicines used for Arthrosis
9.5. Anti-inflammatory Enzymes
9.6. Medicines acting on Bones and Calcium Metabolism

GROUP 10 - Antialergics
10.1. Antihistamines
10.2. Corticosteroids
10.3. Sympathomimetics

Table 1.2. Pharmaceutical emerging pollutants, according to the NORMAN Network (8)

(R)-O-Desmethyl Naproxen	Difloxacin	Metoprolol
17-alpha-Estradiol	Diphenhydramine	Mevastatin
17-alpha-Ethinylestradiol	Domperidone	Minocycline
17-beta-Estradiol	Doxepine	Nadolol
Acebutolol	Doxorubicin	Nafcillin
Acecarbromal	Doxycycline (anhydrous)	Nandrolone
Aceclofenac	Doxycycline (monohydrate)	Naproxen
Acemetacin	Enoxacin	Neomycin B
Acetaminophen (paracetamol)	Enrofloxacin	N-Methylphenacetine
Acetazolamide	Epirubicin	Nordiazepam
Acetylsalicylic acid (aspirin)	Erythromycin	Norfloxacin
Acyclovir	Escitalopram	Novobiocin
Albuterol	Esomeprazole	Ofloxacin
Albuterol sulfate	Estriol	Oleandomycin
Alclofenac	Estrone	Omeprazole
Allobarbital	Estrone sulphate	Oxacillin
Alprazolam	Ethosuximide	Oxalinic acid
Amitryptiline	Etofibrate	Oxazepam
Amobarbital	Famotidine	Oxprenolol
Amoxicillin	Fenfluramine	Oxytetracycline
Ampicillin	Fenofibrate	Paroxetine
Anthracen-1,4-dione	Fenofibric acid	Penicillin G
Apramycin	Fenoprofen	Penicillin V
Aprobarbital	Fenoprofen calcium salt dihydrate	Pentobarbital
Atenolol	Fenoterol	Pentoxifylline
Azithromycin	Flucloxacillin	Phenazone
Baclofen	Flumequine	Phenobarbital
Baquiloprim	Fluorouracil	Phenylbutazone
Betamethasone	Fluoxetine	Phenytoine
Beta-sitosterol	Fluvoxamine	Pindolol
Betaxolol	Furosemide	Pipamperon
Bezafibrate	Gemfibrozil	Pravastatin
Bisoprolol	Gentamicin	Prednisolone
Bromazepam	Glyburide (glibenclamid; glybenzcyclamide)	Primidone
Butalbital	Hexobarbital	Propranolol
Caffeine	Hydrochlorothiazide	Propyphenazone
Carazolol	Hydrocodone	Ranitidine
Carbamazepine	Ibuprofen	Roxithromycin
Cefacetrile	Ibuprofen 1-hydroxy	Salbutamol
Cefalexin	Ibuprofen 2-hydroxy	Sarafloxacin
Cefalonium	Ifosfamide	Secobarbital
Cefapirin	Imapramine	Secobarbital sodium
Cefazolin	Iminostilbene	Sertraline
Cefoperazone	Indomethacin	Simvastatin
Chloral hydrate	Iohexol	Sotalol
Chloramphenicol	Iomeprol	Spectinomycin
Chlorobutanol	Iopamidol	Spiramycin
Chlortetracycline	Iopromide	Streptomycin
Cholesterol	Ivermectin	Sulfadiazine
Ciprofloxacin	Josamycin	Sulfadimethoxine
Citalopram	Kanamycin sulfate	Sulfadoxine
Clarithromycin	Ketoprofen	Sulfamerazine
Clenbuterol	Lamotrigine	Sulfamethazine
Clofibric acid	Lansoprazole	Sulfamethoxazole
Clotrimazole	Levetiracetam	Sulfapyridine
Cloxacillin	Lidocaine	Taloxa
Codeine	Lincomycin	Temazepam
Cotrimoxazole	Lithium carbonate	Terbutaline
Crotamiton	Loratadine	Tetracycline
Cyclophosphamide	Lorazepam	Tiamulin
Cyclophosphamide (anhydrous)	Lovastatin	Tilmicosin
Danofloxacin	Marbofloxacin	Timolol
Dantrolene	Mebeverine	Tolfenamic acid
Dapsone	Meclofenamic acid	Tramadol
Daunorubicin	Medazepam	Trimethoprime
Dexamethasone	Mefenamic acid	Tylosin
Diatrizoate	Meprobamate	Valnemulin
Diazepam	Mestranol	Valproic acid
Diclofenac	Metformin	Verapamil
Dicloxacillin	Methicillin	Zolpidem
Diethylstilbestrol	Methylphenobarbital	

1.2.1.2. Consumption: Worldwide, in Europe and in Portugal

Still in 2004, the global consumption of drugs (as total pharmaceutical formulation) produced and used by humans was estimated to be 100,000 metric tons (t) per year, corresponding to a worldwide average per capita consumption of approximately 15 $g.cap^{-1}.a^{-1}$ (13). However, according to statistical studies conducted in the United States of America (USA) and in Sweden regarding industrialized countries, it was foreseen that the consumption of active pharmaceutical substances would still rise up to the range between 50 and 150 $g.cap^{-1}.a^{-1}$. Moreover, these two surveys showed that ca. 95% of the total consumption of pharmaceutical substances corresponded to less than 50 of the 130 compounds investigated (14, 15).

The trends on pharmaceuticals use clearly vary between different countries. For instance, in Germany, the amount of active compounds in use was estimated to be around 3,000, though this number is certainly underestimated due to over-the-counter (OTC) drugs, i.e., drugs acquired without medical prescription, besides those procured illegally. Likewise, prescription drugs are generally sold in quantities at least one order of magnitude lower than non-prescription drugs (5). In contrast to Germany, sales in the USA for compounds such as carbamazepine and ciprofloxacin have shown a trend of decreasing use between the years 1995 and 2001, whereas metoprolol use has been increasing in both countries (16). Several publications regarding consumption data in England, Denmark and Australia, have also displayed an intensive use of pharmaceutical products. Nonetheless, despite being possible to establish a similar consumption pattern for many pharmaceuticals between several EU countries, obvious differences can also be spotted when comparing specific pharmaceutical products among different countries (17, 18).

Besides the considerable variation over different countries, pharmaceuticals consumption also varies over time, with the introduction of new products in the market, and according to the season. For example, the use of compounds such as metoprolol, carbamazepine, diclofenac, iomeprol and ciprofloxacin, has been increasing in Germany between 1996 and 2001. On the contrary, the consumption of drugs such as gemfibrozil, naproxen and erythromycin has been decreasing over the same time period (5). Regarding seasonal differences, a study in Switzerland has shown that, during the winter season, loads of macrolide antibiotics in sewage treatment plants (STPs) were two times higher than in summer. Nevertheless, these differences can be attributed to either lower elimination

rates of pharmaceuticals in STPs and/or sewer, due to minor biological activity, or to the higher input in winter (19).

As far as veterinary medicines are concerned, according to the European Federation of Animal Health (FEDESA), the annual consumption of antibiotics in the EU (including Switzerland) in the year 1999 was in total 13,288 t with 29% (3,850 t) for veterinary medicine (therapy and prophylaxis), 6% (800 t) as antibiotic feed additives and 65% (8,600 t) in human medicine. In the particular case of Switzerland, in 1997, before the banning of veterinary growth promoters, approximately 90 t of antibiotics were used, with 38% (34 t) in human medicine and 62% (56 t) in veterinary medicine. In 1999, after the banning of the growth promoters, antibiotics consumption dropped 33% in feeds, between 1995 and 2000, to a level of 17.3 t annually. On the other hand, the annual volume of antibiotics used in human medicine remained fairly constant (19).

In Portugal, the data on the environmental prevalence and distribution of pharmaceutical compounds is still quite scarce. However, there are clear trends concerning pharmaceuticals consumption over the last few years, as corroborated by the results provided by INFARMED – the Portuguese National Authority of Medicines and Health Products, IP. INFARMED is a Government agency accountable to the Health Ministry, whose objective is to monitor, assess and regulate all activities relating to human medicines and health products for the protection of Public Health (20).

Table 1.3 presents a list of the 30 most marketed pharmaceutical compounds in Portugal, over the years 2005, 2007 and 2009. As one can observe, paracetamol highlights in the first position of the ranking for the three years, while alprazolam, simvastatin and metformin also find place in the “top 10” for the three years. Moreover, all other 26 pharmaceutically active substances remain in the group of the 30 most commercialized over the studied years, with slight modifications in position. A few exceptions include alendronic acid, amlodipine, ambroxol, glibenclamide and nifedipine, which from 2005 to 2007 were replaced in this ranking by acetylsalicylic acid, glucosamine, clopidogrel, irbesartan and perindopril. On the other hand, from 2007 to more recently in 2009, the association ethinylestradiol+gestodene, glucosamine, acarbose and atorvastatin were also outpaced by levothyroxine, tamsulosin, betahistine and rosuvastatin.

Table 1.3. Pharmaceutical compounds ordered from the highest to the lowest sales amount in Portugal, over the years 2005/2007/2009, according to INFARMED (20)

Ranking	2005		2007		2009	
	Active Substance	Packages	Active Substance	Packages	Active Substance	Packages
1	Paracetamol	3 863 422	Paracetamol	3 520 490	Paracetamol	3 642 302
2	Alprazolam	2 801 120	Alprazolam	2 208 290	Simvastatin	2 941 467
3	Nimesulide	2 155 332	Simvastatin	2 144 523	Metformin	2 429 287
4	Diclofenac	2 019 393	Amoxicillin + Clavulanic acid	2 091 167	Alprazolam	2 280 686
5	Trimetazidine	1 974 870	Metformin	2 011 843	Amoxicillin + Clavulanic acid	2 239 124
6	Amoxicillin + Clavulanic acid	1 897 584	Trimetazidine	1 992 384	Trimetazidine	2 102 559
7	Simvastatin	1 787 970	Diclofenac	1 947 175	Omeprazole	2 029 495
8	Metformin	1 745 421	Lorazepam	1 888 586	Ibuprofen	2 016 009
9	Indapamide	1 724 022	Indapamide	1 775 043	Lorazepam	1 909 169
10	Lorazepam	1 665 454	Omeprazole	1 650 237	Diclofenac	1 817 520
11	Omeprazole	1 459 827	Ibuprofen	1 633 842	Acetylsalicylic acid	1 778 370
12	Bromazepam	1 452 038	Nimesulide	1 622 152	Indapamide	1 743 762
13	Ibuprofen	1 438 448	Gliclazide	1 342 691	Nimesulide	1 510 441
14	Diazepam	1 364 734	Bromazepam	1 207 555	Gliclazide	1 355 017
15	Ethinylestradiol + Gestodene	1 199 641	Diazepam	1 146 901	Influenza vaccines	1 343 266
16	Gliclazide	1 187 588	Furosemide	1 146 044	Furosemide	1 312 465
17	Alendronic acid	1 122 161	Ethinylestradiol + Gestodene	1 125 664	Bromazepam	1 196 396
18	Influenza vaccines	1 004 026	Acetylsalicylic acid	1 107 377	Flavonoids	1 170 030
19	Zolpidem	981 630	Influenza vaccines	1 028 351	Clopidogrel	1 153 693
20	Furosemide	968 630	Flavonoids	1 017 510	Losartan + Hydrochlorothiazide	1 139 107
21	Amlodipine	957 691	Zolpidem	980 308	Diazepam	1 130 220
22	Acarbose	945 264	Glucosamine	969 137	Bisoprolol	1 126 119
23	Azithromycin	938 614	Losartan + Hydrochlorothiazide	943 100	Zolpidem	1 078 175
24	Ambroxol	935 946	Clopidogrel	942 154	Irbesartan + Hydrochlorothiazide	1 021 011
25	Flavonoids	932 684	Bisoprolol	901 661	Levothyroxine	963 390
26	Glibenclamide	907 202	Acarbose	893 544	Tamsulosin	951 188
27	Atorvastatin	778 436	Azithromycin	892 528	Perindopril	941 067
28	Nifedipine	764 028	Irbesartan + Hydrochlorothiazide	884 087	Betahistine	934 658
29	Losartan + Hydrochlorothiazide	763 278	Atorvastatin	842 300	Rosuvastatin	913 081
30	Bisoprolol	717 100	Perindopril	823 971	Azithromycin	912 055

In conclusion, all these results indicate that there are significant seasonal and regional differences of pharmaceutical loads, which consequently affect their concentrations in the aquatic compartment. Thus, such variations must be always considered for a proper environmental risk assessment, without ever disregarding the fact that, in the water cycle, the variety of chemical compounds is further enlarged by metabolites formed in the human body, through microbial activity or via diverse environmental and/or artificial transformation processes (21, 22).

1.2.1.3. Pollution Sources, Environmental Occurrence and Fate

Pharmaceutical compounds are ubiquitary substances, often persistent and bioaccumulable in the environment, namely in the aquatic compartment. As the majority of organic micropollutants, the environmental contamination by pharmaceuticals is mainly of anthropogenic origin (10).

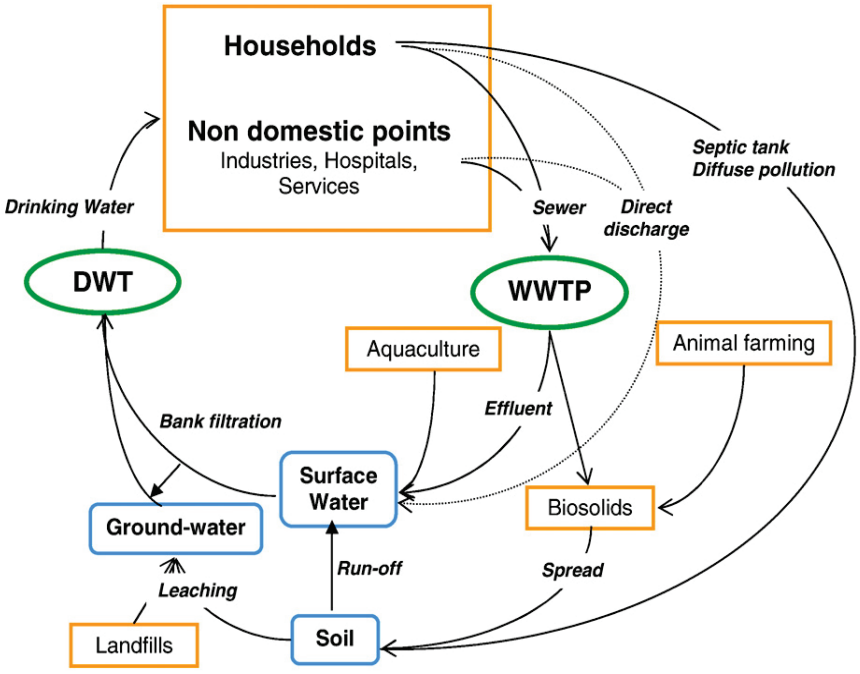

Fig. 1.2. Representative sources and routes of pharmaceutical compounds in the environment (*from Mompelat et al.* (10)).

As according to Fig. 1.2, after intake pharmaceuticals can be partially metabolized and both the native compound and/or its metabolites are excreted via urine and/or faeces, through the sewer network as urban wastewater, until reaching the wastewater treatment plants (WWTPs), or they can be also released directly into septic tanks, as it is usual in countryside households (23). Direct release can occur as well by improper disposal of unused or expired drugs, thrown directly in toilets, from manufacture spill accidents or, in the case of veterinary pharmaceuticals, via application in aquaculture (24). On the other hand, indirect release may happen by way of animals topically treated and mainly via run-off and leaching through fields from manure spreading to agricultural fields and livestock wastes (25, 26).

However, it is consensual that the most significant entry route for pharmaceuticals into the aquatic environment is the release from WWTPs. Since there is no unit specifically designed to remove these compounds, the treatment processes used by most WWTPs seems to be inefficient and, along with the treated wastewater, these pollutants are released to the aquatic environment (27, 28). Afterwards, and once surface waters can be physically closely linked to groundwaters, they can contaminate one another, even after soil or bank filtration (29). Finally, and before drinking water distribution, the ultimate elimination step of pharmaceuticals in raw water may take place during the treatment processes at water treatment plants (WTPs) (Fig. 1.2).

Hence, large amounts of pharmaceuticals can be discharged into the aquatic environment after being used, which will further enable their detection in wastewater, surface water, groundwater and even drinking water, at ng L^{-1} to several μg L^{-1} levels (30, 31). Nonetheless, and since the occurrence patterns of pharmaceuticals in WWTPs are related to local production and/or sales figures, as previously described in section 1.2.1.2., regional surveys need to be conducted (32). Furthermore, environmental conditions in the receiving body vary a lot across latitudes.

According to data in already published literature, the main therapeutic classes drawing the attention of the scientific community are non-steroidal anti-inflammatory drugs (NSAIDs), lipid regulators, antibiotics and sex hormones, as presented in Fig. 1.3. For example, Brown et al. (33) studied the environmental fate of ofloxacin, a fluoroquinolone antibiotic, first detected at 35.5 μg L^{-1} in an Albuquerque (New Mexico, USA) hospital effluent, while in the Albuquerque WWTP only 410 ng L^{-1} and 110 ng L^{-1} were found, in the influent and effluent, respectively. Finally, in the Rio Grande River, which receives the

WWTP effluent, ofloxacin was already below the detection limit. On the other hand, NSAIDs usually present the higher concentrations in surface waters, ranging from few ng L^{-1} up to ca. 20 μg L^{-1}, being diclofenac, paracetamol and ibuprofen the most quantitatively found (34).

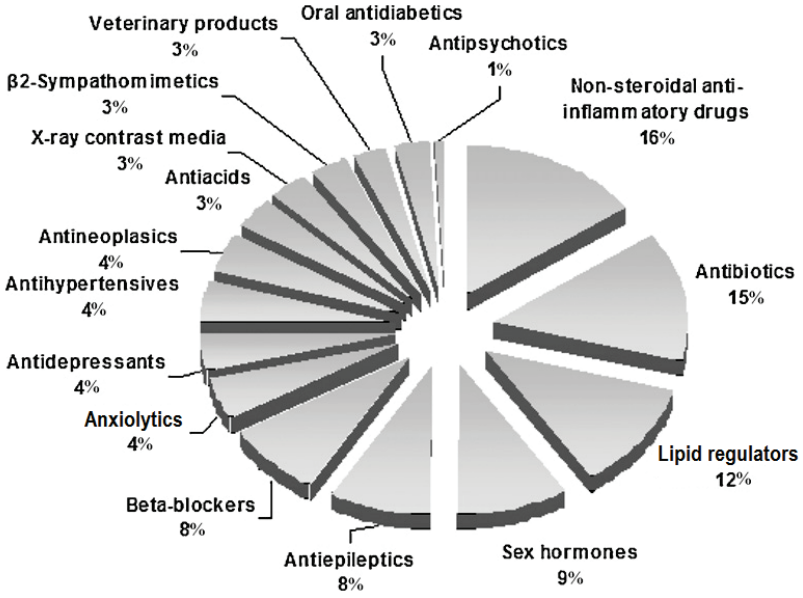

Fig. 1.3. Therapeutic classes of pharmaceuticals detected in the environment (expressed in relative percentage). Data collected from 134 articles published between 1997 and 2009 (*adapted from Santos et al.* (9)).

Groundwater contamination by pharmaceutical compounds is much less reported. However, few were already detected, such as paracetamol at 211 ng L^{-1} in French wells and carbamazepine up to 465 ng L^{-1} between 5-10 m below ground in bank filtration transects in Germany (35, 36). Besides, ibuprofen, salicylic acid, gemfibrozil, naproxen, indomethacin and bezafibrate were also found in effluents of septic tanks in Ontario, Canada, up to 2150, 480, 430, 300, 4 and 12 ng L^{-1}, respectively (23).

With regard to drinking water, results are even scarcer and most countries (if any) do not have monitoring programs to routinely test drinking water for pharmaceuticals, due to practical difficulties. Thus, the majority of the occurrence data comes from targeted research projects/investigations and ad hoc surveys (37). For instance, studies in the USA conducted by Benotti et al. (38) have detected very low concentrations of pharmaceuticals in finished drinking water, with the highest value of 40 ng L^{-1} reported for meprobamate.

In the Netherlands, however, antibiotics, antiepileptics and β-blockers have been detected at concentrations up to 100 ng L^{-1} in the drinking water supply (39). It is estimated that, to date, there have been detected between 15 and 25 pharmaceutical compounds in drinking water all over the world (37).

As previously stated, pharmaceutical compounds can be metabolized in humans or animals organism, then both native compounds and metabolites can reach the environment and originate different transformation products, under various physico-chemical and biological processes, as in WWTPs or waterworks. Thus, the designation of "by-products" (BPs) was created to comprise both the excreted metabolites and the environmental transformation products (10). The fate and behaviour of pharmaceuticals and their BPs in the environment still requires further elucidation. Nevertheless, it is understandable that their concentration levels in receiving waters can be attenuated first by dilution and then adsorption on suspended solids and sediments, colloids or other organic matter. They may also undergo biotic and/or physico-chemical transformations, namely direct and indirect photodegradation reactions, besides, hydrolysis, oxidation/reduction, isomerisation, etc. (40, 41). These phototransformation processes will be further discussed on chapter 4 of the present thesis.

1.2.1.4. Risk Assessment and (Eco)Toxicity

Results of toxicology studies have revealed that some pharmaceuticals are suspected to have direct toxicity towards certain aquatic organisms. Moreover, their continual but undetectable effects can slowly accumulate, finally leading to irreversible changes on both wildlife and human beings (42, 43). It is essential to foster the study on ecotoxicological hazards, to go through a comprehensive evaluation of the toxicity effects on non-target organisms, using tests for both acute effects (e.g., measuring mortality rate) and chronic effects (e.g., exposing to different concentrations of a chemical over a prolonged period of time and measuring growth index or reproduction rate) (44). These latter are particularly scarce, possibly owing to their complex experimental procedure (45).

There is a huge concern associated to the environmental contamination with antibiotics and the possible development of resistance mechanisms by bacteria. This could subsequently compromise public health in terms of treatment effectiveness. Chemical risk assessment methods for substances found in water involve establishing different points of

departure (PoD) or toxicological endpoints such as the predicted environmental concentration (PEC), predicted no-effect concentration (PNEC), no-observed effect concentration (NOEC), lowest observed effect concentration (LOEC), half-maximal lethal concentration (LC_{50}) and/or half-maximal effective concentration (EC_{50}) (9, 37). For instance, Yamashita et al. (46) evaluated the inhibition of the algae *P. subcapitata* by levofloxacin and clarithromycin and the results obtained showed that the latter presented higher toxicity, with an EC_{50} of 11 μg L^{-1} and a LOEC and a NOEC of 6.3 and 3.1 μg L^{-1}, respectively. On the other hand, Isidori et al. (47) tested five antibiotics (oxytetracyclin, sulfamethoxazole, ofloxacin, lincomycin and clarithromycin) on aquatic organisms from different trophic levels (bacteria, algae, rotifers, crustaceans and fish). Results showed that the antibiotics were less active against rotifers, crustaceans and fish, and that while acute toxicity levels were in the order of mg L^{-1}, chronic toxicity became visible at concentrations in the order of μg L^{-1}, mainly for algae.

Another therapeutic class raising great awareness is sex hormones, extremely active biological compounds capable of inducing intense therapeutic effect at very low doses. Within this group, estrogens are the most commonly found in the environment, existing either as natural or synthetic substances and acting as endocrine-disrupting compounds (48). For example, Kidd et al. (49) developed a 7-year experiment which demonstrated that the chronic exposure of fathead minnow to 5-6 ng L^{-1} of ethinylestradiol (EE2) led to feminization of males fish and altered oogenesis in females. EE2 is a synthetic estrogen present in oral contraceptive pills with proved estrogenic effects in fish.

In the group of NSAIDs, 28-days chronic toxicity tests conducted on the rainbow trout (*Oncorhynchus mykiss*) by exposure to only 1 μg L^{-1} of diclofenac resulted in cytological changes in the liver, kidneys and gills (50). Regarding the toxicity of lipid regulators such as statins, larval and adult grass shrimp (*Palaemonetes pugio*) 96-hours exposure to simvastatin resulted in a LC_{50} of 1.18 and 10 mg L^{-1}, respectively (51). Also the widely used antiepileptic carbamazepine showed to be carcinogenic to rats, though not presenting mutagenic properties in mammals. Furthermore, it proved to be lethal to zebrafish at 43 μg L^{-1} and induce sub-lethal changes in *Daphnia* sp. at 92 μg L^{-1} (52).

Finally, it must be highlighted that pharmaceutical compounds do not occur alone in the environment, but in mixtures of different active substances, their metabolites and transformation products, which may display potentially additive, antagonistic and even synergistic effects among them and with other naturally occurring compounds (53).

1.2.1.5. Legislation

Nowadays, there is still a big hiatus in legislation concerning the environmental contamination by pharmaceutical compounds. This gap is probably due to the scarcity of studies and available data which could allow a coherent profiling and a precise approach to this issue.

Up until 1992, there were no requirements imposed by the EU legislation towards the pharmaceutical industry, demanding the inclusion of environmental risk assessment data to obtain the Marketing Authorization of a new pharmaceutical product. This was first achieved through Directive 92/18/EEC (54), but only regarding veterinary products. Consequently, EMEA published a Note for Guidance (55), establishing the guidelines for the accurate risk assessment of veterinary medicinal products. The extension of this procedure to pharmaceutical products for human use was performed by the EC through Directive 2001/83/EC, later amended by Directive 2004/27/EC (11). These documents required the Marketing Authorization of a new pharmaceutical product for human use to be accompanied by a proper environmental risk assessment study, according to the guidelines set out by EMEA. Evaluation of the environmental impact of both human and veterinary medicines embraced two phases: Phase I, concerning the environmental exposure assessment to pharmaceutical compounds and/or their metabolites; Phase II, comprising the study of their effects and fate in the environment. This second phase was still sub-divided into two parts: Tier A, regarding the evaluation of potential effects and fate of the respective pharmaceuticals and/or metabolites, and Tier B, focusing on their potential effects on fauna and flora within specific environmental compartments. However, only pharmaceutical products for human use presenting a PEC in surface waters equal to or above 0.01 μg L^{-1} are requested to undergo Phase II studies (56) (Fig. 1.4).

In the USA, concerns over the environmental presence of pharmaceutical compounds have long been raised. At the moment, regulation established by the USA Food and Drug Administration (FDA) requires the inclusion of the environmental risk assessment study in order to obtain the Marketing Authorization, according to the Guidance for Industry - Environmental Assessment of Human Drug and Biologic Applications (57). Nonetheless, this study is only mandatory for pharmaceuticals with PEC superior to 1 μg L^{-1} (Fig. 1.5).

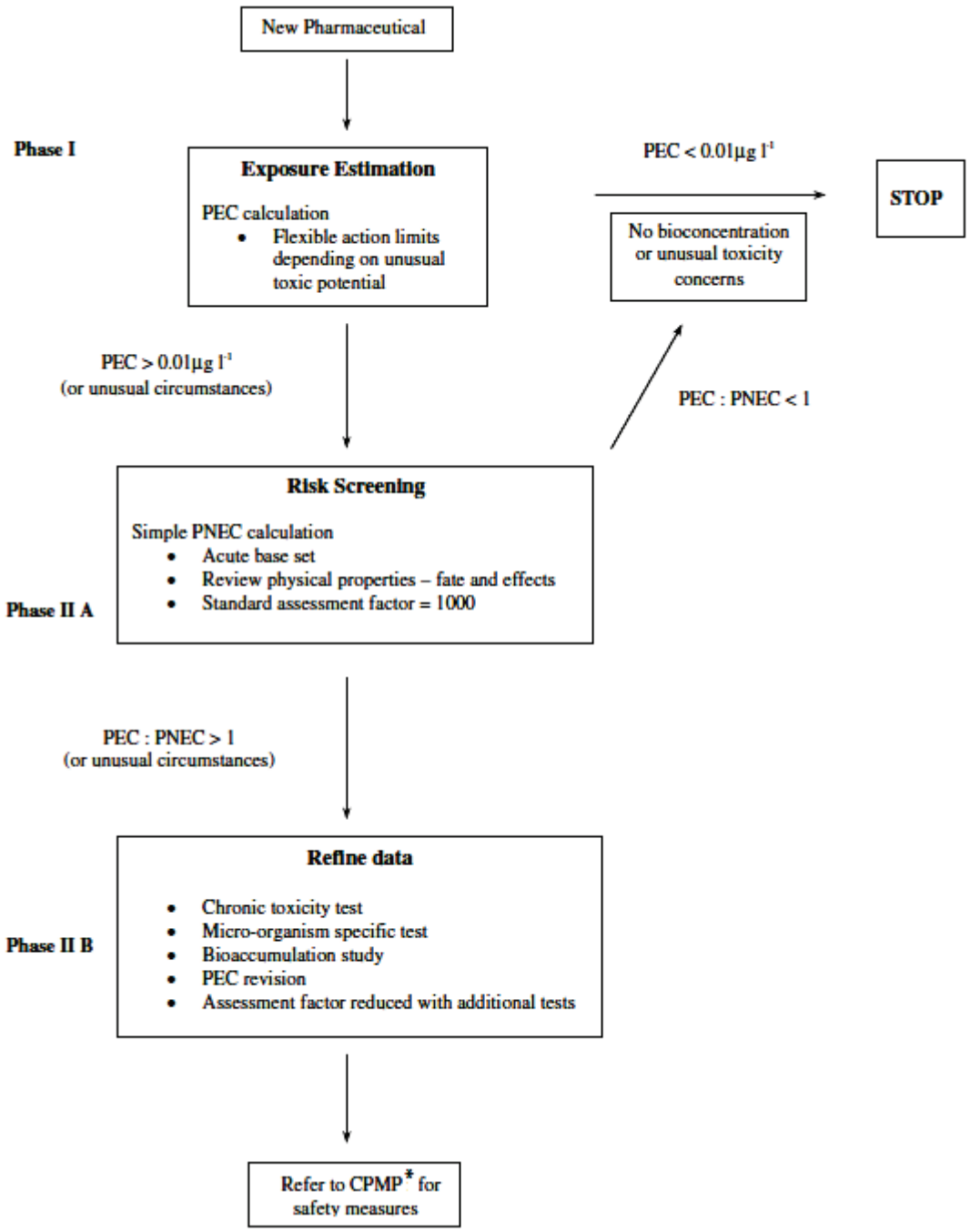

Fig. 1.4. Graphical representation of EMEA guidelines (58).

* **Committee for Proprietary Medicinal Products** (CPMP) was the former designation for the EMEA Committee.

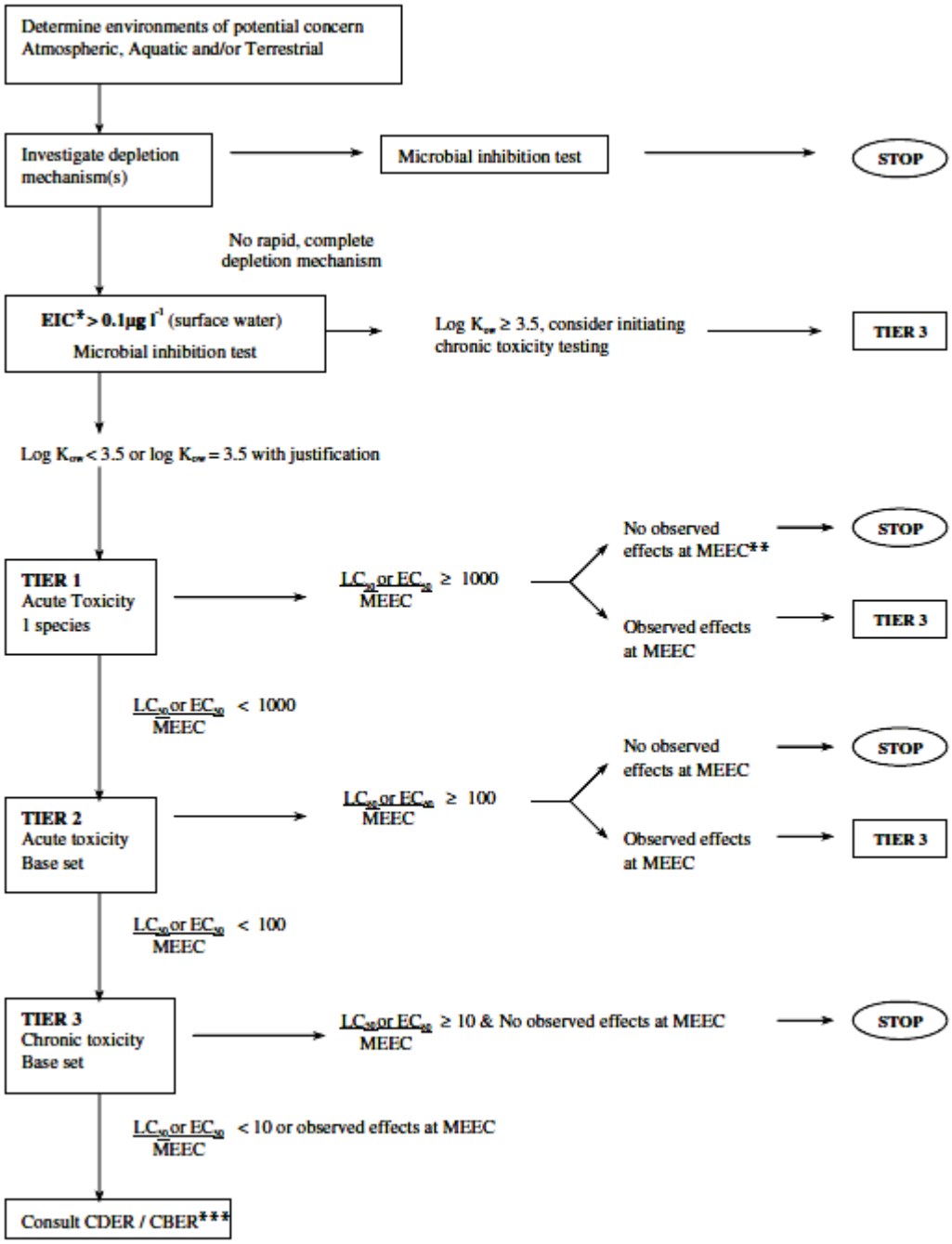

Fig. 1.5. FDA tiered approach to fate and effects testing (58).

* **Expected Introductory Concentration** (EIC);

** **Maximum Expected Environmental Concentration** (MEEC);

*** **Centre for Drug Evaluation and Research** (CDER)/ **Centre for Biologics Evaluation and Research** (CBER).

1.3. Thesis

1.3.1. Main Objectives

Considering the state-of-the-art *a priori*, as well as the main knowledge gaps and analytical obstacles, briefly exposed in the preceding sections, the ultimate purpose of this Ph.D. work was to significantly contribute to the enlightening of the problematic concerning the aquatic compartment contamination by pharmaceutical residues. This implies the development of novel analytical and experimental approaches to streamlining the detection, quantification and removal of pharmaceuticals from water samples, consequently providing new data on the impact and fate of these compounds in the environment. Research holds an important role in the support of new legal decisions, helping to reach a balance between all the actors involved, including official authorities, environmental managers and industry officers. The restrictive legislation that will be adopted at EU level will oblige to spend huge financial budgets and technical resources.

Bearing it in mind, a multidisciplinary strategy was adopted with the aim of help finding answers to the key-topics in environmental studies: occurrence, behaviour and toxicity.

Hence, the following tasks were addressed within this Ph.D. project:

1) Development of a multiresidue method for the analysis of pharmaceutical compounds in water, using an adequate sample preparation/extraction procedure, followed by the analysis through liquid chromatography–tandem mass spectrometry (LC-tandem MS or LC-MS/MS or LC-MS^2).

   Sample preparation processes are crucial for a good separation of target analytes from the remaining sample compounds. Basically, they comprise the following steps: *i)* extraction; *ii)* concentration; *iii)* cleansing; *iv)* extract injection. Nowadays, a wide variety of adsorbents/coatings is commercially available, allowing the efficient and selective extraction of compounds, within a broad range of polarities. These solid-phase adsorbents will be thoroughly studied during this work.

   One of the biggest limitations in the analysis of emerging pollutants relies on the lack of analytical methodologies able to detect and quantify contaminants present in low concentrations. One prerequisite for the quality assessment and monitoring of wastewaters, surface and drinking waters, is the capability to develop multiresidue methods, capable of providing rapid and accurate results. Even though there has been an increase of such methodologies reported in literature over the past few years, there

is still the need to improve both sensitivity and selectivity in the case of more complex matrices as wastewaters.

2) Participation in inter-laboratory exercises (ILEs) promoted by the Joint Research Centre (JRC) and organized under the scope of European projects, with the purpose of establishing a valid system for quality control and methods' harmonization.

3) Monitoring campaigns encompassing WWTP samples and receiving surface water bodies, impact assessment and further evaluation of the constraints influencing a representative sampling plan.
   During the last few decades, the scientific community has started reporting the presence and concentrations of pharmaceutical residues in WWTP effluents, surface waters, its fauna and flora. In the case of WWTPs, the great variability of results achieved is usually attributed to the type of treatment process employed, as well as its location, social/economic factors, most commercialized medicines and other demographic parameters.
   Regarding sampling processes, either grab or composite samples can be collected using automatic samplers, according to the results obtained after preliminary exploratory examinations. Recently, passive sampling is considered a very promising technique. However, it is still in a preliminary experimental phase.

4) Study of the photodegradation kinetic of several pharmaceutical compounds, at lab-scale and pre-industrial pilot scale.
   The influence of several factors must be taken into consideration, such as temperature, pH, presence of dissolved and particulate material and radiation source. The results attained may be of great relevance for the conception and design of future WTPs and WWTPs.

5) Identification of phototreatment by-products and elucidation of the involved photo(cata)lytic degradation mechanisms.
   Herein, there is the need to take profit of advanced high-resolution techniques, such as ultra-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-QToF-MS), which enable an accurate highly sensitive non-target screening approach.

1.3.2. Organization

The presented Ph.D. thesis is organized in chapters, each one dealing with a different topic and including its own bibliographic references.

In Chapter 1, "General Introduction", a broad overview is presented concerning water quality policy and the recent problematic of emerging water micropollutants. Special emphasis is given to pharmaceutical compounds, including their therapeutic classification and identification of main environmental contamination sources. Moreover, a brief review on pharmaceuticals' environmental distribution, occurrence and fate, as well as their consequent (eco)toxicological effects, already demonstrated in different species, is also displayed. A final note regarding the still incomplete legislation on this issue is presented in the end.

In Chapter 2, "Analytical Method Development for Detection and Quantification of Pharmaceuticals in Waters", the different stages of a multiresidue analytical method for pharmaceuticals' quantification are approached. From the optimization of the cleanup and extraction procedure, to the selection of the most suitable separation and detection techniques, different aspects are taken into consideration, according to the specific target analytes and the nature and complexity of samples' matrix. This chapter also includes the publication "*Cleanup strategies and advantages in the determination of several therapeutic classes of pharmaceuticals in wastewater samples by SPE-LC-MS/MS*", which reports on the work conducted during the development, optimization and validation of the analytical method, applied to the detection and quantification of diverse pharmaceutical compounds in wastewater samples.

In Chapter 3, "Monitoring of Pharmaceuticals in Municipal Wastewaters and Surface Waters", the monitoring results achieved with the developed method on Febros WWTP, and its impact on Febros river and later on Douro river, are assessed regarding the pharmaceutical content. Furthermore, different sampling methodologies are comparatively evaluated. This chapter also includes the scientific paper in Portuguese language "*Effects of treated domestic effluents and tributaries on the contamination of Douro river with pharmaceutical compounds – Mitigation processes*", concerning the results aforementioned. Finally, the participation in two inter-laboratory exercises, achieved results and withdrawn conclusions regarding the method reliability are also reported.

In Chapter 4, "Photo-Remediation of Contaminated Waters using Advanced Oxidation Processes (AOPs)", various advanced oxidation processes for pharmaceuticals' mitigation in water samples are described. Special attention is given to heterogeneous photocatalytic processes, while the influence of different experimental variables on the overall treatment is also studied. This chapter also includes two published papers "*Photolytic and TiO_2-Assisted Photocatalytic Oxidation of the Anxiolytic Drug Lorazepam (Lorenin® pills) under Artificial UV Light and Natural Sunlight: A Comparative and Comprehensive Study"* and "*Suspended TiO_2-Assisted Photocatalytic Degradation of Emerging Contaminants in a Municipal WWTP Effluent using a solar Pilot Plant with CPCs*", concerning the optimization of TiO_2-assisted photocatalytic degradation processes of lorazepam, a recalcitrant anxiolytic drug, and later application to the treatment of a municipal WWTP effluent, contaminated with diverse pharmaceuticals.

In Chapter 5, "Phototreatment Mechanisms and By-Products Structural Elucidation", lorazepam photo(cata)lytic degradation mechanisms are enlightened, while the resulting by-products are also identified and structurally elucidated. This chapter is mostly composed by the publication "*Lorazepam Photofate under Photolysis and TiO_2-assisted Photocatalysis: By-products' Identification and Evolution Profiles during Phototreatment of a Contaminated WWTP Effluent*".

In Chapter 6, "Occurrence of Emerging Pollutants' By-Products in Water Resources", a résumé of a broad review regarding emerging pollutants' by-products environmental occurrence in different water sources is presented.

In Chapter 7, "Final Conclusions and Remarks: Work Novelty, Knowledge Gaps and Future Research", the key achievements of the work are presented, as well as the main knowledge lacunas and missing scientific evidences which may be filled in and enlightened during some future experiments' proposals.

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Chapter 2

Analytical Method Development for Detection and Quantification of Pharmaceuticals in Waters

2.1. Multiresidue Methods for the Analysis of Pharmaceutical Compounds

Given the hundreds of tonnes of pharmacologically active substances consumed per year, the exponentially increasing amount of identified metabolites and transformation products (TPs) resulting from different natural and/or artificial physico-chemical and biological degradation processes, the hypothesis of finding complex mixtures or "cocktails" of these pollutants in the environmental aquatic compartment seems well-founded. Consequently, there is a strong need to search for a wide range of pharmaceuticals and respective by-products, potentially contaminating different environmental matrixes. Taking into account all the resources and time expenses involving this task, the newly developed analytical methods must be capable to simultaneously determine trace levels of the largest amount of compounds possible, thus allowing to reduce the number of cleanups and extraction steps. Such methods are addressed as multiresidue or multiclass methods, contrary to analytical methods encompassing only one class of pollutants, or eventually specific to one compound only (1). Therefore, multiresidue analytical methods constitute a prerequisite to provide reliable figures on the occurrence, partition, removal and fate of pharmaceutical contaminants in the environment, reducing monitoring costs and simultaneously enabling to increase its frequency and spread (2).

In the attempt to simplify the analytical work, the rational selection of target analytes to be included in the multiresidue method also constitutes one extremely important criterion. In this Ph.D. work, and since pharmaceuticals' environmental occurrence is highly

conditioned by their consumption levels, region- and time-specificity were two significant factors considered during the selection process. It would obviously be a waste of time and resources to monitor the presence of pharmaceuticals little or not at all commercialized in our country, whose environmental occurrence in real matrices would thus be unlikely. Hence, the pharmaceutical compounds encompassed in our study (**Paper 1**) were chosen according to data provided by the Portuguese National Authority of Medicines and Health Products, IP (INFARMED), belonging to the list of the 30 most marketed pharmaceuticals in Portugal, over the last few years (please refer to section 1.2.1.2). They represent 9 different therapeutic classes, including analgesic and antipyretic drugs, anti-inflammatories, lipid regulators, antibiotics, anxiolytics, antidepressives, diuretics, cardiotonics and anti-ulcer agents. These therapeutic groups are also within the most studied, reported and consumed worldwide.

Several multiresidue analytical methods have already been described in literature, some for the detection of specific therapeutic categories (3, 4), others aiming at a wider range of compounds (5, 6). Nevertheless, the set-up of a multiresidue method implies taking into consideration all different stages of the analytical process, i.e., not only assuring that the separation and detection systems allow the identification of all target analytes, but also that these can undergo a common pre-treatment process (e.g., preservation and sample extraction), more complicated when applied to complex matrixes such as wastewater samples.

2.2. Extraction and Concentration of Water Samples

According to a review study by Liska (7), two major target areas of interest can be distinguished during the development of a method for environmental organic trace analysis: *i)* sample preparation and *ii)* analytical separation and detection. While remarkable progress has been achieved during several decades concerning the second area, sample preparation has been in the shadow for quite longer time. Only after highly sensitive analytical systems became common standard for environmental analysts did they realise that samples' preparation was an important breaking factor in the general progress of environmental analysis. Nonetheless, this stage is usually highly time-consuming (8), comprising less automated processes during which many errors can take place, carrying along more or less significant deviations on the accuracy and precision of the results (9).

The use of an appropriate sample handling technique is thus a must in an analysis of organic micropollutants in water. The main goal is to achieve a sample extract enriched in target analytes and free of other matrix components, as far as possible. To do so, three key-steps must be conducted: *i)* extraction of trace amounts of target analytes from the sample matrix, *ii)* concentration of those analytes, and *iii)* removal of other substances which may be co-extracted and simultaneously concentrated, consequently hampering the efficiency of the method (7, 10).

Every effort concerning the improvement of this analytical stage may result in significant increases in the yield and quality of the results obtained (9). The most relevant sample pre-treatment techniques, some of which employed during this Ph.D. work, will be discussed in the following sections.

2.2.1. Solid-Phase Extraction

2.2.1.1. Fundamentals and Applications

The first experimental applications of solid-phase extraction (SPE) started approximately five decades ago (7). From then on, hundreds of scientific papers have described various developments and applications of SPE in water analysis (11-13), being currently one of the most frequently used techniques for cleanup and pre-concentration of pharmaceuticals from water samples (14-17).

The isolation process comprises the selective retention of analytes in a solid adsorbent, which can in turn be eluted with an organic solvent. Analytes are selectively trapped on the adsorbent mainly through hydrogen bonds, Van der Waals forces, hydrophobic effects, π-electron interactions and cation- or anion-exchange processes (18). A scheme of SPE procedure is shown in Fig. 2.1.

SPE major goals are the extraction, concentration and elimination of interfering components. Nevertheless, other advantageous uses of SPE have been found out: *i)* storage of analytes in the adsorbed stage, most useful for less stable compounds in the aqueous phase or volatile substances, *ii)* fractionation of the sample extract in different groups of compounds and *iii)* derivatization of analytes in the cartridge (10). However,

SPE also has several drawbacks, such as the loss of more polar compounds during sample percolation, the co-extraction of matrix interferences, filters clogging, a noticeable time consumption for the extraction of large water volume, a significant consumption of organic solvents, an incomplete desorption of target analytes, the loss of volatile compounds and the incomplete recovery of the dry extract (1, 18).

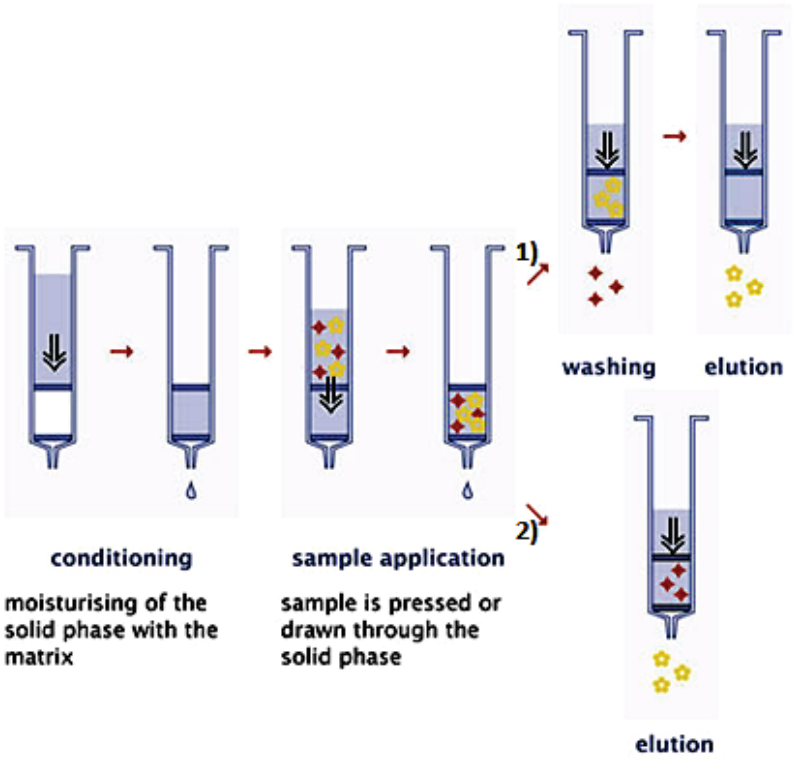

Fig. 2.1. Illustration of the SPE process: ✦ - interferences; ★ - target analytes. 1) *Retention of the analyte*: analyte molecules are enriched on the adsorbent > interfering components and solvent molecules (matrix) are not retained > remaining interfering components are washed from the adsorbent > the analyte is removed from the adsorbent by elution with a suitable solvent (e.g., methanol, tetrahydrofuran (THF), -propanol);
2) *Retention of interfering components*: analyte molecules show no interaction with the adsorbent > interfering components and solvent molecules (matrix) are retained > analyte molecules are "washed" from the adsorbent > the solid phase is simply used to "filter" the sample (19).

2.2.1.2. Adsorbents

Several SPE adsorbents have been developed. Among them, the most adequate for the extraction of organic compounds are the reverse phase sorbents, from the conventional

alkyl-modified silica materials (C8 and C18 non-polar phase), to the more recent polymeric sorbents, which improve the retention of polar compounds due to the high surface area and cross-links (e.g., poly(styrene-divinylbenzene) (PS-DVB)), and carbon materials (e.g., graphitised carbon black (GCB)) (18, 20).

Polymeric sorbents present different functional groups combined with a polymeric skeleton, allowing more than one kind of interaction between analytes and the sorbent (hydrophilic, hydrophobic and π- π interactions) (18). Some examples of these sorbents are displayed in Table 2.1.

Table 2.1. Commercial brands of SPE extraction columns constituted by polymeric adsorbents, frequently utilized in the extraction of pharmaceutical compounds from water samples

Cartridge commercial designation	Adsorbent composition	Application	Reference
Strata-X	Polydivinylbenzene resin chemically modified with piperidone groups	Extraction of non-steroidal anti-inflammatory drugs (NSAIDs) from surface and groundwaters	(21)
Evolute ABN	Surface modified polystyrene divinylbenzene polymer	Extraction of human pharmaceuticals from river waters	(22)
Oasis HLB (**H**ydrophilic-**l**ipophilic-**b**alanced reversed-phase sorbent for acids, bases and neutrals)	Hydrophobic-lipophilic copolymer of divynilbenzene and vinylpirrolidone	Extraction of veterinary pharmaceuticals from wastewaters	(23)
Oasis MCX (**M**ixed-mode **c**ation e**x**change sorbent for bases)	Poly(divynilbenzeneco-N-vinylpirrolidone) copolymer on which strong-cation exchanging sulfonic groups are convalently bonded	Extraction of basic/neutral pharmaceuticals and illicit drugs from surface waters	(15)
Oasis MAX (**M**ixed-mode **a**nion e**x**change sorbent for acids)	Poly(divynilbenzeneco-N-vinylpirrolidone) copolymer on which strong-anion exchanging quaternary ammonium groups are convalently bonded	Extraction of human pharmaceuticals from wastewaters	(24) (**Paper 1**)

The recent trends in the development of SPE (and cleanup) adsorbents contemplate the extraction of increasingly polar compounds and wider polarity ranges, thus extending the scope of SPE and further supporting the improvement of multiresidue methods. Some recent developments in this field will now be presented.

Monolithic (particle free) SPE is a recent strategy to deal with selectivity and sensitivity issues regarding liquid chromatography-mass spectrometry (LC-MS) bio-analytical methods. In contrast to SPE cartridges, monolithic stationary phases are presented in the format of SPE discs (available in 15 different sorbent chemistries, such as C2,C8 and C18) (25) and 96-wells plates, more easily automated. These materials present several advantages: *i)* low cost, *ii)* mechanical robustness and high stability, *iii)* no void volumes, *iv)* easy control of the porous properties of adsorbents, *v)* high hydraulic permeability and *vi)* dominance of the convection over the diffusion mechanism of mass-exchange under dynamic conditions that allow the separation at extremely high flow rates (26).

Extraction selectivity may also be increased using *molecularly imprinted SPE* (MISPE). Molecularly imprinted polymers (MIPs) are synthetic polymers with a predetermined selectivity for a given analyte or group of structurally related compounds. They enclose specific recognition sites which are complementary in shape, size and functional group, to the analyte or analytes of interest (18, 26). MIPs have already been synthesised for several emerging pollutants, including pharmaceuticals and endocrine disrupting compounds (EDCs) (27).

Immuno-affinity chromatography (IAC) is a technique based on the affinity between antibodies and antigens, mainly applied as a cleanup technique in food and biological analyses. Although presenting high selectivity and enabling the better cleanup attained for polar compounds in complex matrices, its main drawbacks include small breakthrough volumes and low stability against solvents, strong pH and temperature (26, 28).

Restricted access materials (RAMs) are frequently used as pre-columns in column-switching LC systems. They consist on a support type which allows direct injection of complex samples by limiting the accessibility of interaction sites within the pores only to small molecules (26).

Most recently, new nanomaterials have been exploited as well to integrate new extraction and cleanup technologies (29).

Besides being used for sample extraction (Fig. 2.1 -1)), SPE can also be applied to sample cleanup (Fig.2.1-2)); in this case, normal phase adsorbents such as silica, alumina and florisil are usually preferred (10) (**Paper 1**).

It must also be noticed that pH control plays an important role in SPE, since it governs the dissociation of ionisable compounds, therefore affecting their hydrophobicity and interaction with the sorbent. For instance, when using a reverse phase sorbent, the retention of a compound increases for pH values favouring their non-ionised form (21).

Lately, the development of on-line SPE coupled to LC-MS/MS methodologies has further increased the overall method robustness. Besides saving time on the analysis, it offered an alternative as a more environmentally friendly method for pharmaceutical determinations in waters. In fact, on-line SPE requires low sample and solvent volumes (14).

2.2.2. Solid-Phase Microextraction

Solid-phase microextraction (SPME) was introduced by Pawliszyn and co-workers (30), in 1989, representing a significant step forward in terms of simplification and miniaturization of the analytic process.

SPME represents a solvent-free sampling technique, often used in the extraction of organic micropollutants from water samples. It employs a fused-silica fibre coated on the outside with an appropriate stationary phase. SPME fibres are commercially available in a "syringe-like" format, which allows its protection during septum puncture (30). Volatile analytes can be emitted from the sample and isolated from the headspace or adsorbed by direct immersion into the liquid sample and concentrated in the fibre coating. SPME association with gas chromatography-mass spectrometry (GC-MS) is overwhelming comparatively to LC-MS (31, 32). Therefore, after the extraction, thermal desorption in the hot GC injection port usually follows (33).

Fig. 2.2 shows a schematic representation of a SPME set-up and operating modes, as well as the composition of a SPME fibre.

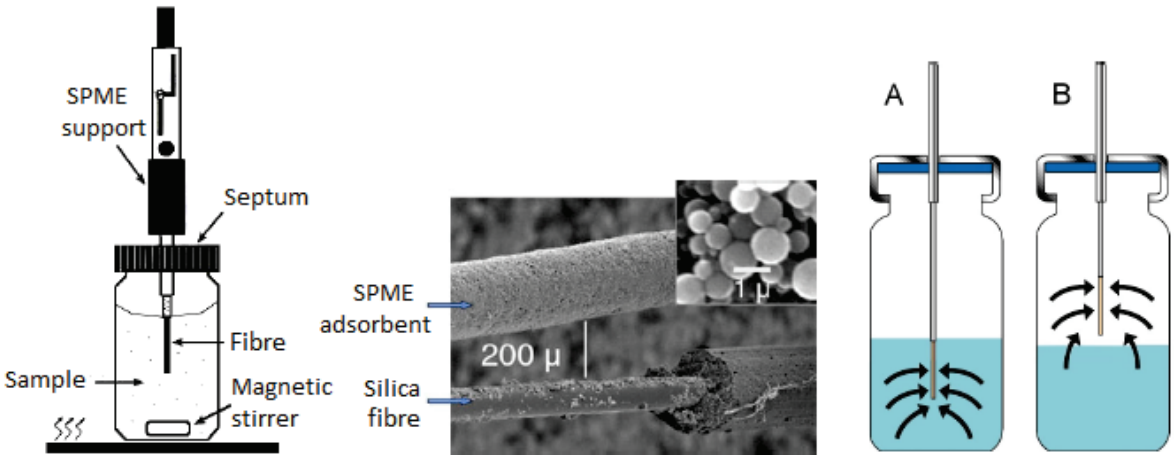

Fig. 2.2. SPME components: on the left – scheme of a SPME set-up; in the centre – detail of a SPME fibre; on the right – operating modes of SPME: A – immersion, B – headspace.

SPME is associated with strong matrix effects and, consequently, complications in quantification. Moreover, the variability of detection limits for several analytes in dependent upon the equilibrium between the fibre coating and the matrix (33). Different factors can influence the efficiency of the SPME process: *i)* the type of adsorbent polymer (34), *ii)* sample stirring (35), *iii)* sample temperature (9), *iv)* sample pH (34), *v)* sample ionic strength (36) and *vi)* the duration of the extraction (37).

On the other hand, SPME presents as main advantages its simplicity, low cost and low time consumption, high selectivity and sensitivity, when coupled to adequate detection methods (37). It integrates, in only one continuous process, sampling, extraction, concentration and introduction of target analytes in the chromatographic system for separation and detection. All these pros, along with its "solventless" character, make of SPME a rather promising extraction technique (37, 38).

SPME successful application on the extraction of various emerging contaminants, including pharmaceuticals, from water samples has also been reported in several studies (39-42).

2.2.3. Other Extraction Techniques

Apart from SPE, which is the most used technique in pharmaceutical analyses, SPME has also earned great acknowledgment in this field, presenting further increasing development potential. On the contrary, more conventional techniques are now less employed. Such is the case of liquid-liquid extraction (LLE), which presents several drawbacks, including the emulsion formation, the use of large volumes of solvents, environmentally unfriendly, high time consumption and high cost. Liquid-phase microextraction (LPME) is a solvent-minimised sample pre-treatment modality of LLE, requiring some μL of organic solvent to concentrate analytes (43). Nevertheless, LPME shows a low precision (44).

Other more recent extraction techniques include dispersive liquid-liquid microextraction (DLLME) and stir membrane liquid-liquid microextraction (SM-LLME), both presenting high extraction efficiencies (18).

2.3. Separation and Detection of Pharmaceutical Compounds

In order to determine very low concentration levels of pharmaceutical compounds in waters, the appropriate sample pre-treatment is fundamental. Afterwards, separation, detection, quantification and confirmation of analytes follow. The separation of organic compounds is usually achieved either by liquid or gas chromatography, according to analytes' polarity, volatility and decomposition at high temperatures. While volatile or semi-volatile compounds may be analysed by GC, more polar or thermolable non-polar compounds are analysed by LC, with no need of prior derivatization. Generally, every compound is amenable to LC, although not all are directly and easily detected by the available detectors (10).

Fast and high resolution LC systems have been developed, without compromising resolution and separation efficiency. They include liquid chromatography at ultra high pressures, using sub-2-μm particle packed columns (UHPLC) (**Paper 5**), the use of monolith columns, the use of fused core columns and high-temperature liquid chromatography (HTLC) (26).

Nowadays, the detection at sub-ppt concentrations is practically routine for many organic contaminants, including pharmaceutical compounds in water and wastewater, whereas some methods can even detect few hundred fentograms of some analytes (45). These impressive improvements in method detection limits are mostly attributed to the use of hyphenated chromatography-mass spectrometry techniques, which currently constitute the methods of election for the analysis of trace organic contaminants in environmental samples (26). Fig. 2.3. presents some examples of pharmaceutical compounds usually detected in water and wastewater, using these advanced analytical methods (46).

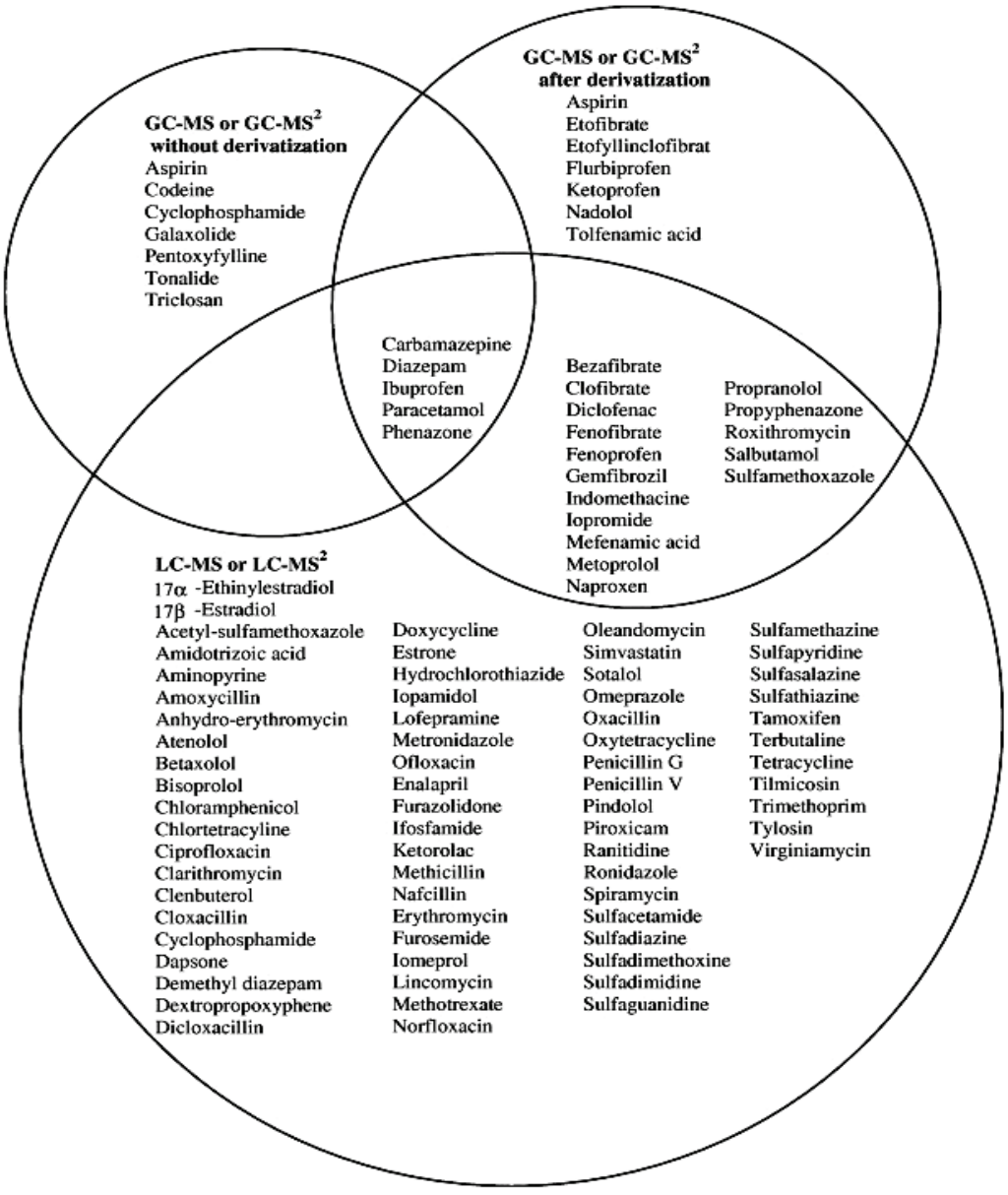

Fig. 2.3. Analytical methods applied for the most common pharmaceutical compounds present in water and wastewater (*from Fatta et al.* (46)).

Regarding mass spectrometry (MS), it is an instrumental technique based on the separation of ions according to their mass-to-charge ratios (*m/z*), in vacuum, in the gas phase. After ionization, the (pseudo)molecular ions formed can undergo further fragmentation and all these formed ions are then separated in the mass spectrometer according to their *m/z* and are detected. A mass spectrometer is composed by three fundamental parts: an ion source, a mass analyser and a detector (Fig. 2.4) (33).

Several ionisation methods are available. Hence, the fitness of the ion source will depend on the polarity, molecular weight and thermal lability of the analytes, as well as the complexity of the sample to be analysed. Concerning mass analysers, they can vary from quadrupole, quadrupole ion trap, linear quadrupole ion trap and magnetic sector, representing scanning instruments, to other type of mass analysers, such as time-of-flight (ToF) (Fig. 2.5), Fourier-transform ion cyclotron resonance analyser and orbitrap, which belong to the group of non-scanning instruments. Afterwards, the detector allows to monitor the ion current and finally records the data as mass spectra (33).

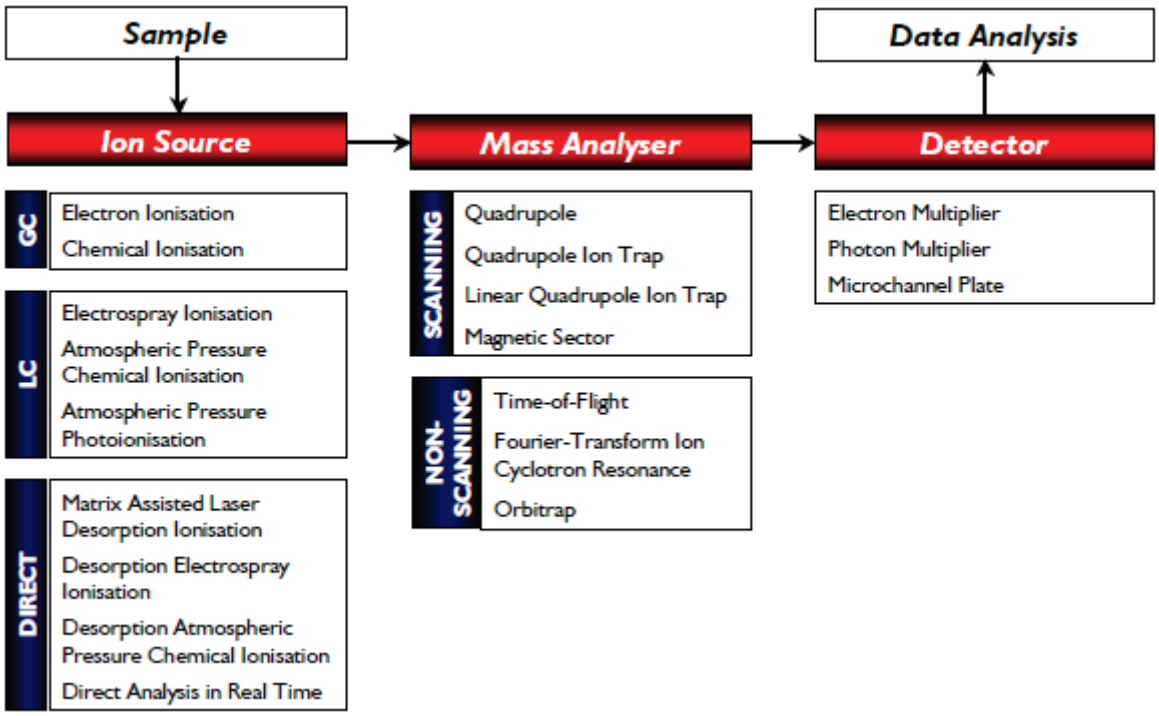

Fig. 2.4. Overview of mass spectrometric techniques (*from Cajka et al.* (33)).

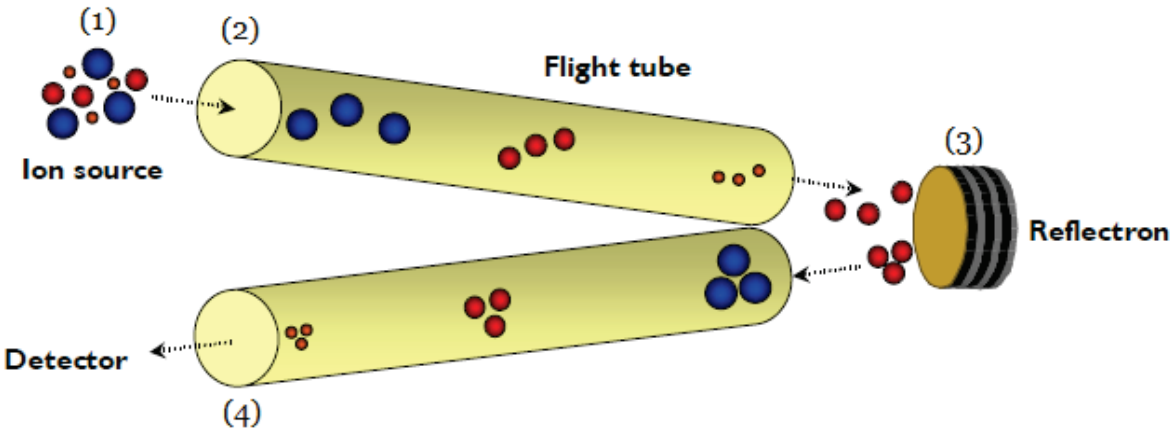

Fig. 2.5. Time-of-flight mass analyser. (1) Pulse of ions from the orthogonal accelerator (spatial focusing); (2) separation of ions according to their flight times; (3) focusing of the kinetic energy of ions; (4) separation of focused ions according to their flight times, dependent on their weight (*adapted from Cajka et al.* (33)).

Full-scan sensitivity, a low-femtomole level of sensitivity, high-mass resolution and mass accuracy (mass errors below 2 mDa or 5 ppm) can be achieved with QqToF-MS (47). Consequently, these instruments are often the first choice in studies devoted to the elucidation of degradation mechanisms of organic emerging pollutants, under environmental conditions. Several strategies can be employed for the identification of degradation products, taking profit of exact mass measurements, tandem mass fragmentation and typical "diagnostic ions". This subject will be further addressed in Chapter 5, applied to the photo(cata)lytic degradation of the anxiolytic drug lorazepam (**Paper 5**).

Herein follows **Paper 1**, which reports on the analytical approaches and obtained results during the optimization of a multiresidue method for the determination of several pharmaceutical compounds in wastewaters, giving particular emphasis to the cleanup step. Another work on this topic was also published by our group, although this one more specifically oriented to cleaner river waters (Annex II).

2.4. Paper 1 – Cleanup strategies and advantages in the determination of several therapeutic classes of pharmaceuticals in wastewater samples by SPE-LC-MS/MS

Published Scientific Paper:

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Anal Bioanal Chem (2011) 399:807–822
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ORIGINAL PAPER

Cleanup strategies and advantages in the determination of several therapeutic classes of pharmaceuticals in wastewater samples by SPE–LC–MS/MS

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Abstract This work describes the development and validation of an offline solid-phase extraction with simultaneous cleanup capability, followed by liquid chromatography–(electrospray ionisation)–ion trap mass spectrometry, enabling the concurrent determination of 23 pharmaceuticals of diverse chemical nature, among the most consumed in Portugal, in wastewater samples. Several cleanup strategies, exploiting the physical and chemical properties of the analytes vs. interferences, alongside with the use of internal standards, were assayed in order to minimise the influence of matrix components in the ionisation efficiency of target analytes. After testing all combinations of adsorbents (normal-phase, ion exchange and mixed composition) and elution solvents, the best results were achieved with the mixed-anion exchange Oasis MAX cartridges. They provided recovery rates generally higher than 60%. The precision of the method ranged from 2% to 18% and 4% to 19% (except for diclofenac (22%) and simvastatin (26%)) for intra- and inter-day analysis, respectively. Method detection limits varied between 1 and 20 ng L^{-1}, while method quantification limits were <85 ng L^{-1} (both excluding ibuprofen). This analytical method was applied to gather preliminary results on influents and effluents of two wastewater treatment plants (WWTPs) located in the urban region of Porto (Portugal). Typically, paracetamol, hydrochlorothiazide, furosemide, naproxen, ibuprofen, diclofenac and bezafibrate were detected in concentrations ranging from 1 to 20 μg L^{-1}, while gemfibrozil, simvastatin, ketoprofen, azithromycin, bisoprolol, lorazepam and paroxetine were quantified in levels below 1 μg L^{-1}. These WWTPs were given particular attention since they discharge their effluents into the Douro river, where water is extracted for the production of drinking water. Some sampling spots in this river were also analysed.

Keywords Pharmaceuticals · Wastewater · Cleanup · MAX cartridges · Liquid chromatography–tandem mass spectrometry (LC–MS/MS) · Surface water

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Introduction

Emerging pollutants are defined as compounds that are not currently covered by the existing legislation in the area of water quality, whose environmental impact is not yet sufficiently studied and which are thought to be potentially harmful to environmental ecosystems and human health [1]. They encompass a wide range of products, including pharmaceuticals, personal care products, fragrances, detergents, plasticisers, flame retardants, pesticides and several other classes (NORMAN FP6 Project, http://www.norman-network.net/index_php.php?module=public/about_us/emerging&menu2= public/about_us/about_us#substances).

The environmental occurrence of pharmaceutical products is known since the 1970s, first in the USA and almost one decade later in the UK (England) [2]. Concerns about the

hazards associated with long-term exposure for non-target organisms and for human health are emerging. Nevertheless, this is probably due not only to the increasingly disseminated use of these compounds, on both human and veterinary levels, but mainly to the development of more selective and sensitive analytical techniques, enabling detection limits as low as a few nanogrammes per litre, that allowed revelation of new contaminants.

The major sources of environmental pharmaceutical contamination are considered to be wastewater treatment plants (WWTPs), which are not prepared for the complete removal or degradation of such micropollutants, as mentioned in several studies [3–5]. However, pharmaceuticals can reach the environment after transport and distribution via different routes, for instance, through septic tanks commonly used in countryside households, which are susceptible to release the pharmaceutical compounds into groundwaters [6]. Other direct and indirect pathways include the improper disposal of unused or expired drugs, anthropogenic activities like aquaculture, manufacture spill accidents and, most importantly, via run-off and leaching from fields where manure and livestock wastes were applied [7]. Despite the fact that the two most studied aqueous compartments are wastewater and surface water [4, 8–10], where the majority of the pharmaceutical compounds have been found, some publications do also report their presence in groundwater and even drinking water [11, 12].

One of the best known detrimental effects of the pharmaceutical substances in the environment is the disruption of the endocrine system in wild species, affecting their growth, physiology and reproduction. The selection of multi-resistant strains of pathogenic microorganisms is also a threat to humans and ecosystems, resulting from the uncontrolled use of antibiotics and deficient treatment of effluents [13, 14]. However, even more worrying than pollution by isolated substances is the presence of cocktails of parent substances, metabolites and degradation products, for which toxicity studies are even more scarce [15]. Therefore, simple, efficient, high-throughput and broad-spectrum/multi-residue methods are requested in order to provide a great amount of data necessary for pollution characterisation and decision making.

The main goal of this work was to simultaneously determine 23 pharmaceuticals of diverse chemical nature, in Portuguese wastewaters, through the development and validation of a solid-phase extraction (SPE) followed by a liquid chromatography–(electrospray ionisation)–ion trap mass spectrometry (LC–(ESI)–MS/MS) method. Despite the fact that research for understanding the fate of pharmaceuticals in WWTPs has now been intensively performed [1, 3, 7], specific regional surveys still need to be considered since the occurrence pattern of pharmaceuticals in WWTPs is normally related to local production or sales figures. Furthermore, notwithstanding the diversity of analytical procedures already described [2, 8, 10], a more methodical approach, namely regarding wastewater samples' preparation and cleanup, is indispensable. An efficient cleanup procedure appeared crucial to obviate matrix interferences, the main cause of inaccuracy in the analysis of wastewater samples.

We have undertaken a systematic effort with this aim, testing several adsorbents such as silica gel, Florisil and graphitised carbon black (GCB) (normal-phase), as well as strong anion exchange (SAX) and weak anion exchange (WAX) (NH_2–) and mixed-anion exchange (MAX). Selective wash of the SPE extracts with increasing percentages of MeOH in water and filtration with narrow pore polyester filters (until 0.22 μm) were also attempted. To the authors' knowledge, such a methodical cleanup evaluation for the analysis of challenging analytes as pharmaceuticals has not been published in literature so far, but the optimised method looked extremely promising, even for a group of compounds with so diverse physicochemical properties.

Pharmaceutical compounds hereby encompassed belong to nine different therapeutic classes, including analgesic and antipyretic drugs, anti-inflammatories, lipid regulators, antibiotics, anxiolytics, antidepressives, diuretics, cardiotonics and antiulcer agents. They are among the most marketed pharmaceuticals in Portugal, over the last few years (according to the National Authority of Medicines and Health Products, IP), and the corresponding therapeutic groups are within the most studied, reported and consumed worldwide [2, 7].

As part of a wider effort, this work also aimed to preliminarily evaluate the impact of pharmaceuticals present in effluents to the contamination load into Douro river, one of the main Portuguese rivers and source of raw water for the production of drinking water supplied to a huge metropolitan population in Porto urban region.

Experimental

Chemicals, stock solutions and materials

The studied pharmaceutical compounds and respective therapeutic groups are presented in Table 1.

All pharmaceutical standards used were of analytical grade purchased from Fluka, Sigma and Riedel-de-Haën (Sigma-Aldrich, Spain), except the benzodiazepines, which were supplied by LGC Standards (Barcelona, Spain). The isotopically labelled compounds paracetamol-D4 (100 μg mL^{-1}) and fluoxetine-D5 (99%, 1 mg mL^{-1}), used as internal standards, were purchased from LGC Standards and Sigma-Aldrich (Spain), respectively.

Individual stock standard solutions were regularly prepared in methanol, at the approximate concentration of 500 mg L^{-1}

Table 1 Characteristics and therapeutic classes of the studied pharmaceutical compounds

Therapeutic group	Pharmaceutical	Molecular formula	Molecular weight	CAS no.
Analgesic and antipyretic drugs	Paracetamol	$C_8H_9NO_2$	151.2	103-90-2
Non-steroidal anti-inflammatory drugs (NSAIDs)	Diclofenac	$C_{14}H_{10}Cl_2NNaO_2$	318.1	15307-79-6
	Ibuprofen	$C_{13}H_{18}O_2$	206.3	15687-27-1
	Ketoprofen	$C_{16}H_{14}O_3$	254.3	22071-15-4
	Naproxen	$C_{14}H_{14}O_3$	230.3	22204-53-1
	Nimesulide	$C_{13}H_{12}N_2O_5S$	308.3	51803-78-2
Lipid regulators	Bezafibrate	$C_{19}H_{20}ClNO_4$	361.8	41859-67-0
	Gemfibrozil	$C_{15}H_{22}O_3$	250.3	25812-30-0
	Simvastatin	$C_{25}H_{38}O_5$	418.6	79902-63-9
Antibiotics	Azithromycin	$C_{38}H_{72}N_2O_{12}$	749	83905-01-5
	Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	331.4	85721-33-1
Anxiolytics	Alprazolam	$C_{17}H_{13}ClN_4$	308.8	28981-97-7
	Bromazepam	$C_{14}H_{10}N_3BrO$	316.2	1812-30-2
	Diazepam	$C_{16}H_{13}N_2ClO$	284.7	439-14-5
	Lorazepam	$C_{15}H_{10}N_2Cl_2O_2$	321.2	846-49-1
	Zolpidem	$C_{19}H_{21}N_3O$	307.4	82626-48-0
Antidepressives	Fluoxetine	$C_{17}H_{18}NF_3O$	309.3	54910-89-3
	Paroxetine	$C_{19}H_{20}NFO_3$	374.8	61869-08-7
Diuretics	Furosemide	$C_{12}H_{11}N_2ClO_5S$	330.7	54-31-9
	Hydrochlorothiazide	$C_7H_8N_3ClO_4S_2$	297.7	58-93-5
	Indapamide	$C_{16}H_{16}ClN_3O_3S$	365.8	26807-65-8
Cardiotonics	Bisoprolol	$C_{18}H_{31}NO_4$	325.4	66722-44-9
Antiulcer agents	Omeprazole	$C_{17}H_{19}N_3O_3S$	345.4	73590-58-6

and stored at −18°C in the dark. A mixture of all pharmaceutical standards at 5 mg L^{-1} was prepared every 4 months. A working standard solution containing 500 μg L^{-1} of all pharmaceuticals prepared in methanol/water (25:75, *v*/*v*) was run daily before each sample batch. Internal standards were added to the sample extracts in the final concentration of 500 μg L^{-1}.

Baker-analysed methanol for LC–MS was supplied by J.T. Baker (Deventer, the Netherlands), and ultrapure Milli-Q water was obtained from a Milli-Q apparatus (Millipore, Molsheim, France). Formic acid (50%), hydrochloric acid (37%), *n*-hexane, dichloromethane, chloroform, ethyl ether, ethyl acetate, acetonitrile, acetone, tetrahydrofuran, isopropanol and methanol (99.8%) were obtained from Fluka, Sigma and Riedel-de-Haën (Sigma-Aldrich, Spain).

Several solid-phase adsorbents were tested, including Oasis HLB (200 mg, 6 mL) and Oasis MAX (500 mg, 6 mL) cartridges from Waters Corporation (Ireland), LC-Silica (500 mg, 3 mL), LC-Florisil (1 g, 6 mL), LC-SAX (500 mg, 3 mL) and LC-NH_2 (500 mg, 3 mL) cartridges, all Supelclean (Supelco) from Sigma-Aldrich and GCB Resprep EPA Method 521 (1 g, 6 mL) cartridges from Restek (Bellefonte, USA).

Regarding surface water samples, Bakerbond spe 1°, 2°-amino (NH_2/NH) columns (250 mg, 3 mL) provided a light cleanup prior to the extraction with Bakerbond H_2O-philic (200 mg, 6 mL) cartridges, both from J.T. Baker. GF/C 1.2-μm glass microfibre and 0.45-μm mixed cellulose ester filters were purchased from Whatman (Dassel, Germany), and 0.22-μm polyester syringe filters CHROMAFIL Xtra were from Macherey-Nagel (Germany).

Sample collection and pre-treatment

Wastewater samples included influents and effluents from two WWTPs discharging to Douro river, one of the biggest Portuguese rivers, crossing the country in the northern region. Surface water samples were collected from strategic points along the river, considering its tributaries and effluents' discharge points.

All were grab samples, collected in amber glass bottles, previously washed with Neodisher LM3 (SE) detergent (Dr. Weigert, Hamburg, Germany) and then rinsed thoroughly with Milli-Q water.

Every sample was collected in duplicate (2.5 L for wastewaters and 1 L for surface waters) and brought to the laboratory in ice-packed containers.

Upon arrival, all samples were immediately vacuum-filtered through a 1.2-μm glass microfibre filter and then through a 0.45-μm mixed cellulose ester filter. Finally, they were stored at 4 °C until analysis.

The Douro river samples were collected at a 1-m depth, every 3 months, over the year of 2009, according to a planned sampling scheme. To further characterise the nature of the samples, pH, specific conductivity and dissolved oxygen parameters were measured in loco, during each sampling campaign. Samples were also stored at 4°C until analysis.

Sample preparation: cleanup and SPE

In the optimisation of the cleanup and extraction processes of wastewater samples (50 mL for influents and 100 mL for effluents), many adsorbents, elution solvents and techniques were engaged. The recovery efficiency was checked at three different concentration levels of the 23 pharmaceuticals by extracting various wastewater samples spiked at 100 ng L^{-1} and 1 and 10 μg L^{-1} each. By recovery, we mean the overall amount of analyte measured by the analytical equipment compared with the spiked amount, including the less than optimal extraction efficiency on the SPE material and matrix effects along the method.

The first endeavoured cleanup strategy was by simple physical filtration of a 500-ng L^{-1} spiked wastewater sample through a 0.22-μm cellulose acetate membrane filter (Fig. 1a). A selective wash of the extracts was also attempted. After sample enrichment onto the cartridges, they were rinsed with 3 mL of 10%, 15% and 20% solutions of methanol in water to remove impurities (Fig. 1b). Other preliminary experiments followed, submitting the extracts previously obtained using Bakerbond H_2O-philic cartridges to adsorption cleanup. Solid-phase extraction was performed with an automated device ASPEC XL system from Gilson (Middleton, USA). Extraction cartridges were preconditioned with 3 mL of methanol and 3 mL of Milli-Q water (pH 2), wastewater samples were then percolated through the cartridges, at a 10-mL min^{-1} flow rate, and they were finally vacuum-dried for 30 min on a vacuum manifold (Analytichem International). Elution was carried out with two aliquots of 3 mL of methanol, with a 1-min delay in between. Afterwards, eluates were evaporated to dryness under gentle nitrogen stream and recovered in 200 μL of methanol, proceeding to cleanup. Normal-phase adsorbents were then evaluated to accomplish cleanup (Fig. 1, c, 1st). LC-Silica cartridges were conditioned with 3 mL of methanol and 3 mL of the elution solvent to be used. The 200 μL concentrate was loaded and manually eluted with two aliquots of 3 mL of elution solvent, with a delay of 1 min in between. Many solvents were assayed, including diverse combinations of pure, mixed and/or acidified (pH 2) *n*-hexane, dichloromethane, chloroform, ethyl ether, ethyl acetate, acetonitrile, acetone, tetrahydrofuran, isopropanol and methanol (MeOH). LC-Florisil and GCB cartridges were also tested, according to the same procedure. Finally, eluates were evaporated to dryness under gentle nitrogen stream and recovered in 200 μL of methanol/Milli-Q water (35:65, *v*/*v*) for LC–MS/MS analysis.

Another cleanup strategy included the use of ion exchange adsorbents, this time prior to the extraction step. Both SAX and WAX (NH_2) were assayed (Fig. 1, c, 2nd). These cartridges were conditioned with 2 mL of methanol and 2 mL of Milli-Q water, followed by the vacuum-forced loading of the wastewater sample (previously filtered with 0.45 μm and adjusted to pH 2 with HCl 37%). Collected samples were subsequently enriched on Bakerbond H_2O-philic cartridges, according to the general SPE procedure described above. At the end, eluates were evaporated to dryness under gentle nitrogen stream and recovered in 200 μL of methanol/Milli-Q water (35:65, *v*/*v*) for LC–MS/MS analysis.

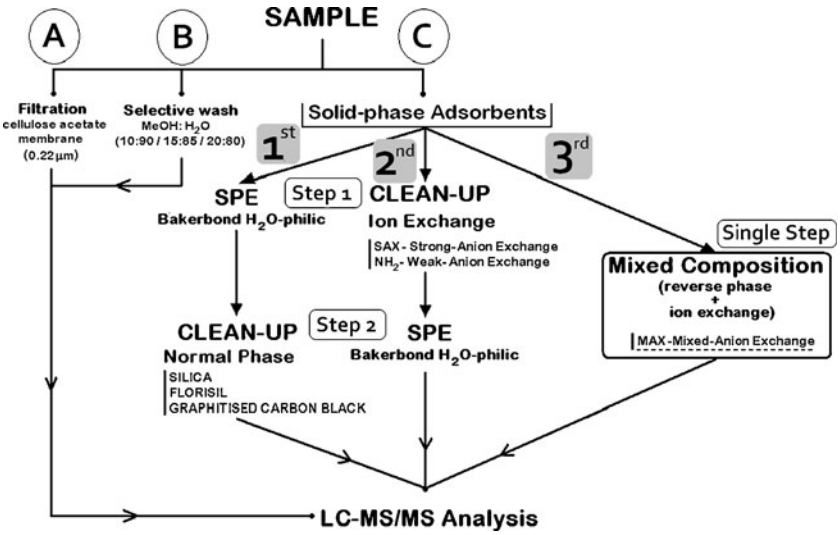

Fig. 1 Schematic diagram of the different cleanup strategies engaged

In the last cleanup approach, mixed-anion exchange Oasis MAX cartridges were used. Simply by following the aforementioned SPE procedure, 0.45-μm filtered and acidified wastewater samples underwent, simultaneously, cleanup and extraction (Fig. 1, c, 3rd). The eluates for LC–MS/MS analysis were treated in the same way as previously described.

The final optimised method included Oasis MAX cartridges used for the cleanup and extraction of 0.45-μm filtered and pH 2 acidified wastewater samples.

With regard to river waters, the extraction protocol included a previous cleanup step of the pH 2 acidified samples (200 mL) with Di-NH_2 cartridges, just on the dirtiest samples, followed by enrichment on Bakerbond H_2O-philic cartridges. Conditioning, elution and reconstitution of the extract before LC–MS/MS analysis followed the procedure already presented.

LC–ESI–MS/MS analysis

Chromatographic analysis and mass detection were performed in an LC–MS/MS system from Varian (Walnut Creek, CA, USA). The high-performance liquid chromatography (HPLC) system consisted of two Varian 210 HPLC pumps coupled to a ProStar 410 autosampler. A Pursuit UPS C18 analytical column (2.1 mm i.d.×50 mm, 2.4 μm) from Varian was used for the chromatographic resolution of the pharmaceutical substances. The equipment was fitted with a 10-μL sample loop. LC mobile phases were 10 mM formic acid in ultrapure Milli-Q water and Baker-analysed methanol.

The gradient programme started with 25% methanol, rising to 75% methanol in 8 min, then to 100% methanol at 10 min and holding until 13 min. At the end of the chromatographic run, the column re-equilibrates to the initial conditions in 1 min and stabilises for 8 min. The flow rate was 300 μL min^{-1}; the column temperature was kept at 35°C and the autosampler at 10°C. The detector was a Varian 500 MS ion trap mass spectrometer. The electrospray ionisation parameters were properly adjusted to obtain the maximum amount of precursor ion entering the ion trap: ionisation mode, drying gas temperature, needle voltage and capillary voltage. Regarding the MS detector, the RF loading voltage and the collision-induced dissociation (CID) voltage were adjusted to obtain the optimum capacity and fragmentation. MS/MS parameters for all 23 analysed pharmaceutical compounds, as well as MS^3 CID voltages and the resulting product ions of some pharmaceuticals (for confirmation purposes), are presented in Table 2. The software used for data processing was the Varian MS Workstation version 6.9.1. A typical MS/MS chromatogram obtained under the optimised conditions is given in Fig. 2.

Validation of the analytical procedure

The analytical figures of merit of the developed method were assessed in order to estimate its efficacy and feasibility for the application to the analysis of real environmental water samples. While the method built to analyse river waters had already been optimised and validated [16], the method developed for the analysis of wastewater samples required full validation.

Recovery experiments were performed on wastewaters, using both influent and effluent samples spiked at the concentrations of 100 ng L^{-1} and 1 and 10 μg L^{-1}. In each case, one blank (non-spiked sample) was subtracted to the respective spiked samples (n=5). The precision of the method was determined by intra-day (repeatability) and inter-day (intermediate precision) analysis of 1-μg L^{-1} spiked samples, in 1 day (n=4) and in 3 non-consecutive days (n=12), respectively. Method detection limits (MDLs) and method quantification limits (MQLs) were determined under the optimised conditions, as the minimum detectable amount of analyte providing a signal-to-noise ratio of 3 and 10, respectively. These values were further corrected according to the matrix effects over each compound, in each sample type (influent and effluent). Instrumental detection limits (IDLs) and instrumental quantification limits (IQLs) were determined by direct injection of decreasing concentrations of the 23 pharmaceutical compounds standard mixture, until reaching the concentration level corresponding to a signal-to-noise ratios of 3 and 10, respectively. The instrumental repeatability was calculated by repeated injections of 50, 100 and 500 μg L^{-1} standard mixtures. The breakthrough volume for each compound was determined by extracting 50, 100, 150 and 200 mL of 1-μg L^{-1} spiked Milli-Q water samples.

The linearity of the method was estimated by fitting a linear least-squares regression. A five-point calibration curve was built based on the following concentrations (in Milli-Q water): 0.01, 0.1, 0.5, 1.0 and 2.0 μg L^{-1}. Another six-point calibration curve, ranging from 0.05 to 100 μg L^{-1}, was also constructed to allow the quantification of some pharmaceuticals present in higher environmental concentrations. The internal standards (paracetamol-D4 and fluoxetine-D5) added to the sample extracts in the concentration of 500 μg L^{-1} were further used for minimising the influence of matrix components and allowing quantification corrections. Matrix effects were also evaluated for each compound.

The quantification of each pharmaceutical was based on the main characteristic MS^2 precursor/product ion transition as given in Table 2. The identification took into consideration their retention time, in comparison with the corresponding reference standard, the qualifiers ratios and, finally, the fitting probabilities against the "laboratory-made"

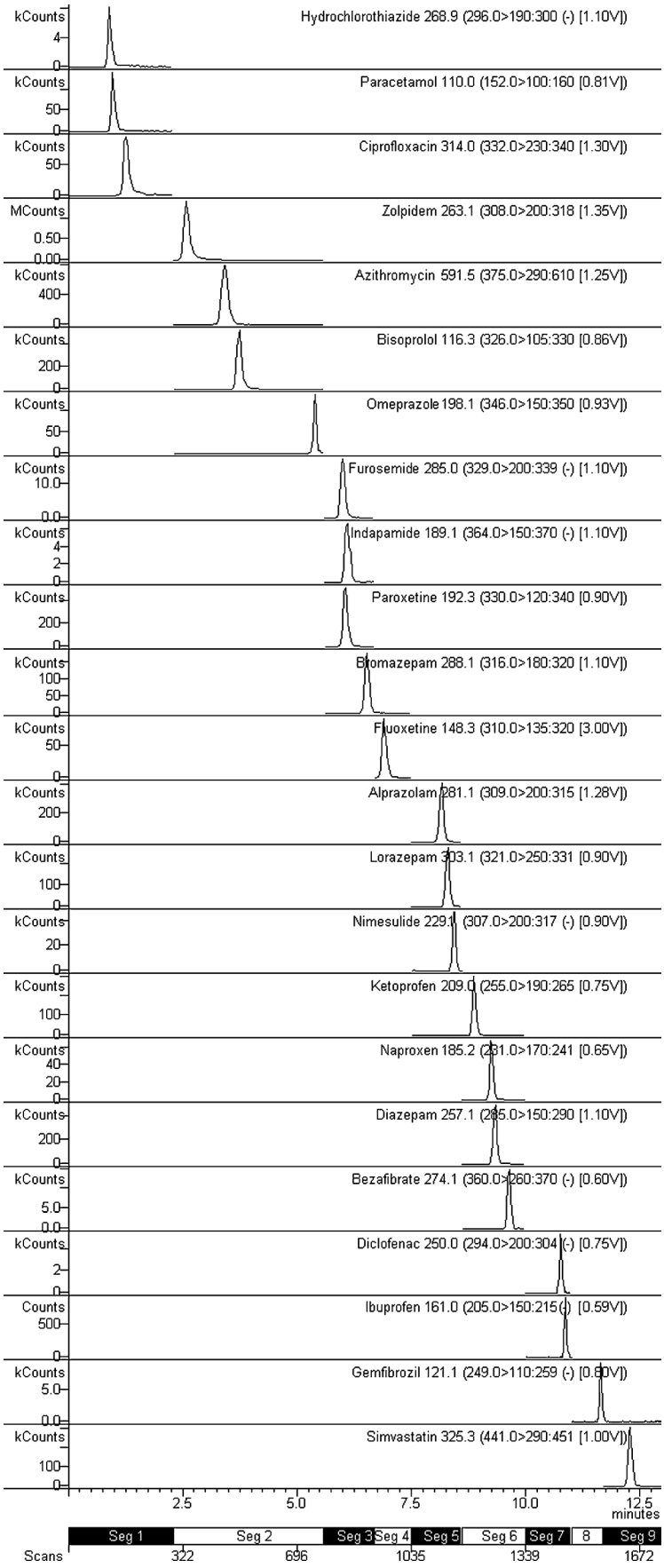

Fig. 2 Representative chromatograms of all 23 pharmaceuticals after injection of a 500-μg L^{-1} standard solution

Table 2 MS/MS parameters for the analysis of target pharmaceuticals

Pharmaceutical	R_t (min)	Precursor ion (*m/z*)	MS^2 product ion (*m/z*)	Ionisation mode	R_t window (min)	Capillary voltage (V)	RF loading (%)	CID voltage I (V)	MS^3 product ion(s) (*m/z*)	CID voltage II (V)
Hydrochlorothiazide	0.87	296	269	ESI-	0.00–2.35	84	128	1.50	*205*, 206	0.73
Paracetamol	0.95	152	110	ESI+	0.00–2.35	54	62	1.00	65, 82, 92, *93*	0.86
Paracetamol-D4	0.97	156	114	ESI+	0.00–2.35	60	54	0.65	–	–
Ciprofloxacin	1.32	332	314	ESI+	0.00–2.35	72	87	0.65	–	–
Zolpidem	2.64	308	263	ESI+	2.35–5.50	102	87	1.35	–	–
Azithromycin	3.72	375	591	ESI+	2.35–5.50	42	90	1.21	398, 416, *434*, 573	1.52
Bisoprolol	3.78	326	116	ESI+	2.35–5.50	80	87	0.79	56, 72, *74*, 98	0.75
Omeprazole	5.26	346	198	ESI+	2.35–5.50	48	86	0.93	–	–
Furosemide	5.87	329	285	ESI-	5.50–6.50	53	117	0.95	*205*	1.22
Indapamide	5.97	364	189	ESI-	5.50–6.50	53	161	1.10	–	–
Paroxetine	6.12	330	192	ESI+	5.50–6.50	78	85	1.06	*123*, 163	0.60
Bromazepam	6.36	316	288	ESI+	5.50–7.30	87	87	1.10	–	–
Fluoxetine-D5	6.52	315	153	ESI+	6.50–7.30	54	85	0.65	–	–
Fluoxetine	6.94	310	148	ESI+	6.50–7.30	49	86	3.00	–	–
Alprazolam	7.89	309	281	ESI+	7.30–8.60	92	87	1.28	–	–
Lorazepam	8.02	321	303	ESI+	7.30–8.60	65	88	0.85	*275*, 277	1.04
Nimesulide	8.26	307	229	ESI-	7.30–8.60	53	103	0.90	–	–
Ketoprofen	8.59	255	209	ESI+	7.30–10.00	75	76	0.75	*105*, 131, 194	0.53
Naproxen	8.93	231	185	ESI+	8.60–10.00	42	77	0.63	153, 154, 155, *170*	0.95
Diazepam	8.97	285	257	ESI+	8.60–10.00	87	81	1.10	–	–
Bezafibrate	9.31	360	274	ESI-	8.60–10.00	63	100	1.44	*154*	0.67
Diclofenac	10.54	294	250	ESI-	10.00–11.00	36	111	0.79	*214*	1.32
Ibuprofen	10.71	205	161	ESI-	10.00–11.00	33	57	0.69	–	–
Gemfibrozil	11.49	249	121	ESI-	11.00–11.70	48	67	0.80	*106*	2.20
Simvastatin	12.19	441	325	ESI+	11.70–13.00	126	66	1.15	295, *310*, 311	1.20

MS^3 base peak ions are in italics

MS^3-spectrum library (positive identification whenever probability percentage >70%).

Results and discussion

Cleanup/SPE

The optimisation of the cleanup/extraction procedures was undertaken with the aim of achieving good recovery rates for all 23 compounds in complex samples as wastewaters and preserve the chromatographic and detection systems.

Initially, a set of different solid-phase extraction materials was evaluated, including modified polystyrene-divinylbenzene and novel polymeric materials, specially prepared for the analysis of pharmaceutical substances. Among them, polymeric sorbents Oasis HLB and Bakerbond H_2O-philic were given particular attention. High extraction efficiencies are usually obtained with Oasis HLB SPE cartridges, due to their hydrophilic and lipophilic balanced characteristics, providing excellent wetting properties of the hydrophilic *N*-vinylpyrrolidone monomer. Besides, they offer the best conditions for simultaneous extraction of acidic analytes from water with no need of sample acidification, as well as neutral analytes of a wide polarity range [17, 18]. Notwithstanding, the Bakerbond H_2O-philic sorbent was selected since it presented specific advantages. After extraction experiments conducted with a 500 ng L^{-1} standard solution in three replicates, it provided a quite good and similar extraction pattern to Oasis HLB, besides allowing the noteworthy boost of simvastatin (Fig. 3). Preliminary experiments also demonstrated that sample acidification with HCl at pH 2 is mandatory to ensure proper retention in the adsorbent of some compounds such as ciprofloxacin, azithromycin, paroxetine, fluoxetine and naproxen.

However, when using spiked wastewater samples instead of Milli-Q water standard solutions, recoveries suffered a significant drawback, therefore demanding a previous cleanup step, also requested due to the high amount of co-extracted matter in the final extract.

The first fractionation attempt consisted of the simple physical filtration of a spiked effluent through a 0.22-μm polyester filter, prior to extraction with Bakerbond H_2O-philic cartridges. Nevertheless, results showed that this process was not capable of preventing ionisation suppression effects, resulting in low recoveries and even loss of

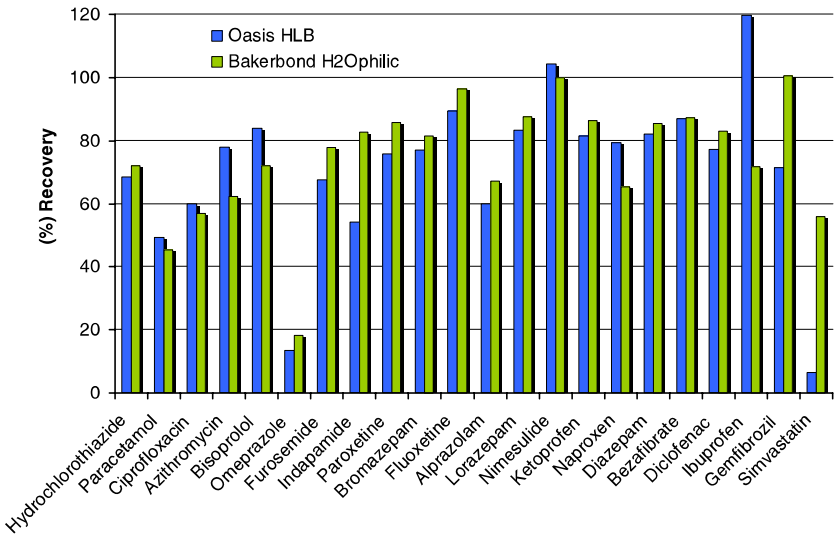

Fig. 3 Recovery rates obtained on the extraction of 500-ng L^{-1} pharmaceuticals standard solutions in Milli-Q water (pH 2) with Oasis HLB and Bakerbond H_2O-philic cartridges (n=3, each)

some compounds, such as indapamide, paroxetine, fluoxetine and ibuprofen.

The next step in setting up a proper cleanup procedure was based on a selective wash approach. Figure 4 displays the recoveries obtained after extracting an influent sample from WWTP 2 using Bakerbond H_2O-philic cartridges, followed by wash with 3-mL volumes with increasing percentage of methanol in water. It became clear that this strategy did not improve the extract visual cleanliness, leading as well to the loss of some compounds.

Normal-phase adsorption of polar interferences was then considered for sample cleanup, using Silica, Florisil and graphitised carbon black sorbents. Different combinations of elution solvents were applied, pure, mixed and/or acidified, in different volumes (usually 3 mL+1 min+ 3 mL), combining and taking profit of their eluotropic strength and wide range of polarities. Figure 5 presents the results obtained in most of the experiments.

Apart from those mentioned in Fig. 5, many other elution solvents/mixtures were tested, including n-hexane, isopropanol and tetrahydrofuran. However, most of them were not even satisfactory, once the aspect of the corresponding cleanup products compromised further analysis. The cleanups using LC-Si cartridges were characterised by dirty extracts and by low efficiency, later on confirmed by low recoveries and absence of several compounds (Fig. 5). LC-Florisil and GCB cartridges gave apparently clean extracts. However, after several trials, none of the solvents was able to elute a considerable amount of the compounds (Fig. 5). Acidified MeOH even led to the formation of a white precipitate, by reacting with the adsorbents. A lower variability between analytes was observed for Florisil and

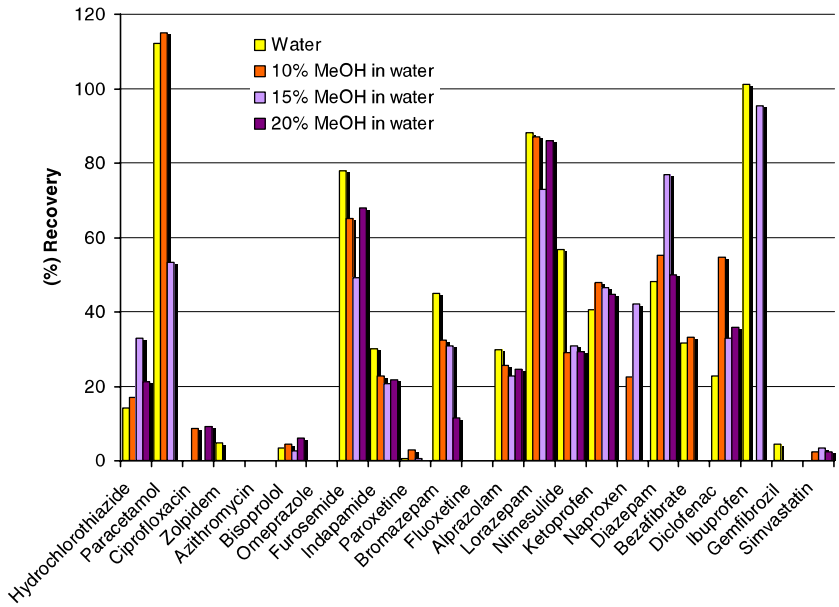

Fig. 4 Recoveries obtained on the extraction of a 500 ng L^{-1} spiked sample (influent) and cartridge wash with 3 mL of the following solutions: Milli-Q H_2O, MeOH/H_2O (10:90), MeOH/H_2O (15:85) and MeOH/H_2O (20:80) (n=3, each)

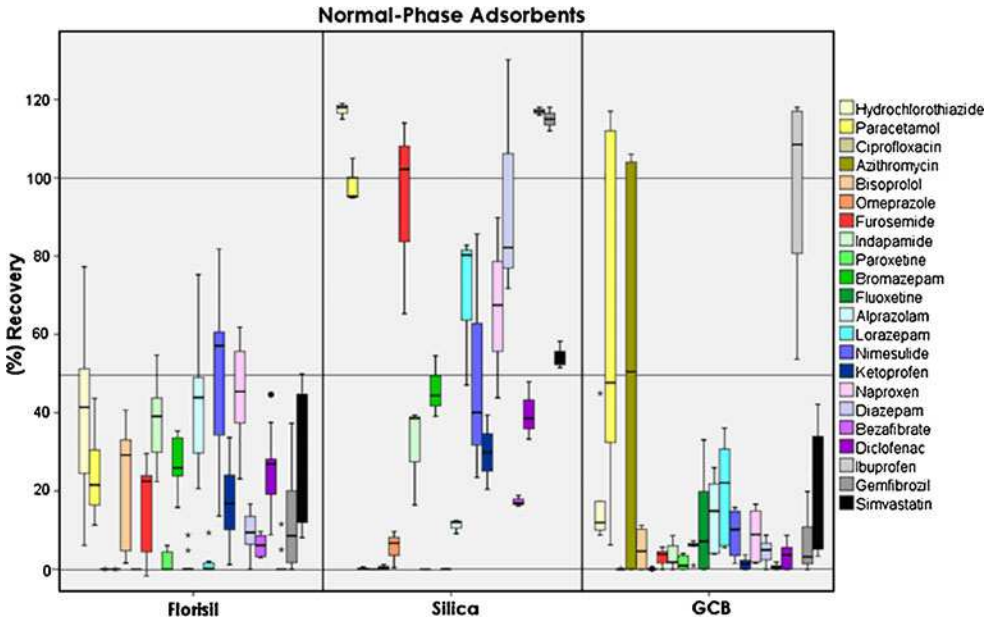

Fig. 5 "Boxes and whiskers" representations of the recovery ranges obtained under different cleanup conditions, after extraction of 1-μg L^{-1} spiked samples with Oasis HLB cartridges. The three normal-phase adsorbents—Florisil, silica and GCB—were used in combination with different elution solvents (n=3, each). Florisil: dichloromethane/MeOH (50:50), ether/MeOH (50:50), ethyl acetate/MeOH (80:20, 60:40, 50:50 and 40:60) and chloroform/MeOH (60:40, 50:50 and 40:60); Silica: acetone, acetonitrile and ethyl acetate; GCB: ethyl acetate/MeOH (75:25), acetonitrile/MeOH (75:25) and acetone/MeOH (75:25), and all three elution mixtures 1% acidified with formic acid *dot*=outlier; *asterisk*=extreme

GCB adsorbents because they strongly and irreversibly retain the analytes (and interferences), resulting in overall low recoveries, conversely to the LC-Si adsorbent.

To exploit ion exchange properties, SAX and WAX (NH_2–) cartridges were then assessed. This included encountering their optimal adsorbent mass and acidification timing, before the cleanup step or prior to the following enrichment on J.T. Baker H_2O-philic cartridges. The performance of cartridges containing 1 g (6 mL) and 500 mg (3 mL) for both SAX and NH_2– adsorbents and also a double-layer 500 mg SAX+500 mg NH_2, prepared in the laboratory, was assessed. Results proved that, to achieve maximal efficiency 500 mg, 3-mL cartridges were sufficient. The acidification of the original sample (to pH~2) was demonstrated to be more effective than the acidification post-cleanup, specifically to compounds such as paroxetine and fluoxetine. By displacing the equilibrium of these two amines to the positively ionised form, it

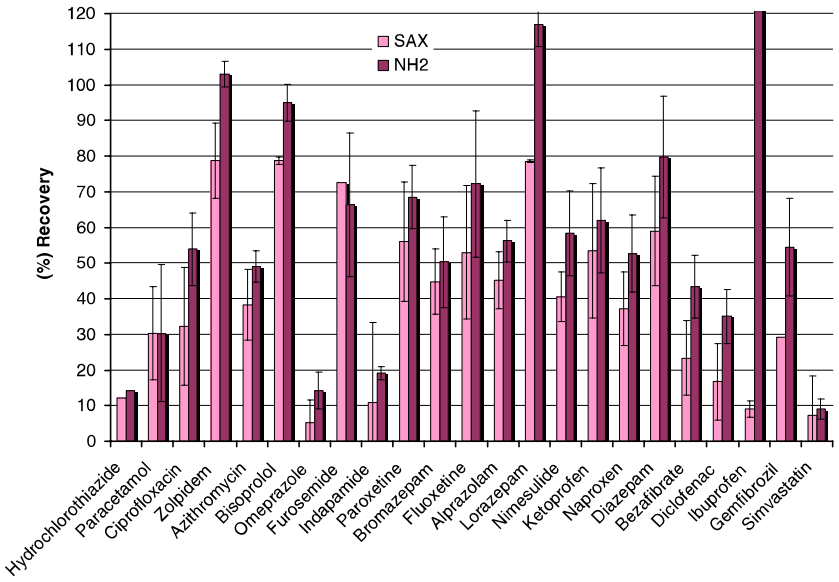

Fig. 6 Recoveries obtained after processing a 1-μg L^{-1} spiked sample (effluent) using either SAX or NH_2 cartridges for the cleanup step, followed by extraction on Bakerbond H_2O-philic cartridges (n=3 each, except in the cases where RSD is not represented, n=2)

probably reduces non-specific hydrophobic retention onto the cleanup adsorbent.

When comparing SAX with NH_2 cartridges followed by enrichment on Bakerbond H_2O-philic cartridges, they both allowed the recovery of all compounds, but the previous acidification of the sample to pH ~2 with HCl is mandatory. Nevertheless, the latter were consistently better in terms of recoveries (Fig. 6). For zolpidem, bisoprolol, lorazepam, nimesulide, diclofenac and ibuprofen, the differences were statistically significant with an advantage for the NH_2 adsorbent.

MAX adsorbent was finally assayed, due to its capacity of simultaneously assuring both cleanup and extraction. Contrary to what could be thought about the (lower) efficiency of a simultaneous cleanup and extraction procedure, the results obtained were quite promising. Oasis MAX is a mixed-mode sorbent consisting of a strong anion exchanger (quaternary amine) and a non-polar *N*-vinylpyrrolidone/divinylbenzene polymer [19]. It has been optimised to achieve higher selectivity and sensitivity for extracting acidic compounds with anion exchange groups, while basic and neutral compounds are retained by the polymeric sorbent. Therefore, the goal was to retain strong acidic impurities in the anion exchange fraction, while non-ionised acidic compounds (due to the pH 2) and basic and neutral compounds could bind to the non-polar adsorbent and be all subsequently selectively eluted with a non-acidified solvent.

Oasis MAX adsorbent provided higher recovery rates for the majority of the compounds (Fig. 7), using MeOH as elution solvent (tetrahydrofuran and isopropanol were also tested, though the results were no better).

The recoveries with MAX cartridges for paracetamol (41%), ciprofloxacin (~100%), indapamide (77%), paroxetine (~100%), bromazepam (68%), fluoxetine (~100%), alprazolam (72%), nimesulide (60%), bezafibrate (92%) and gemfibrozil (98%) were significantly higher than with NH_2 cartridges. According to these results, Oasis MAX cartridges were selected for the preparation of real samples and the enrichment of target pharmaceutical analytes.

Comparing the results herein obtained with those previously reported by other authors using different cartridges [8, 10], we were able to establish a more straightforward method with the use of one single clean-up/extraction step for all 23 pharmaceutical compounds at once. This procedure was demonstrated to be very efficient, using only one cartridge type, without significant losses of any compound. Furthermore, though there are already reports of some of these pharmaceuticals to have been extracted from sewage waters, they either include "families" of compounds with similar physicochemical behaviour [19] or opt for fractionated extraction [20]. In the few published works where MAX cartridges were chosen, the rationale of the extraction procedure was to retain acidic pharmaceuticals in the ionic phase by pH alkalinisation [19] and not the underlying mechanism in this work.

LC–ESI–MS/MS analysis

The optimisation of the chromatographic separation procedure, using a Pursuit UPS C18 analytical column (2.1 mm i.d.×50 mm, 2.4 μm), aimed at allowing the analysis of the whole set of 23 analytes in one single run, without significant loss of sensitivity for any compound, which is likely to occur when analytes measured in positive and negative modes co-elute [18]. A series of preliminary experiments was therefore attempted, using different mobile

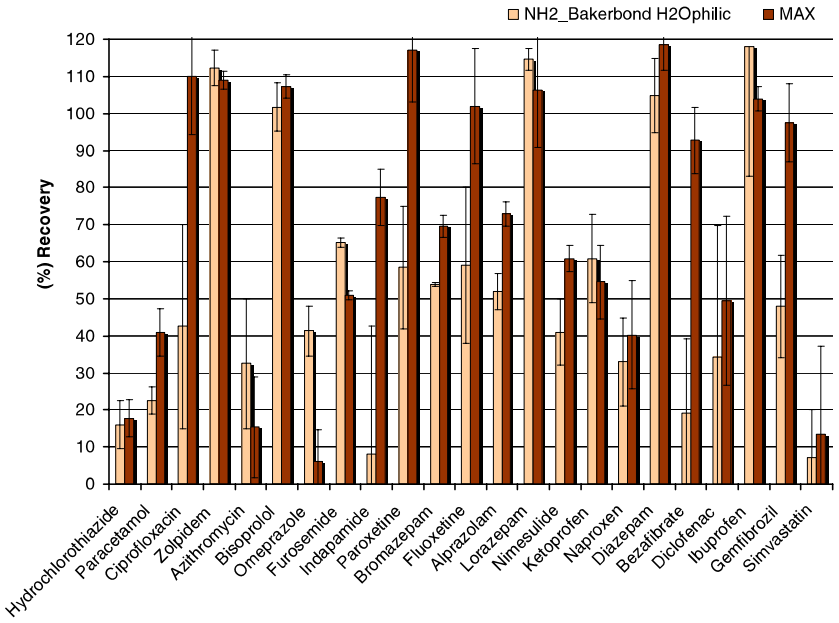

Fig. 7 Comparison of the recoveries obtained on the extraction of a 1-μg L^{-1} spiked sample (influent) using NH_2/Bakerbond H_2O-philic cartridges and MAX cartridges (n=4)

phases, with and without the addition of mobile-phase additives. In the end, the best chromatographic performance was achieved using methanol as the organic phase and 10 mM formic acid in water (pH ~ 2.9) as the aqueous phase, enhancing a fast and efficient separation with a total run time of 13 min and a 23-min cycle between analyses. The peak width at half height ($W_{0.5}$) varied between 4.5 and 11.2 s. The obtained chromatogram is illustrated in Fig. 2. Compounds which ionise in the negative mode suffered minor losses. Indeed, formic acid is found to be a more effective additive for compounds determined in the positive mode [18]. Accordingly, its addition had a paramount importance in the improvement of the ESI+ ionisation efficiency of paracetamol, ketoprofen and naproxen, apart from a major upgrading of peak shape and resolution of ciprofloxacin, azithromycin, bisoprolol, paroxetine and fluoxetine.

In the end, though complete resolution of some analytes could not be achieved, this did not pose a serious threat to the MS/MS analysis, given the good IDL values (Table 4) and the fact that the number of analytes per segment was kept below 5. The mass spectrometry parameters, including operational conditions for fragmentation, have been optimised by flow injection analysis for each compound. They were reported in Table 2, including precursor ions and their product ions, both for quantification and confirmation purposes. Product ions obtained for target analytes were in good agreement with those previously reported [10]. Finally, for quantitative analysis, three criteria were set for positive identification: the correlation of the retention time with the corresponding reference standard, the qualifiers ratios in MS^2 and a fitting probability against the created MS^3-spectrum library >70%.

Performance of the analytical method

The fitness for purpose of the multi-residue analytical method, comprising an extraction step with MAX cartridges and LC–MS/MS analysis, was evaluated for the determination of pharmaceuticals in wastewaters. Several validation criteria

Table 3 Recovery rates obtained at the three spiking levels, tested for each sample type (pH 2), using Oasis MAX cartridges

Pharmaceutical	Recoveries at a lower spiking level		Recoveries at a medium spiking level		Recoveries at a higher spiking level	
	(% RSD) (n=5) WWTP influent	(100 ng L^{-1}) WWTP effluent	(% RSD) (n=5) WWTP influent	(1 μg L^{-1}) WWTP effluent	(% RSD) (n=5) WWTP influent	(10 μg L^{-1}) WWTP effluent
Hydrochlorothiazide	x	37[a] (21)	15 (12)	17 (5)	10 (18)	9 (10)
Paracetamol	x	40 (24)	35 (9)	41 (7)	45 (6)	32 (5)
Ciprofloxacin	76 (13)	136 (13)	68 (15)	109 (17)	120 (7)	176 (3)
Zolpidem	75 (5)	94 (5)	88 (8)	107 (2)	82 (4)	93 (5)
Azithromycin	x	x	12 (9)	15 (14)	12 (44)	15 (14)
Bisoprolol	x	x	92 (3)	135 (4)	95 (4)	104 (4)
Omeprazole	–	–	6 (15)	12 (10)	–	–
Furosemide	48 (3)	x	53 (2)	51 (1)	46 (7)	43 (8)
Indapamide	21 (18)	16 (9)	68 (4)	77 (9)	25 (16)	30 (18)
Paroxetine	48 (11)	74 (18)	84 (16)	129 (14)	92 (5)	110 (5)
Bromazepam	37 (10)	51 (12)	63 (5)	68 (3)	50 (5)	57 (4)
Fluoxetine	62 (11)	90 (11)	81 (13)	115 (17)	85 (6)	99 (1)
Alprazolam	40 (5)	47 (7)	60 (4)	72 (4)	53 (6)	61 (5)
Lorazepam	45 (5)	x	87 (13)	127 (16)	71 (3)	81 (2)
Nimesulide	34 (5)	36 (10)	52 (5)	60 (3)	42 (4)	47 (7)
Ketoprofen	x	x	46 (10)	55 (11)	49 (3)	61 (4)
Naproxen	x	x	42 (6)	40 (9)	26 (8)	23 (3)
Diazepam	58 (13)	62 (5)	92 (7)	145 (15)	67 (2)	73 (3)
Bezafibrate	x	x	89 (17)	92 (10)	55 (7)	54 (10)
Diclofenac	x	x	46 (21)	49 (23)	64 (8)	69 (6)
Ibuprofen	x	x	95 (7)	124 (3)	105 (5)	111 (10)
Gemfibrozil	x	x	89 (11)	98 (10)	93 (5)	102 (8)
Simvastatin	18 (9)	x	15 (20)	12 (24)	22 (16)	17 (9)

x inappropriate spiking level (concentrations present in the sample are higher than the spiking concentration, originating significant errors)

[a] Mean recovery

Table 4 Validation data of the analytical method

Pharmaceutical	Linearity (r^2) 0.01–2 μg L^{-1}	MDL (ng L^{-1})		MQL (ng L^{-1})		Repeatability (%) RSD (n=4) 1 μg L^{-1}		Intermediate precision (%) RSD (n=12) 1 μg L^{-1}		IDL (μg L^{-1})	IQL (μg L^{-1})	Instrumental[a] repeatability (%) RSD (n=4)
		WWTP influent	WWTP effluent	WWTP influent	WWTP effluent	WWTP influent	WWTP effluent	WWTP influent	WWTP effluent			
Hydrochlorothiazide	0.9906	17	15	66	58	11	7	13	12	5	14	9
Paracetamol	0.9982	7	6	25	22	8	8	10	11	4	11	7
Ciprofloxacin	0.9919	2	1	6	4	17	16	12	18	5	16	8
Zolpidem	0.9976	1	1	3	2	4	4	9	5	0.4	1	2
Azithromycin	0.9902	2	2	6	5	8	10	14	14	0.3	1	3
Bisoprolol	0.9992	1	1	3	2	8	3	9	6	0.5	1	1
Omeprazole	–	–	–	–	–	–	–	–	–	1	3	7
Furosemide	0.9916	11	11	34	35	5	17	7	19	4	12	8
Indapamide	0.9823	20	17	65	57	3	8	6	17	3	9	9
Paroxetine	0.9955	1	1	3	3	10	11	13	12	1	3	6
Bromazepam	0.9938	2	2	7	6	3	5	8	13	1	3	5
Fluoxetine	0.9921	1	1	5	4	9	12	10	16	7	23	6
Alprazolam	0.9961	1	1	5	4	2	3	4	7	1	3	8
Lorazepam	0.9981	2	2	7	6	8	15	9	9	2	6	6
Nimesulide	0.9832	2	2	4	3	8	2	9	9	2	6	8
Ketoprofen	0.9988	3	3	9	7	7	8	10	9	1	3	9
Naproxen	0.9973	14	14	47	49	5	7	8	9	9	27	8
Diazepam	0.9974	1	1	3	3	7	8	7	6	2	6	6
Bezafibrate	0.9948	5	5	18	17	13	12	14	15	2	6	1
Diclofenac	0.9927	18	17	84	79	15	18	13	22	3	8	8
Ibuprofen	0.9836	65	62	219	208	12	9	7	12	50	150	9
Gemfibrozil	0.9958	11	10	40	36	8	9	9	14	6	17	5
Simvastatin	0.9943	8	10	29	35	16	16	15	26	1	3	4

[a] Instrumental repeatability values correspond to the average RSD of quadruplicates at the concentrations of 50, 100 and 500 μg L^{-1} standard mixtures of the 23 target compounds

were therefore taken into account, including linearity, sensitivity, precision, recoveries and matrix effects. The performance, expressed in terms of recovery percentages in Table 3, and the remaining validation data, summarised in Table 4, attest to its high efficiency and reliability.

The calibration curves built as described in the "Experimental" section were fit with a linear least-squares regression. Regression coefficients were above 0.99, except for indapamide, nimesulide and ibuprofen. On the whole, they presented acceptable values, if we consider the overall procedure, SPE included.

Regarding MDL values, they ranged from 1 to 20 ng L^{-1} for both influents and effluents, apart from ibuprofen (65 ng L^{-1} for influents and 62 ng L^{-1} for effluents). MQLs remained below 85 ng L^{-1} for both matrixes, again with the exception of ibuprofen (219 and 208 ng L^{-1} for influents and effluents, respectively). These ML values are considered very good for wastewaters, where high concentrations (in the range of 0.1–10 μg L^{-1}) are expected for many of these pollutants [18].

The intra- and inter-day precisions of the global method were also assessed to assure precise quantifications. Relative standard deviation (RSD) values fell between 2% and 18% for repeatability and between 4% and 26% for intermediate precision, for all compounds.

In general, the difference between influent and effluent recovery rates was statistically non-significant. On the whole, they ranged from approximately 50% to 130% for the majority of the compounds. Despite the fact that hydrochlorothiazide, paracetamol, azithromycin, naproxen and simvastatin presented lower recoveries, this was not considered an obstacle to their reliable determination, once they are still considered acceptable for a multi-residue method [21] and the precision was below 15% for the majority of the analytes. The recovery rate of omeprazole is also justifiable due to its limited stability, namely under acidic conditions [8].

The obtained recovery rates might have probably been affected by the presence of co-extracted matrix components, which typically suppress the analyte signal [17]. For this reason, tests were developed to determine the percentage loss during the cleanup and extraction step(s) (samples spiked before and after SPE) and due to the ionisation suppression (extracts spiked prior to HPLC analysis compared to compounds in reconstitution solvent). Results demonstrated that recovery reductions due to matrix ionisation suppression can go up to 80–90% for hydrochlorothiazide and omeprazole, ~70% for paracetamol, 50–60% for indapamide, bromazepam, alprazolam and naproxen, ~40% for furosemide, ketoprofen, bezafibrate, diclofenac and simvastatin, between 10% and 20% for ciprofloxacin, bisoprolol, nimesulide, diazepam and gemfibrozil and finally, <10% for azithromycin, paroxetine, fluoxetine, lorazepam and ibuprofen. Noteworthy are the examples of azithromycin, nimesulide and simvastatin, which despite the fact that suppression effects are not very severe (below 40%), their low recoveries were attributed to losses during the extraction procedure with MAX cartridges.

Finally, the determined breakthrough volumes were above 200 mL for all target compounds, therefore corroborating the chosen sample volumes—50 mL for influents and 100 mL in the case of effluents.

Application to the analysis of real water samples

This multi-residue analytical method was developed with the ultimate purpose of accurately measuring a representative list of 23 pharmaceuticals on wastewaters from WWTPs 1 and 2 (WWTP 1 upstream and WWTP 2 downstream, along Douro river downstream the dam of Lever).

The preliminary results obtained are summarised in Table 5, and an elucidative chromatogram of an influent sample is presented in Fig. 8.

Table 5 Concentrations of target pharmaceuticals detected in influent and effluent wastewaters from WWTPs 1 and 2

Pharmaceutical	Concentrations (ng L^{-1})			
	WWTP 1		WWTP 2	
	Influent	Effluent	Influent	Effluent
Hydrochlorothiazide	6,022	<58[a]	2,763	2,012
Paracetamol	20,074	<22	15,787	<22
Ciprofloxacin	<6	<4	<6	<4
Zolpidem	<3	<2	<3	<2
Azithromycin	571	530	617	836
Bisoprolol	239	187	214	397
Omeprazole	–	–	–	–
Furosemide	2,675	2,430	<34	1,902
Indapamide	<65	<57	<65	<57
Paroxetine	105	60	45	240
Bromazepam	<7	<6	<7	<6
Fluoxetine	<5	<4	<5	<4
Alprazolam	<5	<4	<5	<4
Lorazepam	<7	240	<7	438
Nimesulide	<4	<3	<4	<3
Ketoprofen	122	562	135	446
Naproxen	2,583	2,748	3,474	461
Diazepam	<3	<3	<3	<3
Bezafibrate	1,348	1,797	5,217	343
Diclofenac	431	1,107	1,597	1,429
Ibuprofen	1,992	1,527	3,588	1,088
Gemfibrozil	307	753	290	1,183
Simvastatin	976	197	<29	1,255

[a] Concentration is below MQL

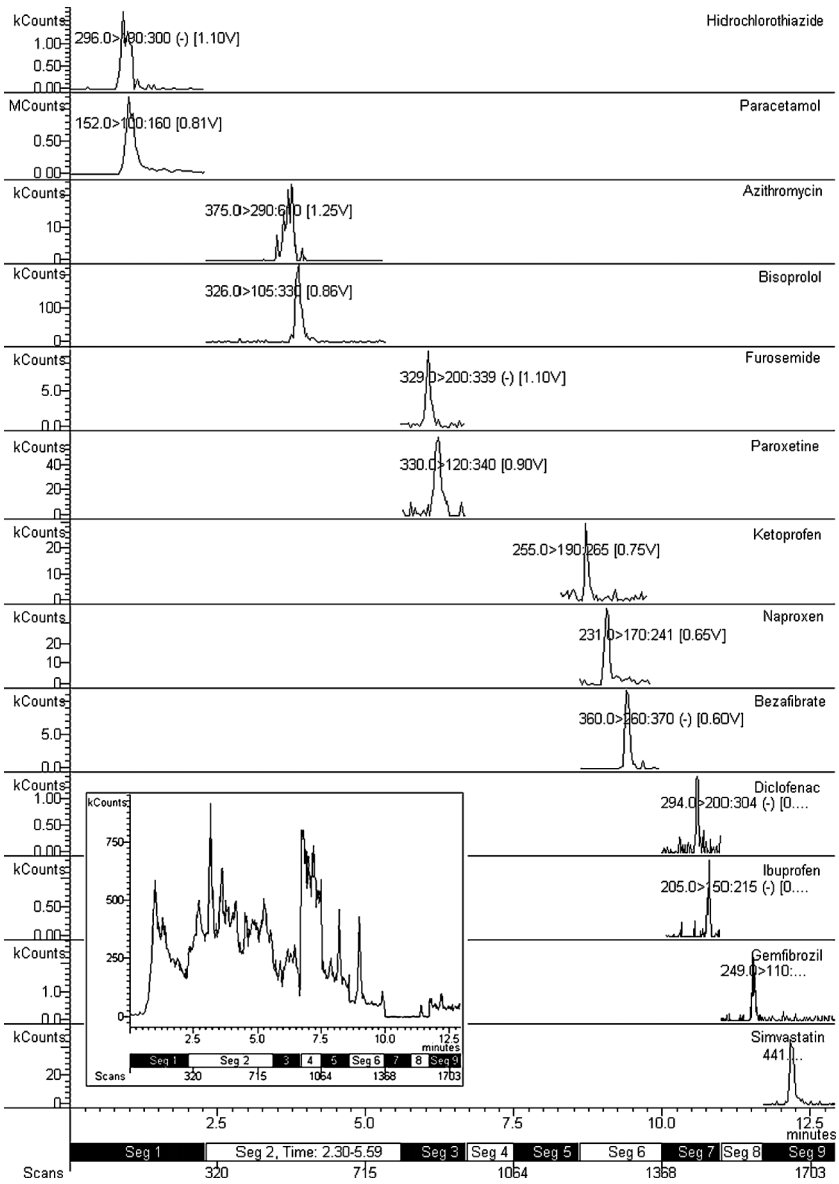

Fig. 8 Chromatogram of a typical influent sample from WWTP 1 contaminated with several pharmaceuticals. The *insert figure* corresponds to the total ion chromatogram

Since the calibration standards were prepared in Milli-Q water, the matrix effects needed to be taken into account when calculating pharmaceuticals' concentrations in real samples. For paracetamol and fluoxetine, matrix effects were corrected with the aid of the corresponding deuterated internal standards (paracetamol-D4 and fluoxetine-D5). The latter also served as marker for retention time stability. Due to the great physicochemical diversity of the 23 pharmaceuticals analysed, these two internal standards were inappropriate to resemble their global behaviour; thus, for the remaining pharmaceuticals, corrections were based on recovery experiments at 500 ng L^{-1} level for each sample batch. After subtracting the non-spiked sample, the total recovery efficiency for each compound in wastewater was used to rectify the concentrations obtained by simple interpolation on the calibration curve.

The main therapeutic groups detected in wastewater samples include anti-inflammatories, analgesics and antipyretics, diuretics and lipid regulators. Among them, paracetamol highlights in concentrations above 10 μg L^{-1}, followed by hydrochlorothiazide, furosemide, naproxen, ibuprofen, diclofenac and bezafibrate, with concentrations in the low microgramme per litre range. Gemfibrozil and simvastatin occurred, on average, on several nanogramme per litre levels (>500), whereas ketoprofen was quantified in lower concentrations. Apart from the mentioned pharmaceutical groups, azithromycin, bisoprolol, lorazepam and paroxetine were also detected, though still in very significant concentrations. Finally, samples were again processed, and the quantified compounds were MS^3-fragmented (see Table 2). Each obtained spectrum was searched against the MS^3-spectrum library previously created, and the fitting probabilities

were >70% for every compound, with the exception of paroxetine and ibuprofen. In the case of paroxetine, its low concentrations probably hampered its MS^3 confirmation, whereas ibuprofen, as predicted due to its low fragmentation output, did not provide a clear MS^3 spectrum.

One very important aspect relates to the fact that, in some cases, the concentrations detected in influent samples were below those obtained in effluents from the same WWTP. Spot sampling is the main reason for this discrepancy, while for certain compounds conversion of conjugated metabolites to the original substances, over the treatment processes, may also increase their concentration [5].

To evaluate the possible impact of the discharges from these two WWTPs in receiving Douro river waters, a sampling plan was carefully designed, including sampling spots of interest upstream, in between and downstream of both WWTPs. Both wastewater and surface water samples were collected at the beginning of the summer of 2009.

In these surface water samples, just a few compounds were detected at trace levels. In one site upstream of WWTP 1, hydrochlorothiazide was present in the concentration of 31 ng L^{-1}, whereas in another site downstream of both WWTPs, paracetamol and ketoprofen were found in the concentrations of 63 and 11 ng L^{-1}, respectively. Others, such as bisoprolol, diazepam and gemfibrozil, were detected upstream of both WWTPs, though their concentrations were below the MQL. The same happened downstream the WWTPs with paracetamol, azithromycin and simvastatin.

Bearing in mind that Douro river has a Portuguese basin of 18,643 km^2 (riverhead in Spain—total of 97,603 km^2) and an average flow of 450 $m^3\ s^{-1}$ (200–710), preliminary conclusions point to a minor impact of the studied WWTPs on the river water quality, in terms of the studied pharmaceutical emerging pollutants, probably due to the enormous dilution factor.

The obtained results for wastewaters are consistent with those previously reported by other researchers in other countries [3, 10, 22, 23].

Conclusions

The developed multi-residue method encompassed one single step of simultaneous cleanup and extraction, achieved with Oasis MAX adsorbent, followed by LC–ion trap–MS/MS detection.

Among the several cleanup strategies exploited in order to minimise the influence of matrix components in the ionisation efficiency of the target analytes, both selective washes with increasing MeOH contents and microfiltration approaches failed, leading to low overall recoveries and loss of some compounds. Regarding the different solid-phase adsorbents tested, LC-Si cartridges were characterised by low cleanup efficiencies and resulting dirty extracts, whereas the other normal-phase adsorbents Florisil and GCB gave apparently clean extracts. However, none of the solvents tested was able to elute a considerable amount of the compounds from these adsorbents. When comparing the ion exchange SAX with NH_2 cartridges, both allowed the recovery of all compounds. Nevertheless, the latter gave consistently better recoveries. Finally, Oasis MAX cartridges were found to be the most suitable to perform wastewater samples' (pre-)treatment, providing higher recovery rates for the majority of the compounds in one single cleanup and extraction step. These cartridges, allied to a broad-spectrum detection method, enabled the quantification of 23 pharmaceutical compounds belonging to different therapeutic and chemical families. The resulting final method can be considered a valuable tool, allowing, on the overall, good recovery rates (50–130%, with few exceptions) and excellent detection limits (1–20 ng L^{-1}).

Pharmaceuticals detected in wastewater samples included paracetamol, hydrochlorothiazide, furosemide, naproxen, ibuprofen, diclofenac and bezafibrate in concentrations above 1 μg L^{-1}, while gemfibrozil, simvastatin, ketoprofen, azithromycin, bisoprolol, lorazepam and paroxetine were quantified in sub-microgramme per litre.

The research must continue, including the receiving surface waters, to provide sufficient analytical information to determine the pathway of these emerging pollutants in the environment and, more importantly, to allow the renewal/construction of more appropriate wastewater treatment facilities, thus providing efficient pharmaceutical removal processes.

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Chapter 3

Monitoring of Pharmaceuticals in Municipal Wastewaters and Surface Waters

As previously mentioned, not many emerging pollutants (EPs) are regulated or included in monitoring programs. In this process, the new legislature within the European Union (EU) – the registration, evaluation and authorisation of chemicals (REACH) system, among other legislative frameworks, will have great importance. In order to acquire the necessary scientific evidence for EPs to be included in such frameworks and future directives, it became necessary to launch several monitoring programs and develop cost-effective and user-friendly strategies for their monitoring in water resources (1).

Since wastewater treatment plants (WWTPs) are considered major contamination sources by EPs, in this Ph.D. work we attempted to evaluate the impact caused by the discharge of a municipal WWTP on Febros river, a small tributary of Douro river, located in the northern region of Portugal, in terms of pharmaceutical content only. To achieve this goal, we employed the developed and optimized methods described in the previous chapter. Afterwards, we further assessed Febros river contribution to the contamination with pharmaceutical residues of Douro river, where it debouches. Douro river constitutes an important water source for the production of drinking water.

This study resulted in the publication of **Paper 2** (please refer to the end of Section 3.4), in Portuguese language. Therefore, its content, main results and conclusions will be displayed along the present chapter.

3.1. Water Sampling

Sampling could be defined as the process of selecting a representative water sample with the appropriate volume to be conveniently transported and handled in the laboratory. It is an integral part of the analytical process, often representing the main contribution to the error of the analytical result (2).

Proper sampling and analytical techniques are of fundamental importance in the characterization of water samples. Firstly, it is essential to ensure that the analysed sample is truly *representative* of the environment sampled. Once this requirement is met, it is then necessary to adopt the appropriate analytical procedures, specifically developed for water or wastewater analyses (3).

The data collected during the sampling plan must be *useful*, thus meeting the objectives of the monitoring plan (4), *reproducible* and *defensible*, i.e., have associated documentation which validates the sampling procedure and provides information concerning the degree of **accuracy** and **precision** of the acquired data. While accuracy measures the closeness of results to the true value, precision measures the reproducibility of the results when the same sample is repeatedly analysed (3, 5).

3.1.1. Sampling Protocol

A detailed sampling protocol must be developed, along with a quality assurance project plan (QAPP) (previously known as quality assurance/quality control - QA/QC), encompassing the following items (4, 5):

i) *sampling plan*: including the number of sampling locations, number and type of samples, frequency and time intervals (e.g., real-time and/or time-delayed samples);
ii) *sample types* and *size*: catch or grab samples, composite samples, integrated samples, separate samples for different analyses, sample volume, etc.;
iii) *sample labelling* and *chain of custody*;
iv) *sampling methods*: indicating the techniques and equipment to be used (e.g., manual, automatic, passive sampling);

v) *sampling storage* and *preservation*: referring the type of containers and preservation methods;
vi) *sample constituents*: consisting of a list of parameters to be measured;
vii) *analytical methods*: consisting of a list of field and laboratory test methods and procedures to be used.

When designing a sampling plan, it is essential to clearly specify the objective of the exercise. For instance, one may aim to estimate maximum or mean concentrations, to detect changes or trends or even to provide a basis for industrial effluent charges. Nevertheless, to attain an accurate assessment of the scenario, it is usually necessary to generate composite samples. Integrated composite samples can be obtained by bulking individual samples collected at known time intervals and in proportion to the appropriate flow of the stream, along a certain period of time. Likewise, in cases when sampling rivers with large channel sections, it is desirable to collect samples at different points across the section and at several depths.

Furthermore, it is always necessary to bear in mind the resources available for both sampling and analysis procedures. Consequently, a realistic level of uncertainty of the results must be set, based upon the established goals (3). Whether high levels of uncertainty lead to unacceptable levels of reliability in the decisions based upon them depends on a rigorous evaluation of the fitness for purpose (6-8).

The potential sources of error during sampling procedures are of diverse origin. Table 3.1 displays some error sources which should be taken into consideration, according to a review study by Madrid and Zayas (2):

Table 3.1. Error sources during sampling procedures

Sampling stage (decision making)	Possible sources of error
Definition and sub-division of the field	Heterogeneity of the sample; spatial and/or temporal change of pollutants: "hot spots"
Sampling method	No representative statistics; skew distribution; contamination or analyte loss
Number of samples	Few replicates; absence of representativeness
Sample mass	Absence of representativeness
Moment of sampling	Seasonal changes; climatic conditions
Experimental conditions	Matrix effects; lixiviation or irreproducible deposits
Bottling	Contamination or extraction by the equipment or container material; volatilization
Storage during sampling	Contamination or losses by volatilization; chemical reactions (change of species)

3.1.2. Materials and Equipments

Ideally, all analyses should be conducted immediately after sample collection, so that the obtained results are a true assessment of the nature of the water sample *in situ*. In the real world, only some analytical determinations are viable on site, such as temperature, pH, conductivity, dissolved oxygen (DO), salinity, among others. These parameters cannot be adequately determined after transportation to the laboratory (9), thus multiparameter probes are usually taken to the field (Fig. 3.1 (a)).

In the case of surface waters, samples are often collected by direct filling of the sample bottle, at ca. 0.5 m depth. For deep water bodies or in places of difficult access, special sampling flasks can be used: these are lowered in a closed state, on a rope or steel cable, and then remotely triggered to open (Fig. 3.1 (b)). Various automatic devices are also available to collect composite samples, being operated on either a time basis or on a flow-proportional basis (Fig. 3.1 (c)). These instruments are of particular interest during the sampling of WWTP effluent discharges, since these are often intermittent in nature. In such circumstances, it is fundamental to fully understand the nature of the operations producing the discharge, in order to draw an appropriate sampling programme and consequently obtain a true picture of the discharge.

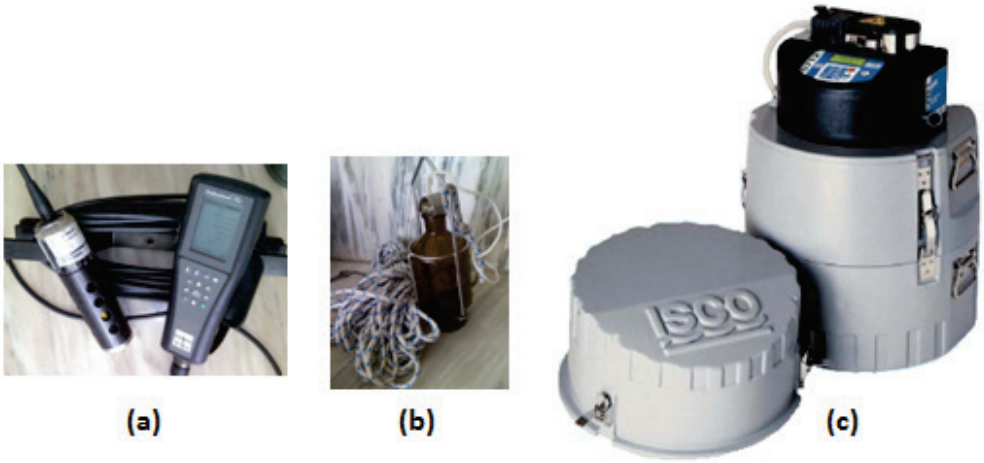

Fig. 3.1. Sampling devices. (a) multiparameter field probe; (b) sampling flask with remotely triggered opening; (c) automatic sampler used to collect and refrigerate grab or composite samples over a 24-h period.

In the last two decades, passive sampling methods have shown much promise as tools for measuring aqueous concentrations of a wide variety of pollutants. In its broadest sense, passive sampling includes any sampling technique based on free flow of analyte molecules

from the sampled medium to a receiving phase in a sampling device, due to a difference between the chemical potentials of the analyte in the two media. Thus, there is no need of any energy source other than this chemical potential difference. The net flow of analyte molecules between both media continues until reaching the equilibrium state or until the sampling period is stopped (10). The main advantages of passive sampling compared to active water sampling, regarding the collection of organic pollutants, include the following aspects: *i)* it is based on diffusion processes (no power supply), *ii)* it occurs *in situ* (selective analyte isolation and pre-concentration (11)) and it is often time-integrative, *iii)* it shows low variability, due to a standardised configuration of the synthetic samplers, and finally *iv)* it mimics the respiratory exposure for aquatic organisms, since, traditionally, it includes a mass transfer and rate limiting membrane(s) in their design (12). Summing up, passive samplers simplify the operations performed at the sampling site.

Table 3.2 presents an overview of some of the most used passive samplers for monitoring of organic pollutants, as reviewed by Namiesnik et al. (13).

Table 3.2. Passive sampling devices for organic contaminants

Sampler	Full Name	Analytes	References
Chemcatcher	Universal passive sampler using Empore disk	Polar and non-polar organics	(14)
Ecoscope	A sampler based on solvent-filled dialysis membranes and chelating sorbent discs	Non-polar organics	(15)
MESCO	Membrane-enclosed sorptive coating	PAHs, PCBs, organochlorine pesticides	(16)
PDB	Passive diffusion bag samplers	Polar organic compounds, VOCs, metals, trace elements	(17)
PISCES	Passive *in situ* concentration-extraction sampler	PCBs	(18)
POCIS	Polar organic chemical integrative sampler	Herbicides and pharmaceuticals with log $K_{OW} < 3$	(19)
SPATT	Solid-phase adsorption toxin tracking	Polar phytotoxins	(20)
SPMD	Semi-permeable membrane devices	Hydrophobic semi-volatile organic compounds	(21)

Lastly, it must be highlighted that the physical, chemical and biological integrity of samples during interim periods between sampling and analysis shall be preserved. Changes in sample composition can be retarded by storage at low temperatures (4 ºC), in the dark. The more polluted a sample is, the shorter the time which can be allowed between sampling and analysis, in order to avoid significant errors (3).

3.2. A Portuguese Case Study

3.2.1. Monitoring Sites

Monitoring water quality is carried out for various purposes and its design will depend on its later uses. Obviously the cost of monitoring has to be related to the ultimate benefit. In our study, both wastewater and surface water samples were analysed in terms of pharmaceutical content. While the first were collected at Febros WWTP, the latter were sampled in Febros river and Douro river.

3.2.1.1. Febros WWTP

Febros WWTP was built in 2003, in the city of Vila Nova de Gaia. It was dimensioned for 80 000 inhabitants, designed to treat a wastewater flow of 39 605 m^3 per day and a load corresponding to 5 334 kg per day in terms of biochemical oxygen demand (BOD_5). Febros WWTP (Fig. 3.2) comprises: an **harrowing/sieving**, **desanding** and **degreasing** system; **2 aeration tanks** with a capacity of 6 053 m^3 each; **3 secondary clarifiers** with a 25 m diameter; **1 thickener** with a diameter of 14 m; **sludge mechanical dehydration** with 2 centrifuges; **sludge storage silo** of 50 m^3 capacity; and, finally, an **odour deodorizing treatment** step using a **column of activated carbon** for 6 380 $m^3 h^{-1}$.

Fig. 3.2. Image of Febros WWTP, which discharges to Febros river, flowing on the right-hand side. In the top-right part of the image one can identify Douro river, dividing the city of Vila Nova de Gaia (left bank) from the city of Porto (right bank) (22).

3.2.1.2. Febros River and Douro River

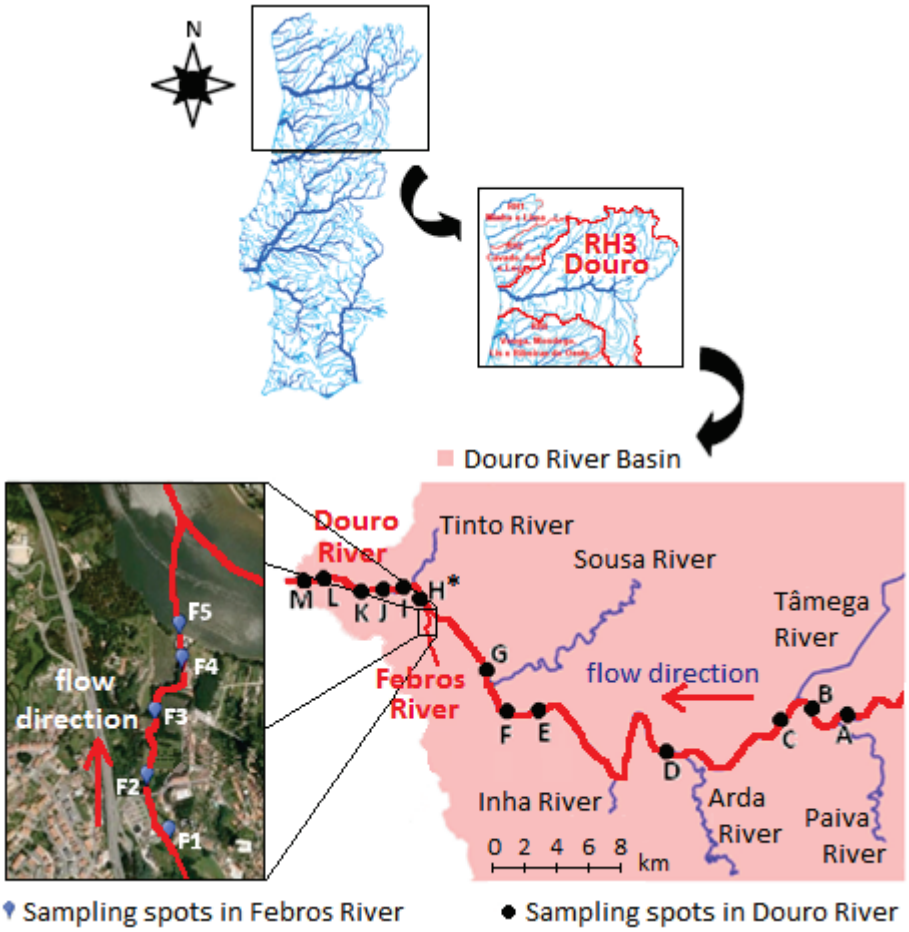

Fig. 3.3. Geographical location of the case study – Portuguese hydrographical region no. 3: Douro.

Table 3.3. GPS coordinates of the sampling spots in Febros river, identified in Fig. 3.3

	Sampling Spots	Latitude	Longitude
Upstream WWTP	F1	41° 7'2.92" N	8°34'11.48" W
Downstream WWTP	F2	41° 7'8.75" N	8°34'14.42" W
	F3	41° 7'15.52" N	8°34'13.24" W
	F4	41° 7'21.04" N	8°34'9.41" W
	F5	41° 7'24.65" N	8°34'9.72" W

* **Sampling Spot H** (latitude 41°08'00.89" N; longitude 8°34'20.77" W): located in Douro River, after the debouching site of Febros River. The remaining sampling spots in Douro River were not part of the present work, but were included in the monitoring campaigns addressed in the study by Gonçalves et al. (Annex II).

The study area is situated in the hydrographical region no. 3 – Douro (Fig. 3.3).

Douro river (Fig. 3.4) is the third longest river in the Iberian Peninsula, with a total length of 927 km. Douro riverhead is located in *Sierra de Urbión*, Sória (Spain), at 2 080 m of altitude, the Portuguese river basin is 18 643 km² (total 97 603 km²) and has an average flow of 450 $m^3 s^{-1}$ (ranging between 200 and 710 $m^3 s^{-1}$). It crosses the northern region of Portugal, flowing towards the Atlantic Ocean, with the river mouth close to the cities of Vila Nova de Gaia and Porto.

Fig. 3.4. Image of Douro river.

In contrast, Febros river (Fig. 3.5) is a small tributary on the left bank of Douro river. Febros riverhead is located in *Seixezelo* and the river mouth is in the *Esteiro de Avintes*. This river also crosses the Natural Park of Vila Nova de Gaia.

Fig. 3.5. Image of Febros river, close to the river mouth.

Hence, due to Febros river low flow and small basin area, a high impact caused by the discharge of the WWTP effluents was predictable from the beginning. Nonetheless, the construction of Febros WWTP proved to be quite advantageous in the remediation of the river. The removal of contaminants of both domestic and industrial origin, in collaboration with the natural city park, allowed Febros river repopulation with a mammal species long disappeared in this river - the otter. On the other hand, a strong dilution effect occurs during Febros river debouching into Douro river. Therefore, its potential impact on Douro river, in terms of pharmaceutical content, was expected to be negligible.

3.2.2. Methodology

3.2.2.1. Sampling

At Febros WWTP, both grab and composite (400 mL per hour, over 24-h) influent and treated effluent samples were collected from May 2009 to March 2011. Concerning surface waters, grab samples were collected in 5 sampling spots in Febros river (1 upstream Febros WWTP and 4 downstream Febros WWTP) and 12 sampling spots in Douro river (7 upstream the debouching point of Febros river and 5 others downstream this point), during two sampling campaigns conducted in 2010 (spring and winter) (please refer to Fig. 3.3 and Table 3.3).

The sampling plan included the collection of 2.5 L sample for wastewaters and 1 L for surface waters (in this case, collected at 1 m of depth), transport to the laboratory in ice-packed containers and subsequent preservation at 4 ºC until analysis (max. 3 days after sampling). Moreover, influent and effluent wastewater samples were filtered using glass fibre filters (Ø 1.2 μm), immediately after arrival to the laboratory.

3.2.2.2. Analytical Method – SPE-LC-MS/MS

The analytical methods developed and optimised for the determination of 23 pharmaceutical compounds potentially present in the collected samples were described in the previous chapter. Briefly, they consisted on an initial cleanup step (optional in the case of surface water samples), followed by a solid-phase extraction (SPE) procedure, in order

to remove most interferences and concentrate the target analytes, and subsequent analysis/quantification by high performance liquid chromatography coupled to mass spectrometry (HPLC-MS).

For wastewater samples, the cleanup and SPE processes were simultaneously carried out using *Oasis MAX* columns. *Oasis MAX* is a mixed-mode sorbent consisting of a strong anion exchanger (quaternary amine) and a non-polar N-vinylpyrrolidone/divinylbenzene polymer. It has been optimised to achieve higher selectivity and sensitivity for extracting acidic compounds with anion exchange groups, while basic and neutral compounds are retained by the polymeric sorbent. In the case of surface water samples, whenever necessary, cleanup was achieved using *Bakerbond 1º,2º-Amino* (weak-anion exchange, WAX) cartridges, while SPE procedure was conducted with *JTBaker H_2Ophilic* extraction columns (23). Besides the sorbent, other extraction parameters were as well optimised, including the pH (pH 2), percolation flow (10 mL min^{-1}) and the breakthrough volume of 50 mL for WWTP influents, 100 mL for WWTP effluents and 200 mL for river waters.

The separation, identification and quantification of pharmaceuticals was performed by HPLC-ESI-MS/MS (ion-trap detector), assuring the unequivocal confirmation of each compound through the comparison with reference spectra obtained for the respective standards.

The final validated analytical method proved to be sensitive, selective, precise, accurate and robust for the determination of the 23 pharmaceuticals in water and wastewater samples. In specific cases, definitive identification was achieved by MS/MS/MS (MS^3).

3.2.3. Results and Discussion

3.2.3.1. Wastewaters from Febros WWTP

Table 3.4 summarises the results obtained after analysis of Febros WWTP composite influent and effluent samples, using the developed pharmaceutical multiresidue method. Pharmaceuticals like paracetamol and hydrochlorothiazide highlighted in significantly high concentrations in the WWTP influent (> 5 μg L^{-1}). Nonetheless, their removal along the treatment processes showed to be quite efficient, as one can confirm by the concentration values in the corresponding WWTP effluent (below the respective method quantification limit). On the contrary, the removal percentage of other pharmaceutical

compounds, such as azithromycin, bisoprolol, furosemide and ibuprofen, was rather low. Moreover, another very important aspect relates to the cases of lorazepam, ketoprofen, naproxen, bezafibrate, diclofenac and gemfibrozil, whose concentrations detected in influent samples were below those obtained in the corresponding WWTP effluents. This apparent incongruence can be justified considering: *i)* the difficulty to establish an accurate correspondence between the sample collected at the intake of the WWTP (influent) and that collected after the treatment, at the exit of the WWTP (effluent) – related to the WWTP typical hydraulic retention time (HRT)); *ii)* the conversion of conjugated metabolites (present in influent samples) to the original substances, over the treatment processes (24).

Table 3.4. Target pharmaceuticals quantified in Febros WWTP influent and effluent

Pharmaceuticals	Concentration (ng L^{-1})	
	Influent	Effluent
Hydrochlorothiazide	6 022	<58*
Paracetamol	20 074	<22
Ciprofloxacin	<6	<4
Zolpidem	<3	<2
Azithromycin	571	530
Bisoprolol	239	187
Omeprazole	-	-
Furosemide	2 675	2 430
Indapamide	<65	<57
Paroxetine	105	60
Bromazepam	<7	<6
Fluoxetine	<5	<4
Alprazolam	<5	<4
Lorazepam	<7	240
Nimesulide	<4	<3
Ketoprofen	122	562
Naproxen	2 583	2 748
Diazepam	<3	<3
Bezafibrate	1 348	1 797
Diclofenac	431	1 107
Ibuprofen	1 992	1 527
Gemfibrozil	307	753
Simvastatin	976	197

* Concentration below the method quantification limit.

With the purpose of better assessing the concentration levels of pharmaceuticals over the WWTP daily cycle, and possibly identifying potentially critical discharge periods, grab effluent samples were collected each hour, for 21-h, using the aforementioned automatic sampler device (Fig. 3.1 (c)), and further analysed.

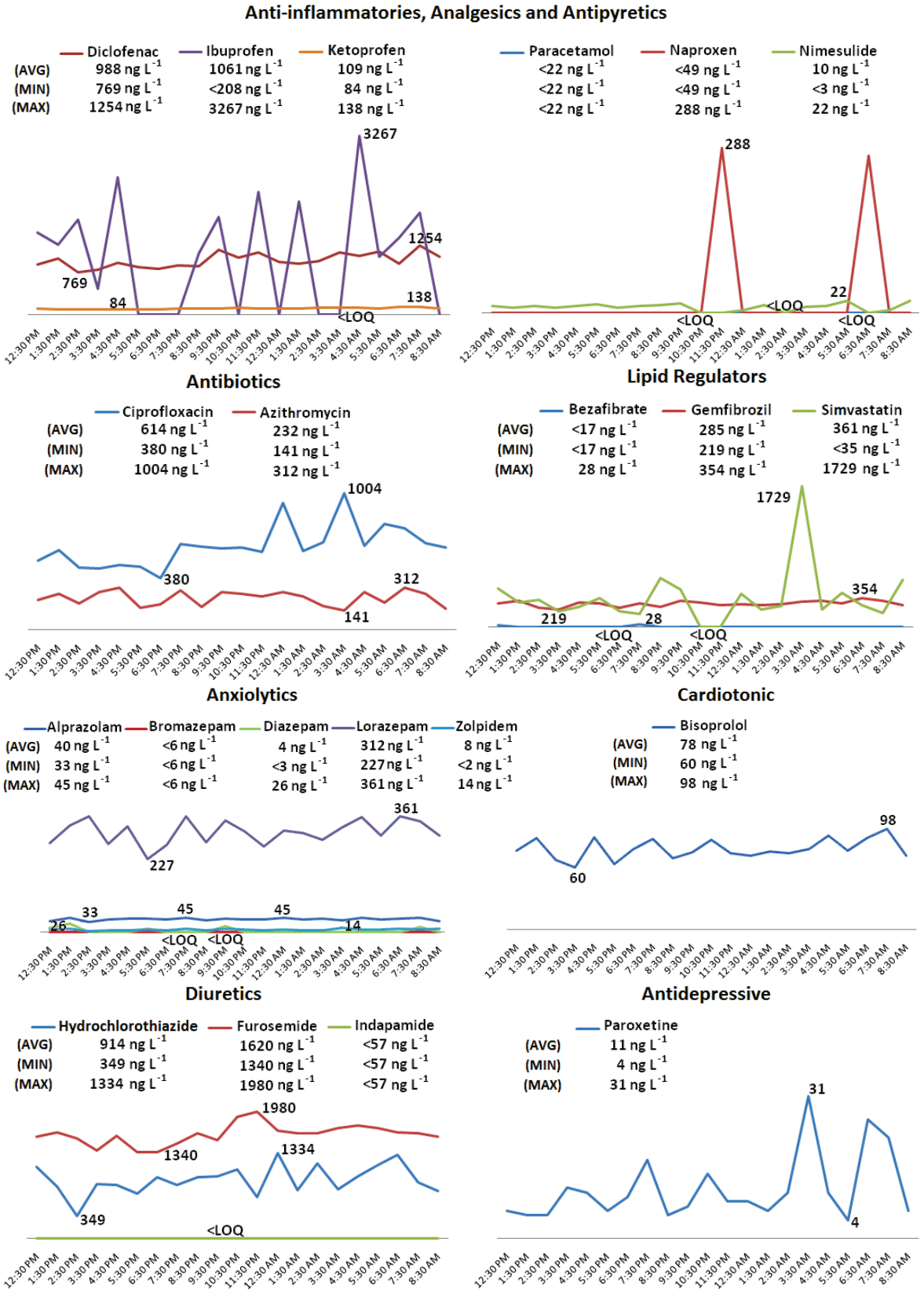

Fig. 3.6. Grab samples of WWTP effluent, collected hourly for a 21-h period, analysed individually. Graphics represent concentration variations for each pharmaceutical over the sampling period. AVG – theoretical average value calculated for a composite sample; MIN – minimum value; MAX – maximum value; LOQ – limit of quantification.

The results obtained, and displayed in Fig. 3.6 revealed that, for the majority of the pharmaceuticals detected in the different effluent grab samples, there were no significant variations in terms of concentration. Hence, we confirmed that composite samples were accurate and representative. The HRT of this WWTP was allowing a proper homogenisation of influent wastewaters and the consequent attenuation of any eventual pharmaceutical discharge peak. Only the anti-inflammatory compound naproxen and the lipid regulator bezafibrate were indicated as <LOQ by analysis of the composite sample, while quantifiable concentration levels were achieved for some grab samples (max. 288 ng L^{-1} and 28 ng L^{-1}, respectively). However, besides being rather low environmental concentrations, these results may denote punctual situations, with no substantial consequence whatsoever on the representativeness of composite samples.

3.2.3.2. Surface Waters from Febros River and Douro River

Concerning Febros river, a significant increase in the concentration levels of bisoprolol and furosemide was observed (approximately 2 and 6 times, respectively – **Paper 2**), when comparing sampling spot F1 (upstream the WWTP discharge point) with spot F2 (immediately downstream the WWTP discharge point) (Fig. 3.3). Moreover, hydrochlorothiazide, azithromycin, diclofenac, gemfibrozil and simvastatin became quantifiable in F2, in concentrations ranging from 40 to 360 ng L^{-1}. These results, in addition to those previously obtained for Febros WWTP effluent (Table 3.4), demonstrate the contribution of the WWTP to Febros river contamination with pharmaceutical compounds. Furthermore, the case of paracetamol must also be given particular attention. Its concentrations in Febros river upstream and downstream the WWTP discharge point were rather similar, which indicted the presence of another anthropogenic contamination source, possibly domestic untreated effluents. It is known that paracetamol is highly degraded over the treatment processes taking place in WWTPs (24).

With regard to Douro river, the potential contribution of Febros river (its left bank tributary) to its contamination with pharmaceutical compounds was not confirmed. In fact, the results achieved for the sampling spots F5 and H (Fig. 3.3) revealed no pharmaceutical residue above its respective LOQ, most likely due to the already predicted high dilution effect, given Douro river huge dimensions.

3.2.4. Conclusions

The main conclusions withdrawn from the present work relate to the importance of WWTPs' role in the environmental dissemination of pharmaceutical compounds. Specifically regarding Febros WWTP, its implication on the pharmaceutical input to Febros river was evidenced by the higher pharmaceutical load in samples collected downstream the WWTP discharge point, when compared to upstream sampling sites. On the other hand, no significant increase in the pharmaceutical content was observed in Douro river after the mouth of Febros river, most likely due to the great dilution factor.

Owing to the overall low removal efficiency attained by most WWTPs regarding pharmaceuticals in particular and emerging organic micropollutants in general, the development and application of more advanced treatment techniques became essential. This matter will be extensively discussed in the following chapter.

Herein follows **Paper 2** (in Portuguese language), concerning the Portuguese monitoring case study presented along this chapter.

3.3. Paper 2 – Effects of treated domestic effluents and tributaries on the contamination of Douro river with pharmaceutical compounds – Mitigation processes

Published Scientific Paper:

Sousa, M.A., Gonçalves, C., Vilar, V.J.P., Boaventura, R.A.R. e Alpendurada, M.F., *Efeitos dos efluentes domésticos tratados e rios tributários no pool de resíduos farmacêuticos do Rio Douro - Processos de Mitigação [Effects of treated domestic effluents and tributaries on the contamination of Douro river with pharmaceutical compounds – Mitigation processes].* Submitted for publication in *Recursos Hídricos* (APRH - *Associação Portuguesa de Recursos Hídricos*), June 2011.

EFEITOS DOS EFLUENTES DOMÉSTICOS TRATADOS E RIOS TRIBUTÁRIOS NO *POOL* DE RESÍDUOS FARMACÊUTICOS DO RIO DOURO – PROCESSOS DE MITIGAÇÃO

Effects of treated domestic effluents and tributaries on the contamination of Douro River with pharmaceutical compounds – Mitigation processes

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Resumo

A presença de compostos farmacêuticos no meio aquático tem vindo a ser motivo de crescente preocupação por parte da comunidade científica, um pouco por todo o mundo. As ETARs são vistas como importantes fontes de contaminação.

Neste trabalho procurou-se avaliar o impacto causado pela descarga do efluente tratado numa ETAR municipal num pequeno afluente do Rio Douro – o Rio Febros – e, em última análise, a contribuição destas duas fontes para o aporte de resíduos farmacêuticos ao Rio Douro (fonte de captação de água destinada ao consumo humano).

O impacto causado pela descarga deo efluente da ETAR no Rio Febros foi notório dado o aumento significativo dos níveis de diversos fármacos no rio, a jusante do ponto de descarga da ETAR. Já a implicação do Rio Febros na contaminação do Rio Douro com fármacos não foi observada, o que se atribuiu ao elevado efeito de diluição verificado.

Finalmente, e como tentativa de prevenção da contaminação do meio aquático com compostos farmacêuticos, foi testada a fotocatálise solar com TiO_2, recorrendo à utilização de uma Instalação Solar Piloto com Colectores Parabólicos compostos (CPCs). Este processo revelou-se bastante promissor uma vez que, além do aumento significativo das taxas de remoção de diversos fármacos, se baseia no uso de uma fonte de energia renovável, tornando-se assim economicamente mais atractivo e amigo do ambiente.

Palavras-chave: fármacos; contaminação aquática; ETARs; instalação solar piloto com CPCs; fotocatálise solar com TiO_2.

Abstract

The aquatic environmental occurrence of pharmaceutical compounds has been increasingly concerning the scientific community worldwide. Wastewater treatment plants (WWTPs) are seen as important contamination sources.

Therefore, in this work we attempted to evaluate the impact caused by the discharge of a municipal WWTP on a small tributary of Douro River – Febros River – and afterwards their overall contribution to the contamination of Douro River (water source for the production of drinking water) with pharmaceutical residues.

The role of Febros' WWTP on pharmaceutical aquatic dissemination was confirmed by the significant increase of several pharmaceutical compounds in Febros water body, downstream the WWTP discharge point. On the other hand, no significant contribution of these potential sources to Douro River's contamination was observed, probably due to the high dilution effect.

As a promising strategy for the mitigation of pharmaceuticals in the aquatic compartment, a solar photocatalysis experiment with TiO_2 was performed and proved to be quite efficient. With the use of a Solar Pilot Plant with Compound Parabolic Collectors (CPCs), besides increasing removal rates, we make use of a renewable energy source, thus becoming more economically attractive and environmentally friendly.

Keywords: pharmaceuticals; water contamination; WWTPs; solar pilot plant with CPCs; solar photocatalysis with TiO_2.

1. Introdução

1.1. Poluentes Emergentes – Grupo dos Compostos Farmacêuticos

A Directiva-Quadro da Água (DQA, Directiva 2000/60/CE), que entrou em vigor a 22 de Dezembro de 2000, mantém-se actualmente como o principal instrumento da Política da União Europeia relativa à Água. Através do estabelecimento de um quadro de acção comunitária, são indicados os protocolos a seguir com vista à obtenção do "Bom Estado Químico e Ecológico de todas as Águas até 2015" e subsequente uso sustentado a longo termo. Esta Directiva salienta também a necessidade de identificação de "Outras Substâncias descarregadas em quantidades significativas na massa de água" e que constituam um potencial perigo para a qualidade da água, além das já conhecidas Substâncias Prioritárias [http://dqa.inag.pt/].

É exactamente neste grupo de "Outras Substâncias" que podemos incluir os designados Poluentes Emergentes (PEs), compostos actualmente não abrangidos por programas de monitorização a nível Europeu, mas que podem ser candidatos a regulamentação futura, dependendo dos resultados de ensaios de ecotoxicidade,

potenciais efeitos sobre a saúde e dados de monitorização da sua ocorrência em diversos compartimentos ambientais. Entre a grande variedade de compostos abrangidos sob esta designação incluem-se os Compostos Farmacêuticos (CFs), cujas diversas acções terapêuticas podem originar desde o desenvolvimento de resistências bacterianas (ex. antibióticos), a interferências com a reprodução, fisiologia e crescimento de espécies aquáticas (ex. desreguladores endócrinos – esteróides, hormonas). Além do mais, trata-se de compostos que podem apresentar baixa toxicidade aguda mas elevada toxicidade crónica, com potencial bioacumulação em diferentes tecidos animais.

Estes poluentes são considerados emergentes não pela emergência do seu aparecimento mas da sua detecção, dada a cada vez maior capacidade analítica, viabilizada por associações de métodos cromatográficos a processos de detecção avançados, nomeadamente a Espectrometria de Massa, possibilitando a obtenção de limites de detecção na ordem dos µg-ng L^{-1} [Sousa *et al.*, 2011].

1.2. Processos Avançados de Oxidação (PAOs)

O uso crescente de xenobióticos como fármacos, fragrâncias sintéticas, pesticidas, drogas de abuso e outros contaminantes está a conduzir ao aumento das concentrações destas substâncias nas águas residuais e superficiais. Os CFs são considerados poluentes "pseudo-persistentes" dado que as suas taxas de transformação/eliminação (0-100%, dependendo do composto e do processo de remoção natural/artificial) são compensadas pela sua re-introdução contínua no meio ambiente [Petrović *et al.*, 2003].

A ocorrência e destino destes contaminantes têm sido objecto de vários estudos ao longo dos últimos anos (Fig. 1). Em Portugal começaram já a surgir registos da presença ambiental aquática de CFs. Desde efluentes hospitalares contendo, por vezes, elevadas concentrações de antibióticos (quinolonas) como ciprofloxacina, enrofloxacina, ofloxacina e norfloxacina, a efluentes domésticos tratados [Seifrtová *et al.*, 2008 e Pena *et al.*, 2010], bem como cursos de água superficiais, incluindo os rios Douro, Leça e Febros, tem sido reportada a presença de diversos CFs, pertencentes a vários grupos terapêuticos, entre eles analgésicos, antipiréticos e anti-inflamatórios, reguladores lipídicos, antibióticos, anti-epilépticos, etc. [Sousa *et al.*, 2011].

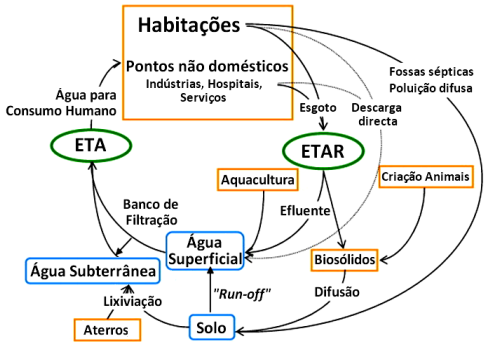


Figura 1. Percurso cíclico dos compostos farmacêuticos nos diferentes compartimentos aquáticos [adaptado de Mompelat *et al.*, 2009].

Dado que a reutilização de água tratada poderá, no futuro, ser a melhor solução para uma gestão sustentada em países mais desprovidos de água, e que uma das preocupações básicas no seu reuso é a presença de microcontaminantes (muitas vezes provenientes das descargas de ETARs municipais), são requeridos processos alternativos e/ou complementares aos tratamentos convencionais, capazes de permitir o aumento da qualidade dos efluentes eliminados e prevenir a contaminação ambiental com CFs.

Neste contexto, os PAOs são considerados processos de tratamento bastante competitivos para a remoção destes compostos, mais ou menos recalcitrantes dada a sua elevada estabilidade química e/ou baixa biodegradabilidade. Os PAOs têm em comum a formação do radical hidroxilo (•OH), altamente reactivo e responsável pela degradação da componente orgânica. Dado o seu poder oxidante não-selectivo, estes radicais são capazes de oxidar e mineralizar quase todas as moléculas orgânicas, originando CO_2, H_2O e iões inorgânicos como produtos finais. Todavia, alguns deles podem apresentar também alguns inconvenientes, tais como altos consumos energéticos (ex. lâmpadas UV, geradores de ozono), quantidades elevadas de oxidantes e catalisadores (ex. H_2O_2, TiO_2), ou ainda a necessidade de ajuste do pH de grandes volumes de amostra (ex. Fenton e foto-Fenton) [Miranda-Garcia *et al.*, 2011 e Oller *et al.*, *in press*].

2. Fármacos no Rio Douro

2.1. Compostos Farmacêuticos

Os fármacos englobados no presente estudo pertencem a doze classes terapêuticas diferentes, incluindo analgésicos e antipiréticos (paracetamol), anti-inflamatórios não esteróides (cetoprofeno, diclofenac, ibuprofeno, naproxeno, nimesulida), reguladores lipídicos (bezafibrato, gemfibrosil, sinvastatina), antibióticos (azitromicina, ciprofloxacina), ansiolíticos (alprazolam, bromazepam, diazepam, lorazepam, zolpidem), antidepressivos (fluoxetina, paroxetina), diuréticos (furosemida, hidroclorotiazida, indapamida), cardiotónicos (bisoprolol) e anti-ulcerosos (omeprazol), entre outros.

Estes CFs encontram-se entre os mais comercializados em Portugal ao longo dos últimos anos, segundo dados fornecidos pelo INFARMED – Instituto Nacional da Farmácia e do Medicamento, e as correspondentes classes terapêuticas fazem parte do grupo das mais estudadas, reportadas e consumidas mundialmente [Sousa *et al.*, 2011].

2.2. Possíveis Fontes de Contaminação – ETAR Municipal e Rio Tributário

O local em estudo situa-se na Região Hidrográfica 3 – Rio Douro (RH3 – Douro, Fig. 2), sendo um dos principais objectivos a avaliação do impacto, no que se refere apenas ao conteúdo em fármacos, da descarga de efluentes tratados pela ETAR de Febros (concelho de V. N. Gaia) no Rio Febros e deste, posteriormente, no Rio Douro, onde desagua.

Enquanto que o Rio Douro é o segundo rio mais extenso da Península Ibérica (com 927 km no total, nasce em Espanha na província de Sória, nos picos da *Sierra de Urbión*, a 2.080

m de altitude, apresentando uma área da bacia de 97.603 km² e um débito médio de 450 $m^3 s^{-1}$, atravessa o norte de Portugal, sendo a foz no Oceano Atlântico, junto das cidades do Porto e V. N. Gaia), já o Rio Febros é um pequeno afluente da margem esquerda do Rio Douro (nasce em Seixezelo e desagua no Esteiro de Avintes, passando também pelo Parque Biológico de Gaia). Assim, é expectável que o possível impacto causado pela ETAR seja mais notório no Rio Febros do que no Rio Douro, onde se prevê um grande efeito de diluição. Todavia, a construção da ETAR mostrou-se desde logo vantajosa na despoluição do Rio Febros de resíduos de origem doméstica e industrial, permitindo inclusivamente a sua repovoação, em colaboração com o Parque Biológico, com uma espécie de mamíferos marinhos há já 20 anos desaparecida deste rio – a lontra [http://www.dn.pt/inicio/ciencia/interior.aspx?content_id=1576557&seccao=Biosfera].

Assim, e como segundo principal objectivo deste estudo, pretende-se também aumentar a taxa de remoção de CFs em efluentes aplicando uma etapa adicional com PAO. Como modelo experimental, usou-se um efluente da ETAR de Febros e uma Instalação Solar com Colectores Parabólicos Compostos (CPCs), à escala piloto, para desenvolver um tratamento de Fotocatálise Solar com TiO_2.

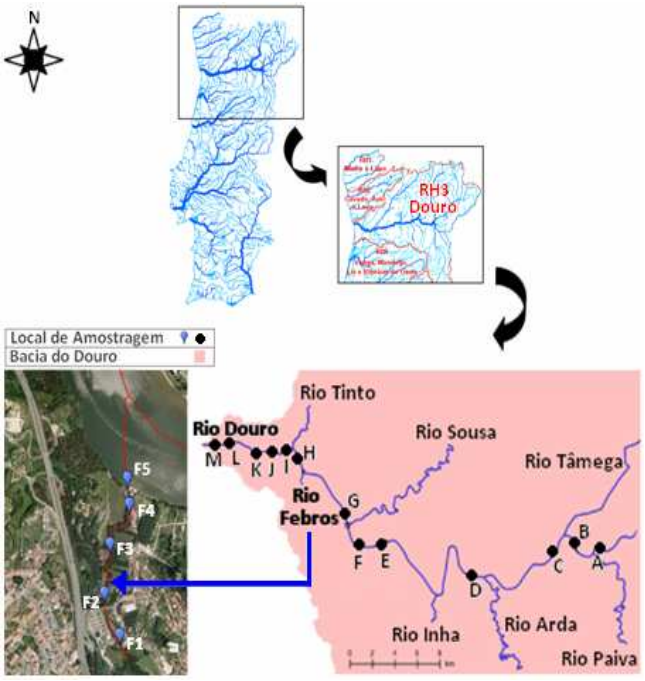


Figura 2. Localização geográfica do local/caso em estudo – Região Hidrográfica 3: Douro.

3. Metodologia Experimental

3.1. Amostragem e Pré-Tratamento das Amostras

O plano de monitorização da ocorrência de CFs em amostras ambientais incluíu, por um lado, afluentes/efluentes da ETAR de Febros (amostras compostas), e por outro lado, águas superficiais dos rios Febros e Douro (amostras simples). Efectuou-se a análise de diversas amostras de água residual à entrada e saída da referida ETAR, desde Maio de 2009 a Março de 2011, bem como de amostras colhidas em 5 pontos do Rio Febros (1 a montante e 4 a jusante do ponto de descarga da ETAR) e 12 pontos do Rio Douro (7 a montante e 5 a jusante da confluência com o Rio Febros), ao longo de 2 campanhas de amostragem realizadas em 2010 (Fig. 2).

O plano de amostragem incluíu a colheita de 2,5 L para águas residuais e 1 L para águas superficiais (neste caso colhida a 1 m de profundidade), transporte até ao laboratório em malas térmicas e posterior conservação a 4 ºC até realização da análise (máx. 3 dias após a colheita). No caso das amostras de afluente/efluente da ETAR de Febros, estas foram filtradas por filtros de fibra de vidro (Ø 1,2 µm) imediatamente após chegada ao laboratório, previamente à conservação a 4 ºC.

3.2. Método Analítico – SPE-LC-MS/MS

Com vista à análise e quantificação dos 23 CFs seleccionados, que pudessem estar presentes nas amostras de água incluídas no programa de monitorização, havia já sido desenvolvido e optimizado pelos autores um método analítico, cf. descrito por Sousa *et al*. (2011). Sumariamente, o método consiste numa primeira etapa de *cleanup* (facultativo no caso das águas superficiais), seguido de extracção em fase sólida (SPE – *Solid-Phase Extraction*), para remoção de interferências e concentração dos analitos em estudo, e posterior análise/quantificação por cromatografia líquida associada a espectrometria de massa (LC-MS – *Liquid Chromatography-Mass Spectrometry*).

Para as águas residuais, ambos os passos de *cleanup* e SPE foram efectuados simultaneamente com o uso de um adsorvente sólido misto (colunas de extracção *Oasis* MAX – *Mixed-Anion Exchange*), constituído por uma mistura entre uma amina quaternária (SAX - *Strong-Anion Exchange*) e uma fase apolar (polímero divinilbenzeno/*N*-vinilpirrolidona). Este adsorvente misto foi optimizado de forma a possibilitar uma elevada selectividade e sensibilidade na extracção de compostos ácidos pelos grupos de troca aniónica, enquanto que os compostos básicos e neutros são retidos na fracção polimérica.

No caso das águas superficiais, o *cleanup* é realizado, sempre que necessário, recorrendo a colunas Bakerbond 1º, 2º - Amino (WAX – *Weak-Anion Exchange*), tendo o SPE sido optimizado com uso de um adsorvente hidrofílico (*JTBaker H_2Ophili*c) [Gonçalves *et al.*, 2011].

A par da optimização do adsorvente de extracção, foram igualmente optimizados outros parâmetros como pH (pH 2), fluxo de percolação (10 mL min^{-1}) e volume de rotura de 50 mL para afluentes de ETAR, 100 mL efluentes de ETAR e 200 mL para águas superficiais.

A separação, identificação e quantificação dos CFs foi conseguida com recurso à cromatografia líquida de alta performance, ionização por Electrospray (ESI) e detecção por espectrometria de massa (detector *ion-trap)*, em modo de operação MS-MS, garantido assim a confirmação inequívoca de cada composto por comparação com os espectros de referência obtidos para os respectivos padrões.

Uma vez optimizadas todas as variáveis experimentais, o método analítico obtido foi validado em termos de sensibilidade, selectividade, precisão, exactidão e

calibração, tendo-se revelado robusto para a determinação de CFs em amostras de água [Sousa *et al.*, 2011].

3.3. Fotocatálise Solar Heterogénea (TiO_2) como Tratamento Terciário de Efluentes Municipais

A versatilidade dos processos avançados de oxidação deve-se à existência de múltiplas reacções capazes de gerar radicais hidroxilo, tais como TiO_2/UV, H_2O_2/UV, Fenton (Fe^{2+}/H_2O_2)/foto-Fenton (Fe^{2+}/H_2O_2/UV)/electro-Fenton /electro-foto-Fenton e ozono (O_3, O_3/UV e O_3/H_2O_2). Todas estas metodologias se tornam economicamente mais atractivas quando existe a possibilidade de utilização da radiação solar como fonte de fotões UV.

Entre os diferentes PAOs, a fotocatálise heterogénea com UV/TiO_2 tem-se revelado bastante promissora na destoxificação de águas residuais. A fotocatálise heterogénea é um processo que ocorre na presença de radiação UV e de um catalisador na fase sólida (fixo ou em suspensão). Na maioria dos métodos, o catalisador utilizado é um semicondutor. A estrutura electrónica destes semicondutores é caracterizada por uma banda de valência completa, de energia mais baixa, e uma banda de condução vazia, de energia mais alta. Estas duas bandas são separadas por uma quantidade de energia que se denomina gradiente energético. Ao absorver um fotão com uma quantidade de energia igual ou superior à do gradiente energético, um electrão da banda de valência ganha energia suficiente para passar ao nível da banda de condução, gerando um par electrão/lacuna ("electron/hole pair" – e-/h+). Contudo, por este ser um estado instável, em que o electrão se encontra excitado, o par electrão/lacuna tem tendência a anular-se, recombinando-se para o estado inicial numa questão de nanosegundos. No entanto, na presença de H_2O e O_2 na superfície do semicondutor, estes podem reagir com o par electrão/lacuna, ocorrendo reacções de oxidação/redução em vez da recombinação, dando origem à formação dos radicais hidroxilo.

O interesse particular no TiO_2 (BGE – *Band Gap Energy* = 3 eV) deve-se às propriedades fotocatalíticas, elevada hidrofilia e reactividade, baixa toxicidade, baixo custo e estabilidade química [Miranda-García *et al.*, 2011 e Oller *et al.*, 2011].

Neste estudo foi utilizada uma Instalação Solar Piloto com Colectores Parabólicos Compostos (CPCs) (Fig. 3) para o tratamento fotocatalítico de um efluente proveniente da ETAR de Febros, usando como catalisador o TiO_2.

A planta encontra-se instalada no terraço do Departamento de Engenharia Química da Faculdade de Engenharia, Universidade do Porto (FEUP). O colector solar é constituído por uma unidade CPC (0,91 m^2) de 4 tubos de vidro borossilicatado (*Schott-Duran type 3.3*, Alemanha, com *cut-off* a 280 nm, diâmetro externo de 50 mm, comprimento de 1500 mm e espessura de 1,8 mm), ligados em série por junções em polipropileno, com reflectores parabólicos em alumínio anodizado, suportados por uma estrutura em alumínio e com uma inclinação de 41º (correspodente à latitude local). A planta solar possui ainda 2 tanques de recirculação (10 L e 20 L), 2 bombas de recirculação (máx. 20 L min^{-1}) e 2 rotâmetros, 5 válvulas em polipropileno e um quadro eléctrico para controlo. A instalação pode ser usada de dois modos: utilizando a área total dos CPCs (0,91 m^2) ou 0,455 m^2 de cada par de tubos individualmente, permitindo a execução em simultâneo de duas experiências independentes, sob as mesmas condições de irradiação solar [Pintor *et al.*, 2011].

A intensidade da radiação UV solar é medida por um radiómetro (ACADUS 85-PLS), instalado com o mesmo

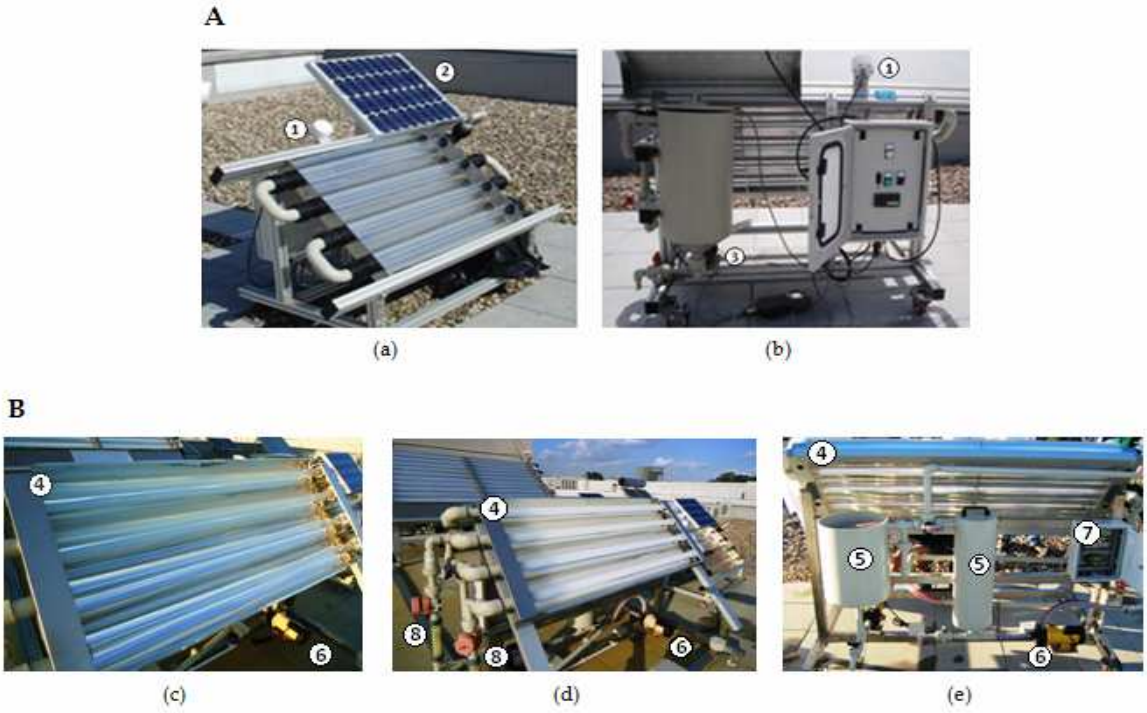


Figura 3. Plantas de Desinfecção Solar à Escala Piloto
A – Planta Solar Piloto com radiómetro: (a) Vista Frente, (b) Vista Trás; 1- Radiómetro, 2- Painel Fotovoltáico, 3- Bateria; B – Planta Solar Piloto utilizada na experiência: (c) CPCs com efluente da ETAR de Febros, (d) CPCs com efluente da ETAR de Febros e 200 mg L^{-1} TiO_2, (e) Vista Trás; 4- Colectores Parabólicos Compostos (CPCs), 5- Tanques de Recirculação, 6- Bombas de Recirculação, 7- Quadro Eléctrico, 8- Rotâmetros.

ângulo de inclinação. As medições da radiação ultravioleta instantânea (W m^{-2}) são obtidas com intervalos de 1 min. e usadas no cálculo da energia UV acumulada ($Q_{UV,n}$, kJ L^{-1}), durante um determinado intervalo de tempo (Δt), através da equação:

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

onde t_n é o tempo correspondente a n-alíquota (s), $V_{t,n}$ o volume total de efluente em n-alíquota (L), A_r a superfície iluminada do colector e $\overline{UV}_{G,n}$ o valor médio de radiação UV solar (W m^{-2}) medida no intervalo de tempo Δt_n [Pintor *et al.*, 2011].

A amostra de efluente da ETAR de Febros foi caracterizada antes do início do tratamento por fotocatálise solar. Os resultados obtidos são apresentados na Tabela 2 (secção 4.3.).

A concentração óptima de TiO_2 determinada foi de 200 mg L^{-1}. As várias alíquotas colhidas ao longo da experiência foram analisadas segundo o método descrito na secção 3.2., com o objectivo de obter as cinéticas de degradação dos diversos fármacos presentes na amostra ao longo das várias horas de exposição solar.

Podem ser encontrados detalhes adicionais sobre o modo de funcionamento e utilização da Instalação Solar Piloto com CPCs, bem como sobre as determinações analíticas seleccionadas/efectuadas para caracterização da amostra em Pintor *et al.*, 2011.

4.Resultados e Discussão

4.1. Águas Residuais – Afluente e Efluente da ETAR de Febros

O método analítico multi-resíduo desenvolvido foi utilizado na análise rigorosa de amostras compostas de afluente e efluente da ETAR de Febros, cujos resultados obtidos se encontram sumariados na Tabela 1.

Compostos como o paracetamol e a hidroclorotiazida destacam-se em concentrações bastante significativas no afluente da ETAR (>5 μg L^{-1}). Todavia, a eficácia dos processos de tratamento utilizados é bastante elevada para ambos os compostos, como se pode comprovar pelos valores obtidos à saída da ETAR (concentrações inferiores aos respectivos limites de quantificação). Já para outros compostos a eficiência de remoção na ETAR não é tão elevada, enquanto que em alguns casos se verifica mesmo um aumento da concentração no efluente, comparativamente ao afluente. Tal pode dever-se, sobretudo, à dificuldade de correlação entre a amostra colhida antes e após o tratamento na ETAR (relacionado com tempo de retenção hidráulica), mas também à possibilidade de não quantificação de alguns compostos à entrada da ETAR pelo facto de se encontrarem na forma conjugada (metabolitos), sendo que à saída se poderão já encontrar na forma original não-conjugada, reconvertida ao longo das etapas de tratamento [Gros *et al.*, 2010].

Tabela 1. Concentrações dos compostos farmacêuticos-alvo detectadas no afluente e efluente da ETAR de Febros

Composto Farmacêutico	Concentração (ng L^{-1})	
	Afluente	Efluente
Hidroclorotiazida	6.022	<58*
Paracetamol	20.074	<22
Ciprofloxacina	<6	<4
Zolpidem	<3	<2
Azitromicina	571	530
Bisoprolol	239	187
Omeprazol	-	-
Furosemida	2.675	2.430
Indapamida	<65	<57
Paroxetina	105	60
Bromazepam	<7	<6
Fluoxetina	<5	<4
Alprazolam	<5	<4
Lorazepam	<7	240
Nimesulida	<4	<3
Cetoprofeno	122	562
Naproxeno	2.583	2.748
Diazepam	<3	<3
Bezafibrato	1.348	1.797
Diclofenac	431	1.107
Ibuprofeno	1.992	1.527
Gemfibrosil	307	753
Sinvastatina	976	197

* Concentração abaixo do Limite de Quantificação do Método

No sentido de conhecer a evolução dos teores de fármacos ao longo do ciclo diário e identificar momentos críticos de descarga destes poluentes, foram realizadas análises, em separado de amostras simples de efluente da ETAR, colhidas de hora em hora, ao longo de 21h consecutivas.

Da análise dos gráficos (Fig. 4) podemos concluir que, para a generalidade dos compostos detectados nas diferentes amostras de efluente da ETAR, não existem variações significativas nas concentrações determinadas, daí que a amostragem composta seja considerada adequada e representativa. O tempo de retenção hidraúlica da ETAR permite uma homogenização do afluente e consequente atenuação de eventuais picos de concentração dos fármacos. Apenas o anti-inflamatório naproxeno e o regulador lipídico bezafibrato são indicados como <LOQ por análise da amostra composta, enquanto que foram obtidos valores quantificáveis para algumas amostras simples (máximos de 288 ng L^{-1} e 28 ng L^{-1}, respectivamente). Todavia, além destas concentrações serem bastante baixas, tratar-se-ão de casos pontuais, sem consequências significativas no que respeita à representatividade da amostragem composta.

4.2. Águas Superficiais – rios Febros e Douro

Um resultado típico obtido nas campanhas de monitorização do Rio Febros encontra-se representado na Fig. 5.

Pode constatar-se que no rio Febros a jusante do ponto de descarga da ETAR (ponto F2, Fig. 2) se verifica um aumento significativo dos níveis de bisoprolol (ca. dobro) e furosemida (ca. 6 vezes superior), enquanto que a hidroclorotiazida, azitromicina, diclofenac, gemfibrosil e

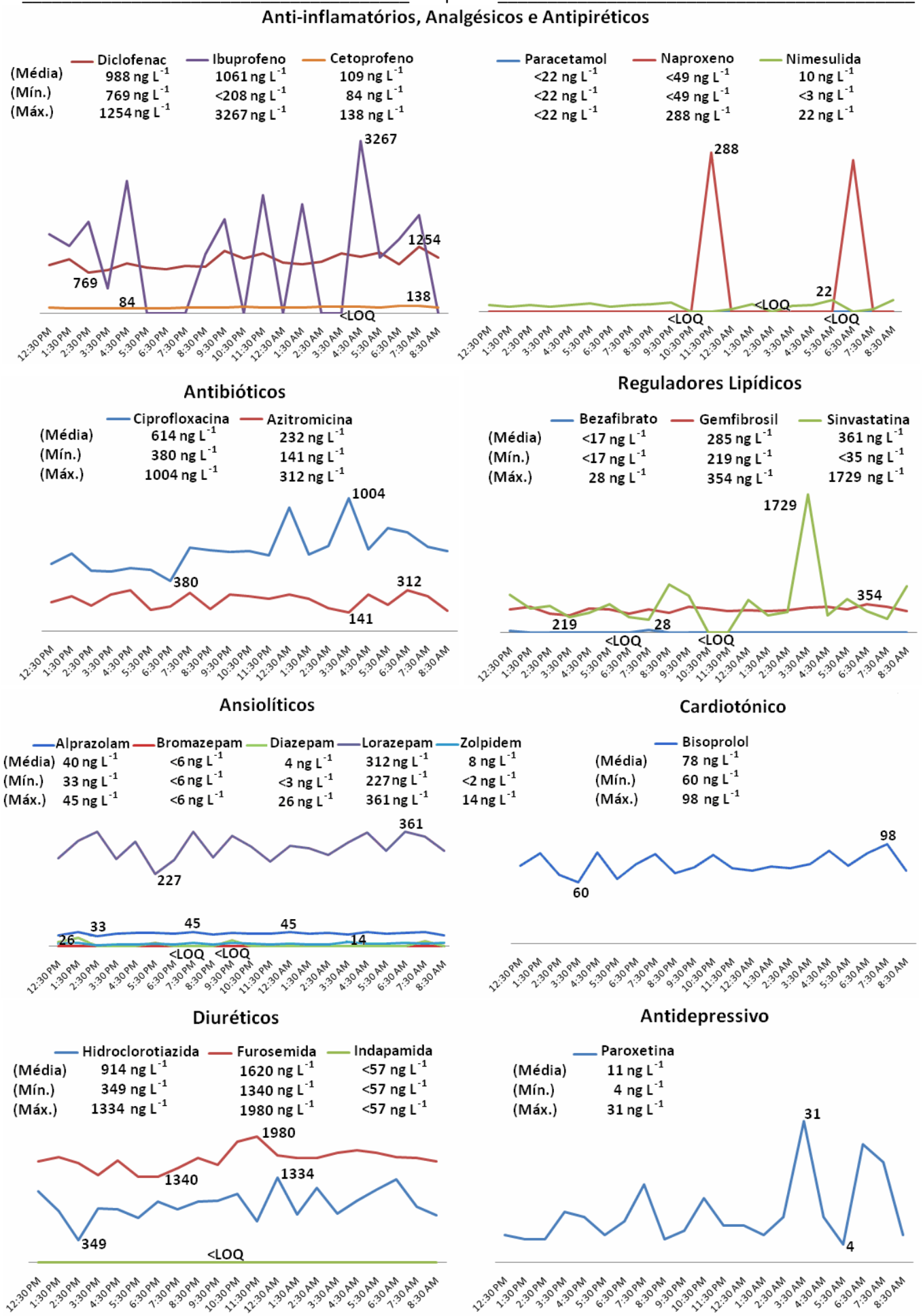


Figura 4. Amostras simples de efluente da ETAR de Febros, colhidas de hora em hora durante um período de 21h, analisadas individualmente. Os gráficos representam a variação da concentração de cada composto durante o período de colheitas. O valor médio indicado corresponde ao valor teoricamente obtido na amostra composta. LOQ – *Limit of Quantification*

Figura 5. Representação gráfica comparativa dos fármacos quantificados no Rio Febros a montante e a jusante do ponto de descarga da ETAR de Febros.

sinvastatina se tornaram quantificáveis, em concentrações entre os 40 e 360 ng L^{-1}. Tais resultados, em complemento com os já obtidos para a análise de efluente da ETAR (Tabela 1), apontam para a contribuição da ETAR na contaminação do Rio Febros com resíduos farmacêuticos. De notar que no caso do paracetamol os níveis detectados no Rio Febros a montante e a jusante da ETAR são semelhantes, indiciando a existência de outra fonte de contaminação antropogénica. È sabido que o paracetamol se degrada durante o tratmento nas ETARs.

Já relativamente ao Rio Douro, o possível impacto do Rio Febros (seu afluente, margem esquerda) no aporte de compostos farmacêuticos ao seu estuário não é visível. De facto, os resultados não revelaram a presença de qualquer um dos fármacos pesquisados acima do respectivo LOQ, provavelmente devido ao grande efeito de diluição verificado, dado o elevado caudal do Rio Douro.

Concluindo, dada a reduzida eficácia de remoção de alguns fármacos por parte da ETAR em estudo, e de outras ETARs em geral, é urgente a aplicação de processos de tratamento mais eficientes na remoção de poluentes emergentes nestas Estações de Tratamento de Água.

4.3. Tratamento do Efluente da ETAR de Febros por Fotocatálise Solar numa Planta Piloto com CPCs

Com o propósito de avaliar a aplicabilidade dos PAOs no tratamento terciário de águas residuais numa ETAR, usou-se como amostra um efluente da ETAR de Febros (Tabela 2) e seleccionou-se a fotocatálise solar com dióxido de titânio como processo de fototratamento.

Nas condições experimentais descritas (secção 3.3.), os resultados obtidos são traduzidos nos gráficos da Fig. 6.

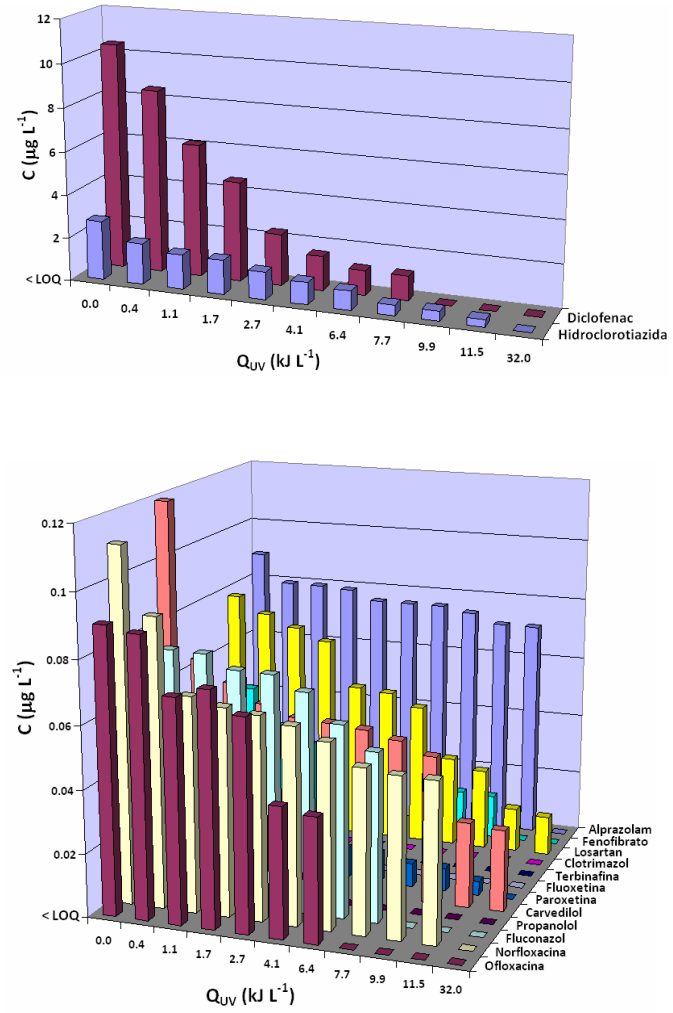
Da análise desta Figura pode concluir-se que o tratamento por fotocatálise com TiO_2 foi eficaz na remoção completa da maioria dos compostos farmacêuticos quantificados no efluente original, recolhido à saída da ETAR de Febros. Exceptuam-se os seguintes compostos: ciprofloxacina, cetoprofeno, losartan[1] e carvedilol[1], cujas concentrações no final do tratamento, embora não inferiores aos respectivos LOQ, eram já significativamente inferiores às concentrações correspondentes iniciais. Nestes casos, o processo fotocatalítico teria de ser ainda optimizado, a nível da concentração do catalisador e/ou da quantidade de energia UV acumulada total necessária.

[1]NOTA: Alguns dos compostos farmacêuticos representados na Fig. 6 não estão incluídos no método descrito na secção experimental. Todavia, a sua inclusão no método foi relativamente simples, seguindo a metodologia de optimização usada para os restantes fármacos.

Tabela 2. Caracterização físico-química da amostra de efluente da ETAR de Febros usada na experiência de fotocatálise solar

Parâmetro (unidades)	**Efluente da ETAR Febros (18.03.2011)**
Côr	n.d.* (dil. 1/20)
Cheiro	n.d. (dil. 1/20)
pH	7,3
Temperatura (º C)	20,0
Turvação (NTU)	115
Condutividade (µS cm^{-1})	555
Oxigénio Dissolvido (mg L^{-1})	2,2
Oxidabilidade (mg L^{-1})	56,2
Carbono Total Dissolvido (mg L^{-1})	37,5
Absorvância a 254 nm (UA)	0,07
Sólidos Suspensos Totais (mg L^{-1})	363
Sólidos Suspensos Voláteis (mg L^{-1})	223
Azoto amoniacal - NH_4^+ (mg L^{-1})	1,2
Nitrato - NO_3^- (mg L^{-1})	23,3
Nitrito - NO_2^- (mg L^{-1})	<0,5
Brometo - Br^- (mg L^{-1})	<0,5
Cloreto - Cl^- (mg L^{-1})	78,5
Fluoreto - F^- (mg L^{-1})	<0,5
Fosfato - PO_4^{3-} (mg L^{-1})	7
Sulfato - SO_4^{2-} (mg L^{-1})	59,8
Fósforo - ^{31}P (mg L^{-1})	3,0
Metais	
Sódio - ^{23}Na (mg L^{-1})	78,8
Potássio - ^{39}K (mg L^{-1})	21,1
Cálcio - ^{44}Ca (mg L^{-1})	274,8

* n.d. – não detectável



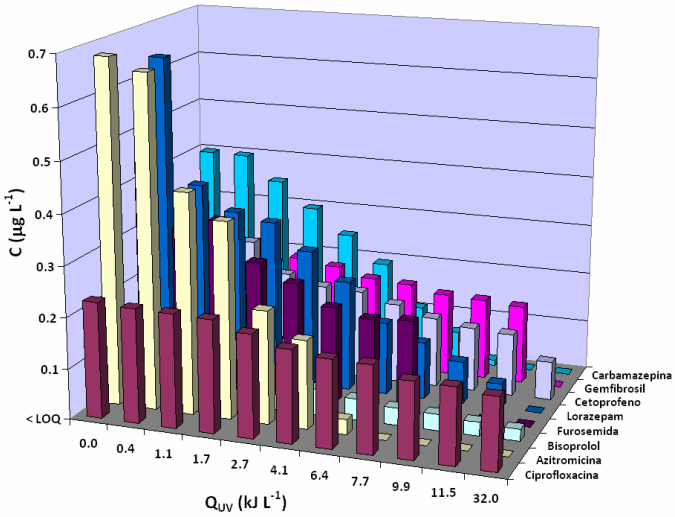


Figura 6. Representação gráfica do decréscimo de concentração dos diferentes fármacos quantificados na amostra de efluente da ETAR de Febros, sujeita ao tratamento fotocatalítico.

5. Conclusões

Do presente trabalho podemos concluir que os compostos farmacêuticos se encontram bastante disseminados no compartimento ambiental aquático, sendo as ETARs uma importante fonte de contaminação.

No caso particular da ETAR de Febros, a sua provável implicação no *input* dum conjunto de fármacos no Rio Febros foi posta em evidência através da comparação dos níveis dos vários compostos em amostras colhidas a montante e a jusante do ponto de descarga da ETAR, sendo estes superiores no segundo caso.

Já no que respeita à contribuição do Rio Febros para os níveis de compostos farmacêuticos no Rio Douro, tal não foi considerada significativa, provavelmente devido ao grande efeito de diluição resultante do elevado caudal deste rio.

Em última análise, este trabalho demonstra ainda a aplicabilidade dos PAOs, em particular da fotocatálise solar com TiO_2, como processo de tratamento terciário com potencial aplicação em ETARs (Fig. 7), surgindo como um método promissor no aumento das taxas de remoção de poluentes emergentes antropogénicos como os compostos farmacêuticos.

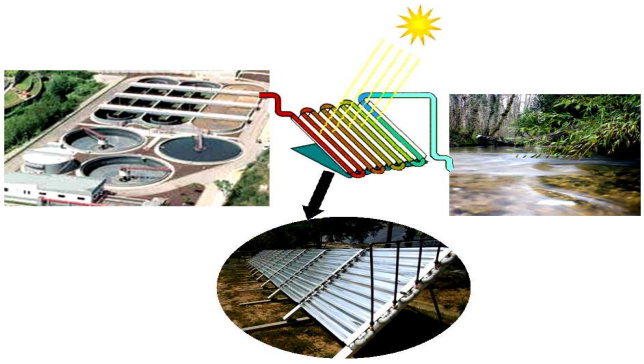


Figura 7. Representação esquemática da possível instalação de uma planta solar com CPCs numa ETAR, como processo de tratamento terciário, precedente à descarga do efluente.

Agradecimentos

O trabalho desenvolvido foi co-financiado por fundos do QREN – ON.2, O Novo Norte através do projecto NORTE-01-0162-FEDER-000023. Os autores agradecem ao IAREN e ao LSRE pelo suporte técnico e instrumental, bem como à Fundação para a Ciência e Tecnologia pela Bolsa de Doutoramento SFRH/BD/44509/2008.

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3.4. Inter-laboratory Exercises: Method Validation and Quality Control

There is currently no standardised practice or protocol for the sampling and analytical determination of pharmaceuticals in water, or any other environmental media, to ensure the comparability and quality of the data generated.

The participation in inter-laboratory exercises (ILEs) allows withdrawing information concerning the overall cycle of the sample, including its reception, analysis and data treatment, ultimately leading to a quality improvement of the results. ILEs can be carried out with three purposes: *i)* to assess the performance of an analytical method, *ii)* to evaluate the competence of a laboratory, or *iii)* to enable the certification of reference materials (25).

Nowadays, European level ILEs, regarding the analysis of frequently used pharmaceuticals in environmental waters, are still scarce. Nevertheless, some joint efforts have already been reported (26, 27).

During this Ph.D. work, we participated in two European inter-laboratory studies, the first promoted by the Joint Research Centre (JRC), a Directorate-General of the European Commission for research, innovation and science (herein addressed as ILE-*FATE_SEES*), and a second one organized under the PHARMAS EU project, by the Advanced School of Public Health (EHESP) (ILE-*PHARMAS*).

ILE-*FATE_SEES* presented as main goal the evaluation of the reproducibility of analytical methods employed in the determination of a list of emerging pollutants (pharmaceuticals included) in WWTP effluents. After 1 L sampling of municipal effluents from two WWTP located in the northern region of Portugal (WWTP from *Viana do Castelo* (VC) and WWTP from *Parada, Maia* (PM)), a certain volume of each sample was shipped to the organizing laboratory in Italy, while the remaining was analysed in our facilities, using the aforedescribed method conceived for the determination of pharmaceutical compounds in wastewaters. Only 12 out of the 23 pharmaceuticals included in our method were present in the list proposed by ILE-*FATE_SEES* organization: diclofenac, ketoprofen, naproxen, ibuprofen, bezafibrate, gemfibrozil, ciprofloxacin, bisoprolol, fluoxetine, paroxetine, alprazolam and zolpidem. Afterwards, the attained results by both laboratories were

statistically evaluated in terms of z-scores. *Z* values were calculated according to the following expression:

$$Z = \frac{X_{lab} - \mathrm{X}}{\sigma} \quad (1)$$

where:

X_{lab} = result for a laboratory;
X = mean values between all laboratories;
σ = standard deviation in the corresponding population.

The most commonly accepted classification establishes that: |z-score| < 2 – satisfactory result; 2 < |z-score| < 3 – questionable result; |z-score| > 3 – unsatisfactory result (28). In a total of 24 analytical determinations (12 pharmaceuticals for WWTP-VC and for WWTP-PM), our results included:

- ✓ 11 determinations with |z-score| < 2: satisfactory results;
- ✓ 4 determinations with 2 < |z-score| < 3: dubious results;
- ✓ 3 determinations with |z-score| > 3: unsatisfactory results;
- ✓ 6 results with pharmaceutical below the respective LOQ.

Even though, the attained results relied on the comparison between two laboratories only. Consequently, more accurate conclusions on the performance of the employed methodologies could only be withdrawn after enlarging the comprehensiveness of the study.

In the ILE-*PHARMAS* exercise, the goal was to evaluate the reproducibility of analytical methods for the measurement of antibiotics in waters (Table 3.5). It was the first initiative organised in Europe in this type of exercises, using different kind of analytical methods and devices. Fourteen laboratories from five countries (Canada, France, Italy, the Netherland and Portugal) participated and a total number of 78 samples were distributed. During the exercise, 2 testing samples (3 bottles of each) prepared from tap water (series A) and river water (series B) were spiked with antibiotics and sent to the participants to be analysed along one month.

Table 3.5. Antibiotics included in the ILE-*PHARMAS* exercise

Class of Antibiotic	Compound	Acronym	CAS number
Fluoroquinolones	Ofloxacin	OFL	83380-47-6
	Ciprofloxacin	CIP	85721-33-1
Sulfonamide	Sulfamethoxazole	SMX	723-46-6
Pyrimidin (associated to sulphonamide)	Trimethoprim	TMP	738-70-5
Macrolide	Erythromycin	ERY	114-07-8

Part of the report of this exercise is displayed in Annex III. In our laboratory, therein identified as Lab. 5, both sample series A and B (tap water and river water, respectively – 3 replicates each) were analysed using the aforedescribed method primarily developed for surface waters, i.e., beginning with 200 mL sample acidification to pH 2, followed by an optional cleanup step using *Bakerbond 1º,2º-Amino* cartridges and subsequent solid-phase extraction with *JTBaker H₂Ophilic* extraction columns (23). Chromatographic and mass spectrometric conditions were optimised for OFL, SMX and TMP, and further included in the analytical method already comprising CIP, whereas ERY was not analysed at our laboratory, due to the unavailability of analytical standard.

The concentrations determined in the 3 replicates of each sample series, A and B, are presented in Tables 3.6 and 3.7.

Table 3.6. Concentration (ng L^{-1}) of antibiotics determined in the three replicates of sample series A (spiked tap water). Each extract was injected twice for LC-MS/MS analysis

Antibiotics	Extraction 1		Extraction 2		Extraction 3	
	Injection 1	Injection 2	Injection 1	Injection 2	Injection 1	Injection 2
Erythromycin	n.a.*	n.a.	n.a.	n.a.	n.a.	n.a.
Ciprofloxacin	<33	<33	<33	<33	<33	<33
Ofloxacin	41.878	38.478	41.611	37.170	45.051	37.958
Sulfamethoxazole	15.766	13.261	-	-	11.517	12.508
Trimethoprim	31.845	32.998	32.659	36.364	33.525	39.222

* n.a. – not analysed

Table 3.7. Concentration (ng L^{-1}) of antibiotics determined in the three replicates of sample series B (spiked river water). Each extract was injected twice for LC-MS/MS analysis

Antibiotics	Extraction 1		Extraction 2		Extraction 3	
	Injection 1	Injection 2	Injection 1	Injection 2	Injection 1	Injection 2
Erythromycin	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Ciprofloxacin	135.034	127.366	121.843	110.450	133.801	154.607
Ofloxacin	259.210	226.503	231.983	209.912	191.808	212.723
Sulfamethoxazole	74.250	86.567	71.857	82.690	68.715	78.851
Trimethoprim	135.034	127.366	121.843	110.450	133.801	154.607

* n.a. – not analysed

The attained z-scores of all participants are illustrated in Fig. 3.7.

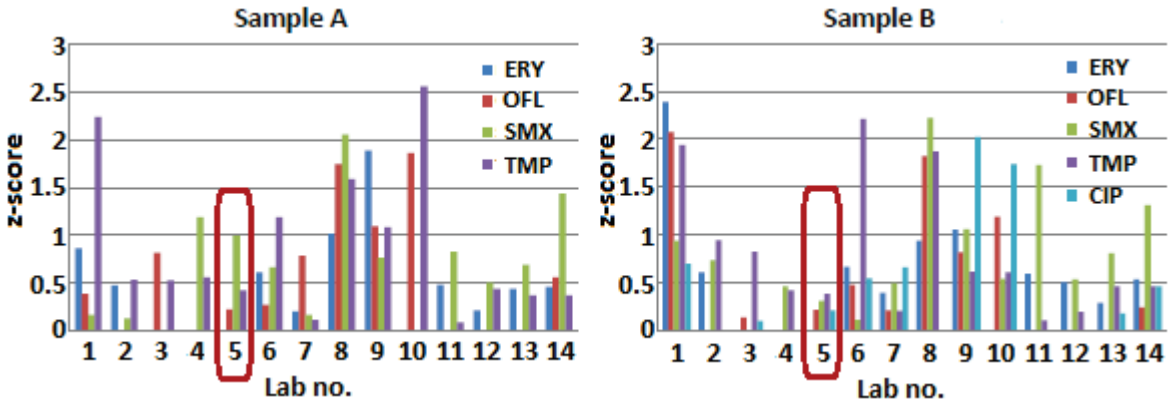

Fig. 3.7. Z-score absolute values (outliers excluded). Sample A – spiked tap water; sample B – spiked river water.

All our results fell in the satisfactory z-score range, which demonstrates the reliability of the developed procedure for monitoring pharmaceuticals in water samples.

In conclusion, ILEs constitute a clear strategy to demonstrate analytical capability (29), which is fundamental to obtain good quality data for future inclusion in framework directives.

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Chapter 4

Photo-Remediation of Contaminated Waters using Advanced Oxidation Processes (AOPs)

Up till this moment, the work herein described consisted on: *i)* the development, optimization and validation of the cleanup–SPE–HPLC-tandem MS multiresidue method for detection and quantification of pharmaceutical compounds in waters (Chapter 2); *ii)* its further application to the analysis of contaminated water samples collected over monitoring campaigns and acquired during inter-laboratory exercises (ILEs) (Chapter 3). Hence, it came the time to tackle the study of remediation procedures aiming for water decontamination. This study resulted in the publication of **Paper 3** and **Paper 4** (please refer to the end of Section 4.2).

As previously mentioned, wastewater treatment plants (WWTPs) are considered the major source of environmental pollution by pharmaceuticals, since the employed conventional treatment processes are inefficient for their complete removal or degradation (1). Once in the environment, such micropollutants can undergo a series of chemical reactions, mainly divided into four types: biotics, photochemicals, hydrolysis and oxidation/reduction (2).

Photochemical processes result from the absorption of the energy contained in solar radiation by organic molecules. This is influenced by factors such as chemical structure, latitude, season and day time. When a compound undergoes a chemical transformation in result of the direct absorption of solar radiation, the process is called direct photolysis. On the other hand, if the photodegradation occurs by means of another compound (e.g., photosensitive natural compounds such as nitrates and humic acids), that absorbs the energy, originating reactive species which will in turn react with the first compound, then the process is entitled indirect photolysis (Fig. 4.1) (2). These are the main processes involved in the degradation of pharmaceutical compounds on shallow surface waters.

However, total mineralization is far from being achieved. Thus, there is an absolute need to develop effective methods for handling these contaminants and potentially toxic compounds, widely dispersed in the environment.

Conventional drinking water decontamination processes involve the combination of flocculation, filtration, sterilization and conservation, with the addition of a limited number of chemicals. On the contrary, normal human sewage water is usually treated in conventional biological processing plants. Despite the fact that biological treatment techniques are well established and relatively cheap, they are often susceptible to toxic compounds which inactivate the degrading microorganisms, thus becoming unable to remove the contaminants. In order to solve this issue, besides reducing emissions, a chemical treatment of contaminated drinking, surface and groundwaters should be pursued, as well as a chemical treatment of wastewaters containing biocides or non-biodegradable compounds (3).

Addressing this problem has recently recalled a tremendous amount of research in the field of robust new water treatment technologies, leading to the complete mineralization of organic pollutants at lower cost and with less energy, while at the same time minimizing the use of chemicals and their subsequent impact on the environment (4). Special attention has been given to advanced oxidation treatment processes.

4.1. Advanced Oxidation Processes

4.1.1. Definition

Advanced oxidation processes (AOPs) are defined as oxidation methods occurring in the aqueous phase and based upon the generation of highly reactive species such as hydroxyl radicals (•OH). These radicals present a high oxidation potential (E_0 = 2.80 V relatively to the normal hydrogen electrode) and are responsible for the oxidation and mineralization of almost any organic molecule, yielding CO_2, H_2O and inorganic ions as final products, due to their strong, unselective oxidative power. Thus, AOPs can be used to chemically decompose pollutants into harmless end-products, otherwise not removed by conventional treatment processes (3, 5). Moreover, these oxidation processes have the increased interest of taking place at room temperature.

Nevertheless, there are species such as CO_3^{2-}, HCO_3^-, SO_4^{2-}, Cl^- and humic acids, which can act as free radicals' inhibitors and significantly reduce the degradation process efficiency, depending on their concentration in the medium. For that reason, some pre-treatment may be required to reduce these species concentration or even eliminate them. In case the treatment process is occurring under acidic conditions, the problem concerning the presence of carbonates or bicarbonates is automatically solved (6).

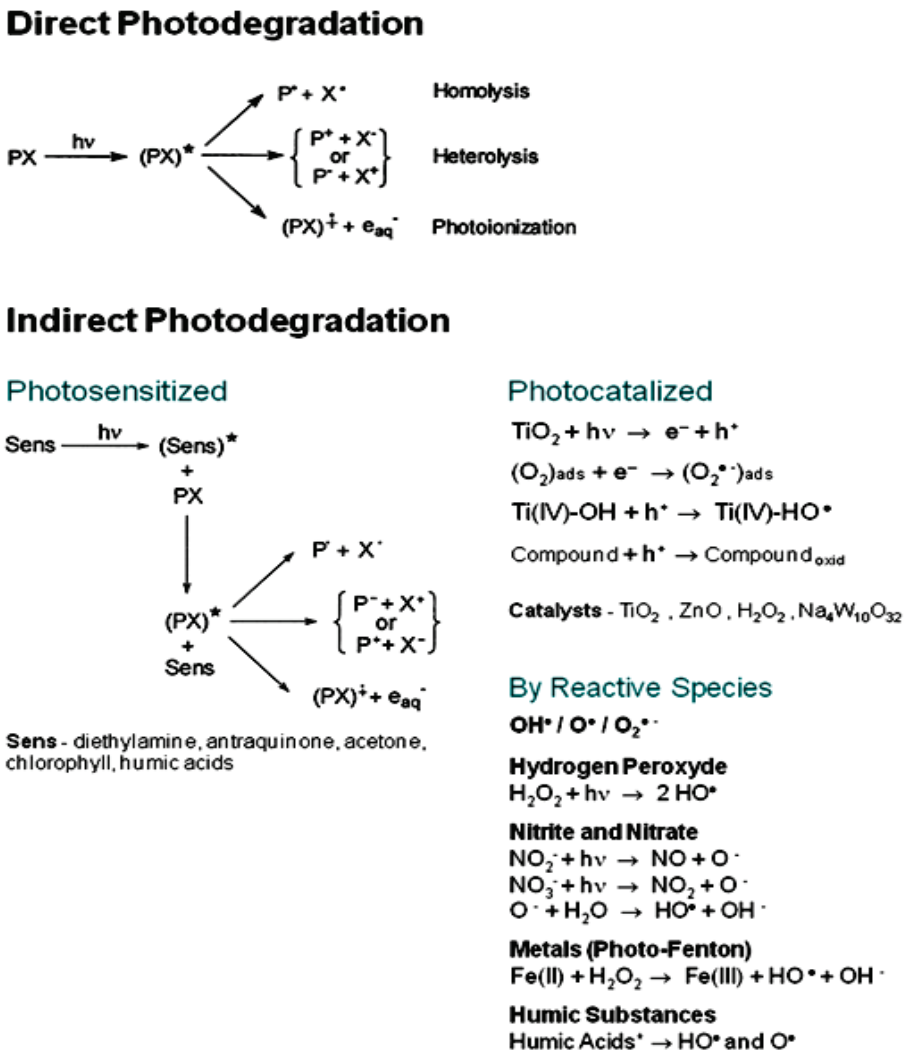

Fig. 4.1. Reactions involved in the processes of direct photolysis, indirect photolysis, heterogeneous photocatalysis and formation of oxygen radical reactive species (*adapted from Burrows et al.* (2)).

4.1.2. Classification and Applications to Water Treatment

According to the *number of phases of the reaction system*, AOPs can be divided in **homogeneous** (single-phase system) or **heterogeneous** (more than one phase system) processes. Moreover, they can also be classified in compliance with the *method used to generate the reactive radical species* as **chemical**, **photochemical**, **electrochemical**, **sonochemical** or **thermochemical** (Fig. 4.2).

HOMOGENEOUS

CHEMICAL	**Ref.**
Ozone in basic medium (O_3/OH^-)	(7)
Ozone and hydrogen peroxide (O_3/H_2O_2) and ($O_3/H_2O_2/OH^-$)	(8)
Homogeneous catalytic ozonation (O_3/catalyst)	(9)
Fenton (H_2O_2/Fe^{2+})	(10)

PHOTOCHEMICAL	**Ref.**
Ozone and ultraviolet radiation (O_3/UV)	(11)
Hydrogen peroxide and ultraviolet radiation (H_2O_2/UV)	(12, 13)
Ozone, hydrogen peroxide and ultraviolet radiation (O_3/H_2O_2/UV)	(14)
Photo-Fenton (H_2O_2/ Fe^{2+}/UV)	(15)

ELECTROCHEMICAL	**Ref.**
Electrochemical oxidation	(16)
Electro-Fenton and photo-electro-Fenton	(17, 18)

SONOCHEMICAL	**Ref.**
Ultrasounds (US)	(19)
Ozone and ultrasounds (O_3/US)	(20)
Hydrogen peroxide and ultrasounds (H_2O_2/US)	(21)

THERMOCHEMICAL	**Ref.**
Wet air oxidation (WAO)	(22)
Supercritical wet air oxidation (SWAO)	(23)
Catalytic wet air oxidation (CWAO)	(24, 25)

HETEROGENEOUS

CHEMICAL	**Ref.**
Heterogeneous catalytic ozonation (O_3/catalyst)	(26, 27)

PHOTOCHEMICAL	**Ref.**
Heterogeneous photocatalysis (TiO_2/UV), ($TiO_2/UV/H_2O_2$) and (O_3/TiO_2/UV)	(12, 28, 29)

Fig. 4.2. AOPs classification, possible combinations and some relevant references.

Many AOPs have recently earned great interest in the field of water decontamination processes, each one presenting its own advantages and disadvantages. Polluted waters vary from industrial effluents with different origins (e.g., agriculture, textile, tannery, etc.), to hazardous effluents from hospitals or pharmaceuticals production facilities, or even to municipal WWTP effluents contaminated with pathogenic agents and recalcitrant pollutants, such as pharmaceuticals and endocrine disrupting compounds (7, 30, 31).

In particular, over the last few decades there has been a paramount increase of studies concerning the use of photochemical AOPs for the effective and sustainable treatment of waters contaminated with pharmaceutical compounds, as one can observe in Fig. 4.3. Indeed, owing to some technological/engineering developments, this growth may also have been propelled by the possibility of using solar radiation as the photon source.

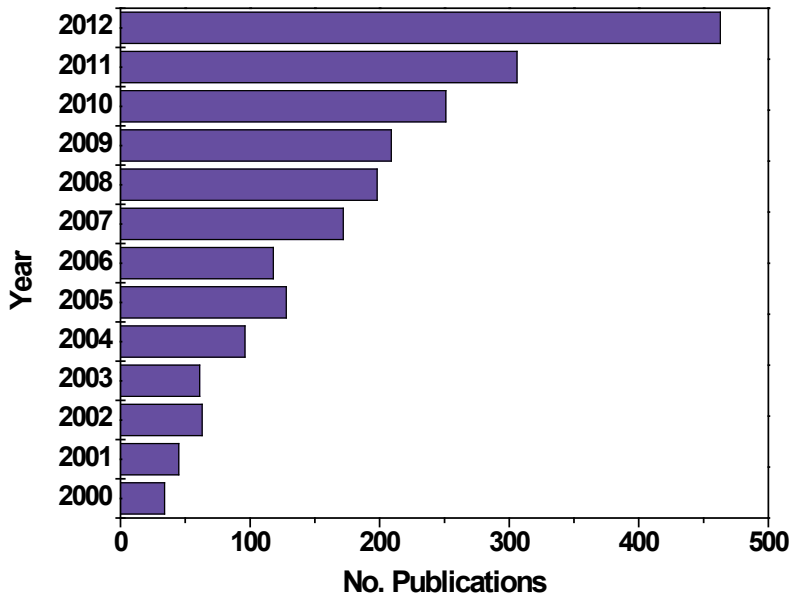

Fig. 4.3. Number of publications relative to photodegradation treatment processes of waters contaminated with pharmaceutical compounds, between 2000 and 2012 (*source: www.sciencedirect.com, using "photodegradation" and "pharmaceuticals" and "water treatment" as keywords*).

Amongst all photochemical oxidation processes that could be chosen, in the herein described Ph.D. work we opted for the heterogeneous photocatalysis, using titanium dioxide (TiO_2) as catalyst (**Paper 3** and **Paper 4**).

4.1.2.1. Heterogeneous Photocatalysis

4.1.2.1.1. Fundamentals

In a photochemical process, light is always a reagent, never a catalyst. The term photocatalysis implies the combination of photochemical process with the use of a catalyst, i.e., it is the "acceleration of a photoreaction by the presence of a catalyst" (3). In the case of heterogeneous processes, the catalyst employed is a semiconductor material like metal oxides.

When a semiconductor (SC) is exposed to radiation with higher energy than the band gap between its valence band (VB – filled electronic band with the highest energy) and its conduction band (CB - empty electronic band with the lowest energy), there is an electron transfer from VB to CB, resulting in the formation of a hole in the valence band (h^+):

$$SC + h\nu \rightarrow SC\,(h^+_{VB} + e^-_{CB}) \quad (1)$$

If the semiconductor is in contact with a liquid electrolyte solution containing redox couple, the formed electron/hole (e^- and h^+) pairs migrate to the surface of the semiconductor particle and charge transfer occurs across the interface, in order to balance the potentials of the two phases (3, 32).

Hydroxyl radicals can be formed by two ways: the valence band hole (h^+_{VB}) can either react with the adsorbed water or the surface OH^- groups on the semiconductor particle:

$$SC\,(h^+_{VB}) + H_2O_{ads} \rightarrow SC + HO^\bullet_{ads} + H^+ \quad (2)$$

$$SC\,(h^+_{VB}) + OH^-_{ads} \rightarrow SC + HO^\bullet_{ads} \quad (3)$$

The semiconductor is recovered when acceptor molecules (A) such as O_2 are adsorbed and react with an electron in the conduction band, while donor molecules (D) such as H_2O are adsorbed and react with a hole in the valence band:

$$SC\,(e^-_{CB}) + A_{ads} \rightarrow SC + A^-_{ads} \quad (4)$$

$$SC\,(h^+_{VB}) + D_{ads} \rightarrow SC + D^+_{ads} \quad (5)$$

It is widely accepted that O_2 plays an important role in these reactions. O_2 can trap conduction band electrons to originate superoxide radicals ($O_2^{\bullet -}$) (Eq. 6). These radicals can react as well with hydrogen ions (H^+) from water splitting process to form $HO_2^{\bullet -}$.

$$SC\ (e^-_{CB}) + O_{2\ ads} + H^+ \rightarrow SC + HO_2^{\bullet -} \rightarrow O_2^{\bullet -} + H^+ \qquad (6)$$

H_2O_2 can also be formed from $HO_2^{\bullet -}$ species:

$$SC\ (e^-_{CB}) + HO_2^{\bullet -} + H^+ \rightarrow H_2O_2 \qquad (7)$$

and hydroxyl radicals may be originated by cleavage of H_2O_2 via one of the following reactions:

$$H_2O_2 + h\nu \rightarrow 2\ HO^{\bullet} \qquad (8)$$

$$H_2O_2 + O_2^{\bullet -} \rightarrow HO^{\bullet} + O_2 + HO^- \qquad (9)$$

$$H_2O_2 + SC\ (e^-_{CB}) \rightarrow HO^{\bullet} + HO^- + SC \qquad (10)$$

Finally, highly reactive hydroxyl radicals will act as oxidants in the degradation of organic compounds (33, 34). A resume of the photocatalytic process detailed by Eqs. (1)-(10) is illustrated in Fig. 4.4.

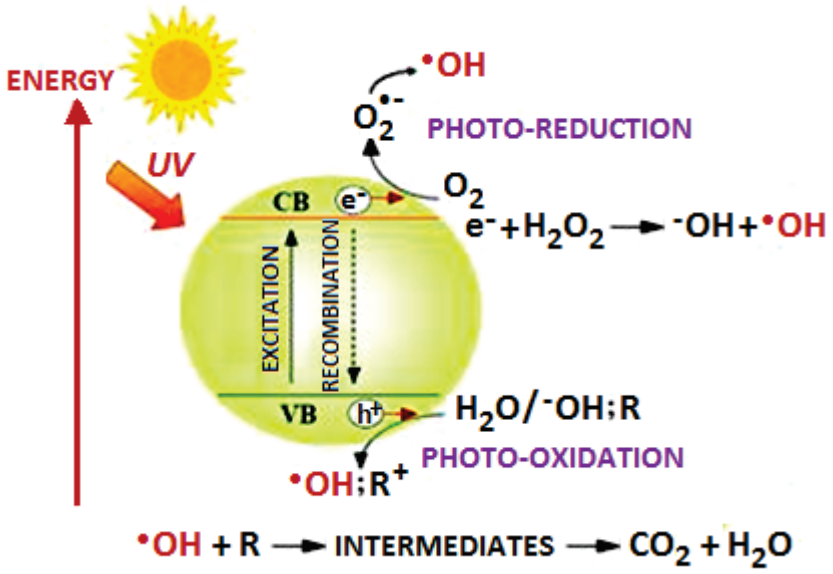

Fig. 4.4. Schematic diagram illustrating the principle of heterogeneous photocatalysis (*adapted from Ahmed et al.* (33)).

4.1.2.1.2. Catalysts

By definition, a catalyst remains unaltered and can be recovered unchanged after many turnovers of the reaction process. In the case of photocatalytic treatment processes, metal oxides and sulphides constitute a large class of suitable semiconductor materials (Table 4.1.).

Table 4.1. Properties of some semiconductor materials (*from Galvez et al.* (3))

Material	Band gap (eV)	Wavelength corresponding to band gap (nm)
$BaTiO_3$	3.3	375
CdO	2.1	590
CdS	2.5	497
CdSe	1.7	730
Fe_2O_3	2.2	565
GaAs	1.4	887
GaP	2.3	540
SnO_2	3.9	318
$SrTiO_3$	3.4	365
TiO_2	3.0	390
WO_3	2.8	443
ZnO	3.2	390
ZnS	3.7	336

Titanium dioxide (TiO_2; white powder; BGE = 3.0 eV) is one of the most commonly used catalysts. As aforementioned, it was also the catalyst chosen for our experimental work (**Paper 3** and **Paper 4**) due to its high reactivity, low toxicity, low price, chemical stability and, above all, its capability of using natural and renewable solar UV energy ($\lambda \leq$ 390 nm). TiO_2 has an adequate energetic separation between the valence and the conduction bands, which can be surpassed by the energy content of a solar photon, thus reducing the cost associated with UV radiation production (3, 35). More specifically, we opted for TiO_2 P25-Degussa (80% anatase and 20% rutile), which is as well the most frequently employed on photocatalytic tests. Anatase and rutile are two distinct crystalline forms of TiO_2, presenting a density of 3.9 and 4.2 g cm^{-3}, respectively.

Titanium dioxide can be used in suspension or, alternatively, immobilized in different supports, such as glass spheres, Teflon or glass fibre. Though in our work TiO_2 was employed in suspension, the immobilized form has proved to be quite efficient, mainly at the industrial scale, where suspended TiO_2 has revealed some problems concerning its separation and regeneration. Due to the small particle dimensions (usually < 0.5 μm), the filtration process becomes rather expensive, thus hindering its application (36).

Studies on the use of this catalyst have also showed that its efficiency can be improved by either increasing the active surface area, redimensioning it to the nanoscopic scale or through the addition of some co-adsorbent materials (e.g., silica, alumina, zeolites or activated carbon) (37-39).

4.1.2.1.3. Variables affecting Photocatalysis

The kinetic behaviour of the photocatalytic degradation reactions of organic pollutants in general, and pharmaceutical compounds in particular, depends on several experimental parameters, which shall be optimized in order to maximize the process efficiency. Such parameters include: *i)* contaminant initial concentration; *ii)* concentration of the catalyst; *iii)* radiation source; *iv)* pH of the reaction medium; *v)* temperature; *vi)* dissolved organic matter (DOM); *vii)* dissolved O_2; *viii)* presence of sensitizers and radical scavengers (3, 40).

i) Influence of Contaminant Initial Concentration

Most authors agree that, for the majority of the contaminants, the influence of the initial concentration of substrate on the photocatalytic degradation rate follows a pseudo-first order kinetic, according to the Langmuir-Hinshelwood (L-H) law.

The photocatalytic reaction rate (r) is proportional to the catalyst covered surface fraction (θ), according to Eq. (11):

$$r = k\theta = k\left(\frac{KC}{1+KC}\right) \qquad (11)$$

where *k* represents the pseudo-first order kinetic constant, *C* the initial concentration of the substrate and *K* the constant of the adsorption balance of substrate at the surface of the catalyst (41).

As oxidation proceeds, less and less surface of TiO_2 particles is covered, as the contaminant is degraded. At total decomposition, the degradation rate equals zero and a decreased photocatalytic rate is to be expected with the increase of accumulated UV energy.

ii) Influence of Catalyst Concentration

The concentration of TiO_2 which maximizes the reaction rate is that corresponding to a maximal particle surface exposed to radiation. When the catalyst concentration is too high, turbidity also rises and hampers the further penetration of light in the reactor. On the other hand, by increasing the number of suspended particles, the probability they agglomerate is higher, leading to a decrease of surface area available for the reaction (3, 41, 42).

In every practical application, the optimum catalyst load must be determined to prevent its excess and ensure the maximal absorption of efficient photons. Therefore, in our experimental work (**Paper 3**), some preliminary tests were conducted comprising a wide range of TiO_2 concentrations (from absence, corresponding to photolysis, to 400 mg L^{-1}), according to the set of different experimental systems employed.

iii) Influence of Radiation Source

The radiation source can affect photocatalysis kinetic according to three factors: the radiation *photon flux,* the radiation *wavelength* and the *quantum yield*.

Regarding the *photon flux*, an increase of radiation intensity generally results in the improvement of the photocatalytic reaction rate, i.e., in the proportional increase of the substrate degradation rate. However, above a certain threshold, the reaction rate becomes independent of the radiation flux, which can be attributed to the recombination of the electron/hole pairs at high radiation intensities (3, 32).

Concerning the radiation *wavelength*, and according to what was previously explained, the adsorption spectrum of the catalyst will condition the useful wavelength range for a certain photocatalytic reaction. In our case-study, TiO_2 just absorbs radiation with $\lambda \leq 390$ nm (please refer to Table 4.1), thus being photo-activated only in the UV spectral region.

As regard to the *quantum yield*, its influence on the photocatalytic process will be later assessed on Section 4.2.2.1.4.

In our initial work (**Paper 3**), two different radiation sources were comparatively tested and optimized: an UV medium pressure mercury lamp (Heraeus TQ 150W) vs. natural solar radiation.

iv) Influence of pH

It is well known that TiO_2 surface is amphoteric and, consequently, its charge is pH-dependent. For TiO_2-P25, used in our work, the zero charge point (pH_{zcp}) is located in the range $5.6 < pH < 6.4$ ($pH \approx 6.3$) (42). For that reason, at lower pH values TiO_2 surface is positively charged, as represented in the following equation:

$$TiOH + H^+ \leftrightarrow TiOH_2^+ \quad (12)$$

On the contrary, if the medium pH is above TiO_2 pH_{zcp}, then the catalyst surface will be negatively charged:

$$TiOH + HO \leftrightarrow TiO^- + H_2O \quad (13)$$

In conclusion, pH will affect both the ionization state of the substrate and TiO_2 surface, thus promoting or hampering the adsorption of organic compounds to the catalyst surface in case they present different or equal charges, respectively. Moreover, it must be highlighted that, under acidic conditions, TiO_2 particles are prone to agglomerate, which will lead to a reduction of active surface area available for the absorption of radiation and adsorption of contaminants.

v) Influence of Temperature

Photocatalytic systems do not require heating and can operate at room temperature, owing to the photonic activation.

The true activation energy (E_t) is null, while the apparent activation energy (E_a) is often very low (a few kJ mol^{-1}) for temperatures in the range of 20°C to 80°C. However, at very low temperatures (-40°C to 0°C), activity decreases and the apparent activation energy increases. On the contrary, at high temperatures (>70-80°C) approaching the water boiling point, the activity decreases for several types of photocatalytic reactions and the apparent activation energy becomes negative (3).

Summing up, the optimal temperature range for photocatalytic processes is located between 20 and 80°C.

vi) Influence of DOM

Apart from contaminants, water samples may contain other dissolved natural organic matter, like humic substances (HS – a class of naturally existing biogenic heterogeneous organic substances, with high molecular weights) (43). These substances will compete for the oxidizing reactive species, such as hydroxyl radicals, and consequently decrease the removal rate of target polluting compounds. The amount of dissolved organic matter is indirectly given by determining the dissolved organic carbon (DOC).

vii) Influence of Dissolved Oxygen

Oxygen is necessary for achieving complete mineralization at the end of photodegradation treatments. Also, it does not seem to compete with other reactives for adsorption onto TiO_2 particles, since oxidation and reduction *loci* are separate. In addition, oxygen may prevent as well the electron/hole recombination process, taking place after TiO_2 irradiation, through the acceptance of the electrons originating from the conduction band.

Oxygen concentration also affects the reaction rate: the photocatalytic reaction occurs faster when oxygen partial pressure increases, and vice-versa. In any case, the difference is not drastic (3).

viii) Influence of Sensitizers vs. Radical Scavengers

Besides the oxidation/reduction reactions taking place at the surface of the catalyst particles, highly reactive radical species can have a different origin. As aforementioned, it is known that nitrates, humic and fulvic acids, often present in fresh waters, may act as sensitizers, giving rise to reactive species such as hydroxyl radicals, singlet oxygen or hydrogen peroxide, which promote indirect photolysis (Fig. 4.1) (44, 45).

On the contrary, there are other substances which can act as radicals' quenchers or scavengers, thus reducing the photocatalytic process efficiency (e.g., CO_3^{2-}, HCO_3^-, SO_4^{2-}, Cl^-) (46).

4.1.2.1.4. Photochemical Reactors

Another major factor affecting waters photocatalytic treatment is the choice of the photochemical reactor. Here, several aspects have to be taken into consideration, including the material structure, the reactor geometry and the system for temperature control.

In our work, three distinct experimental systems were used (**Paper 3**, **Paper 4** and **Paper 5**). The first lab-scale experimental apparatus consisted of a glass immersion photochemical reactor with a water column of 8 cm diameter and 16 cm height. The reactor was loaded with 850 mL of solution, with constant stirring for the total reaction period. It was equipped with a medium pressure mercury lamp Heraeus TQ 150W, with a dominant emission line at 366 nm, placed axially and held in a quartz immersion tube with a surface contact area of ~233 cm^2 - *LsTQUV* (Lab-scale TQ150W-UV prototype) (Fig. 4.5). This system was refrigerated by a continuous tap water flow, which allowed temperature to be properly controlled below 30 ºC.

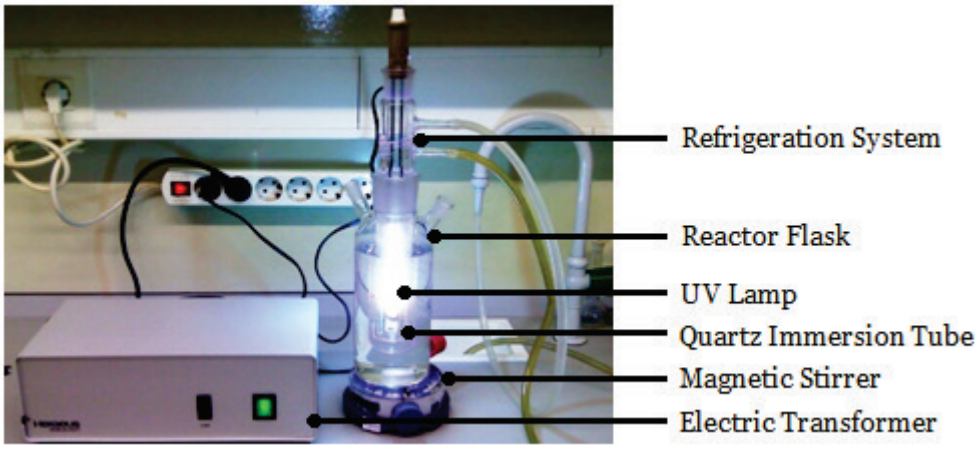

Fig. 4.5. Lab-scale photochemical reactor equipped with a medium pressure mercury lamp Heraeus TQ150W (*LsTQUV*).

The second employed lab-scale prototype was composed by a 5 L beaker, loaded with 4.5 L of solution constantly stirred, and an immersed pyrex glass cylinder placed axially, holding a LYNX S11W blacklight blue lamp with maximal emission at 365 nm. The surface contact area between the pyrex cylinder and the solution was approximately 456 cm^2. The water column was 23.5 cm high with 11 cm diameter - *LsBLBUV* (Lab-scale Blacklight Blue-UV system) (Fig. 4.6).

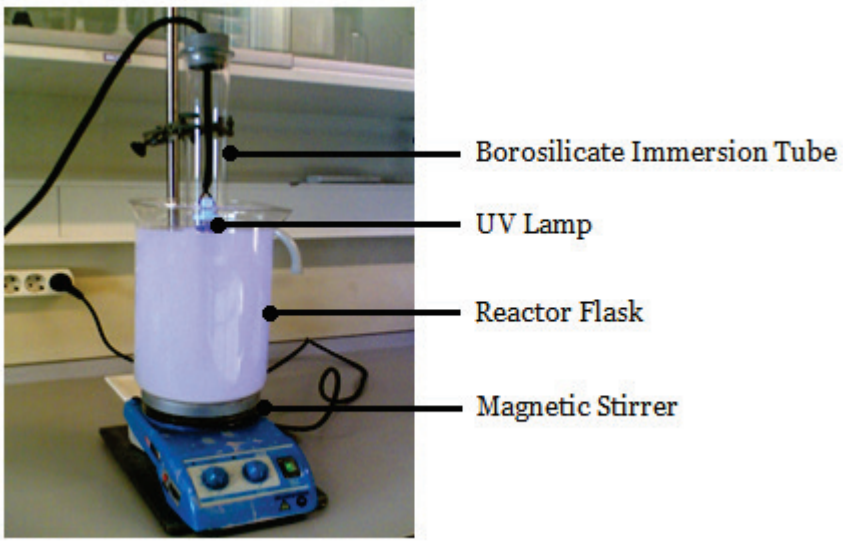

Fig. 4.6. Lab-scale photochemical reactor equipped with a LYNX S11W blacklight blue lamp (*LsBLBUV*).

Furthermore, photocatalytic experiments were also performed, during sunny days, in a Solar Pilot Plant with Compound Parabolic Collectors (CPCs) installed in the roof of the Chemical Engineering Department of the Faculty of Engineering, University of Porto (FEUP), Portugal. This CPC solar collector (0.91 m^2) was composed of four borosilicate tubes (Schott-Duran type 3.3, Germany, cut-off at 280 nm, external diameter 50 mm, length 1500 mm and thickness 1.8 mm) connected in series by polypropylene junctions with their CPC mirrors in anodized aluminum, supported by an aluminum structure and tilted 41º (local latitude) - *SPP-CPCs* (Solar Pilot Plant with CPCs) (Fig. 4.7). The pilot plant has also two recirculation tanks (10 L and 20 L), two recirculation pumps (maximum 20 L min^{-1}), two rotameters, five polypropylene valves and an electric board for process control. It operates in batch mode and can be used in two ways: using the total CPCs area (0.91 m^2) or using 0.455 m^2 of the CPCs area individually, giving the possibility to carry out two distinct experiments at the same time and at the same solar radiation conditions.

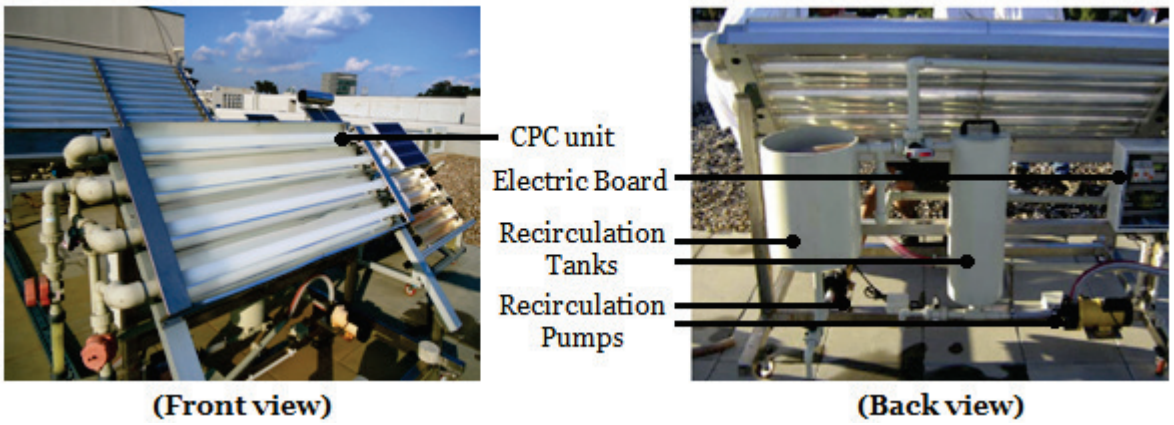

Fig. 4.7. Solar Pilot Plant with CPCs (*SPP-CPCs*).

First of all, and regarding both lab-scale systems, it must be noticed that while one immersion tube was made of quartz (*LsTQUV*), the other one had a borosilicate composition (*LsBLBUV*). This feature is of extreme importance, taking into account the radiation spectral region able to photo-activate TiO_2 ($\lambda \leq 390$ nm). As displayed in Fig. 4.8, quartz transmittance is higher than 80% even for wavelengths as low as ~250 nm. On the other hand, UV radiation does not cross common borosilicate glass. Therefore, the borosilicate used in these experiments had a special treatment, in order to present a cut-off at 280 nm. For the same reason, this borosilicate glass (Schott-Duran type 3.3, Germany, cut-off at 280 nm) was also used for the CPC units of the solar pilot plant (*SPP-CPCs*).

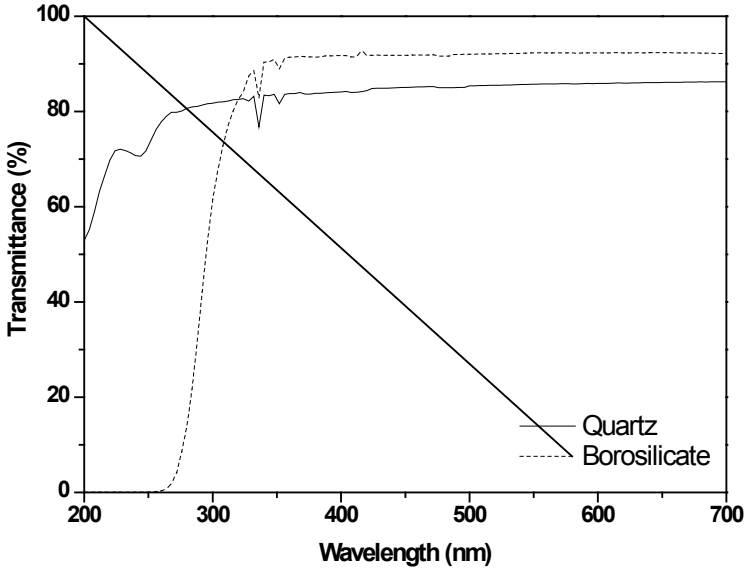

Fig. 4.8. Transmittance spectra of quartz and borosilicate (Schott-Duran type 3.3, Germany, cut-off at 280 nm).

The **photocatalytic performance of a heterogeneous system** can be evaluated by the *quantum yield* (quantum efficiency) of the reaction or through its *apparent kinetic constant*.

The *quantum yield* (Φ) is defined as the ratio between the number of reacting molecules (Δn) and the quantity of photons absorbed by the system (N_a):

$$\Phi = (\Delta n / N_a) \qquad (14)$$

Experimentally, the quantum yield represents the number of radicals originated at the surface of the catalyst by each absorbed photon, i.e., the number of molecules degraded by each photon. If every absorbed photon induces a molecular transformation, then $\Phi = 1$; if $\Phi < 1$, it means there are deactivation processes or other reactions competing with the target one; if $\Phi > 1$, then it indicates there is taking place a series of reactions whose promoter has been excited by a photon (3).

The experimental determination of the quantum yield is rather difficult, forcing the application of indirect methods, which consequently bring in some error.

Apparent kinetic constants constitute another way to assess photocatalysts' efficiency for the degradation of organic compounds, since the great majority of the reactions follow a pseudo-first order kinetic model:

$$C_t = Co\ e^{-kapp\ t} \qquad (15)$$

where C_t is the concentration of the substrate at different reaction times (t), Co is the initial substrate concentration for t = 0 (the instant when illumination is turned on) and $k$app is the apparent pseudo-first order kinetic rate constant (47).

Nonetheless, in order for comparison between artificial UV sources (lamps) and natural solar UV radiation to be possible (**Paper 3**), time (t) was replaced by another parameter: the accumulated UV energy (Q_{UV}, kJ L^{-1}).

In the case of the medium pressure mercury lamp Heraeus TQ150W, with the purpose of calculating the accumulated UV energy as a function of time, it was first necessary to have knowledge of its photon flux. The UV lamp photon flux was determined at different wavelengths with the use of the Hatchard-Parker actinometer (47, 48), one of the most reliable and practical actinometers for UV and visible light up to 500 nm. Under light excitation the potassium ferrioxalate decomposes, according to the following equations:

$$Fe(C_2O_4)_3^{3-} \xrightarrow{h\nu} Fe^{2+} + C_2O_4^{\bullet -} + 2C_2O_4^{2-} \qquad (16)$$

$$Fe(C_2O_4)_3^{3-} + C_2O_4^{\bullet -} \xrightarrow{\Delta} Fe^{2+} + 2CO_2 + 3C_2O_4^{2-} \qquad (17)$$

The quantity of ferrous ions formed during the irradiation period is monitored by conversion to the coloured trisphenanthroline complex (ε = 11,100 L mol^{-1} cm^{-1} at λ_{max} = 510 nm) (Fig. 4.9). The original ferric ions are not appreciably complexed by phenanthroline and the complex does not absorb at 510 nm.

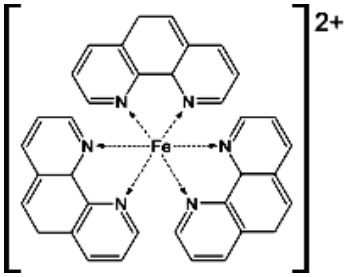

Fig. 4.9. Chemical structure of the complex Fe^{2+} -1,10-phenanthroline (ratio 1:3).

However, it should be noticed that the lamp photon flux (Einstein s^{-1}) corresponds to its total emission spectrum, whereas only radiation with $\lambda \leq 390$ nm is capable of photo-activating TiO_2. Thus, bearing in mind the main emission line intensities up until 390 nm, partial photon fluxes were calculated for each wavelength and the corresponding radiant energy ($E_{\leq 390\ nm}$, J) was determined according to Eq. (18):

$$E = N_A h \frac{c}{\lambda} \tag{18}$$

where E is the energy of electromagnetic radiation, N_A is the Avogadro constant (6.022E23 mol^{-1}), h is the Planck constant (6.626E-34 J s), c is the speed of light in the vacuum (3E8 m s^{-1}) and λ is the respective wavelength. The radiant flux (also known as radiant power) obtained was 15 J s^{-1}, i.e., 15 W.

Afterwards, calculations regarding the accumulated UV energy ($Q_{UV,n}$) took into account the lab-scale photochemical reactor geometry/dimensions and the volume of solution/suspension (surface contact area of ~233 cm^2 and 850 mL of sample, as aforementioned), as well as the time duration of each experiment, according to the following equation:

$$Q_{UV,n} = Q_{UV,n-1} + \Delta t_n \overline{UV}_{G,n} \frac{A_r}{V_t}; \quad \Delta t_n = t_n - t_{n-1} \tag{19}$$

where t_n is the time corresponding to n-water sample(s), V_t is the total reactor volume, A_r is the illuminated surface area and $\overline{UV}_{G,n}$ is the average UV radiation measured in the time interval Δt_n.

Results showed that the UV mercury lamp provided a constant $Q_{UV(total)}$ dose of approximately 3.5 kJ L^{-1} min^{-1} ($Q_{UV(300-400\ nm)} \approx 1$ kJ L^{-1} min^{-1}), except in the first ca. 2 min of lamp warming up (Fig. 4.10).

Similar calculations were performed for the LYNX S11W blacklight blue lamp. However, instead of the actinometrical determination of the photon flux, the average UV radiation intensity was measured with an UV portable radiometer (Kipp & Zonen, model CUV5), which provided the result of 10 W_{UV} m^{-2}.

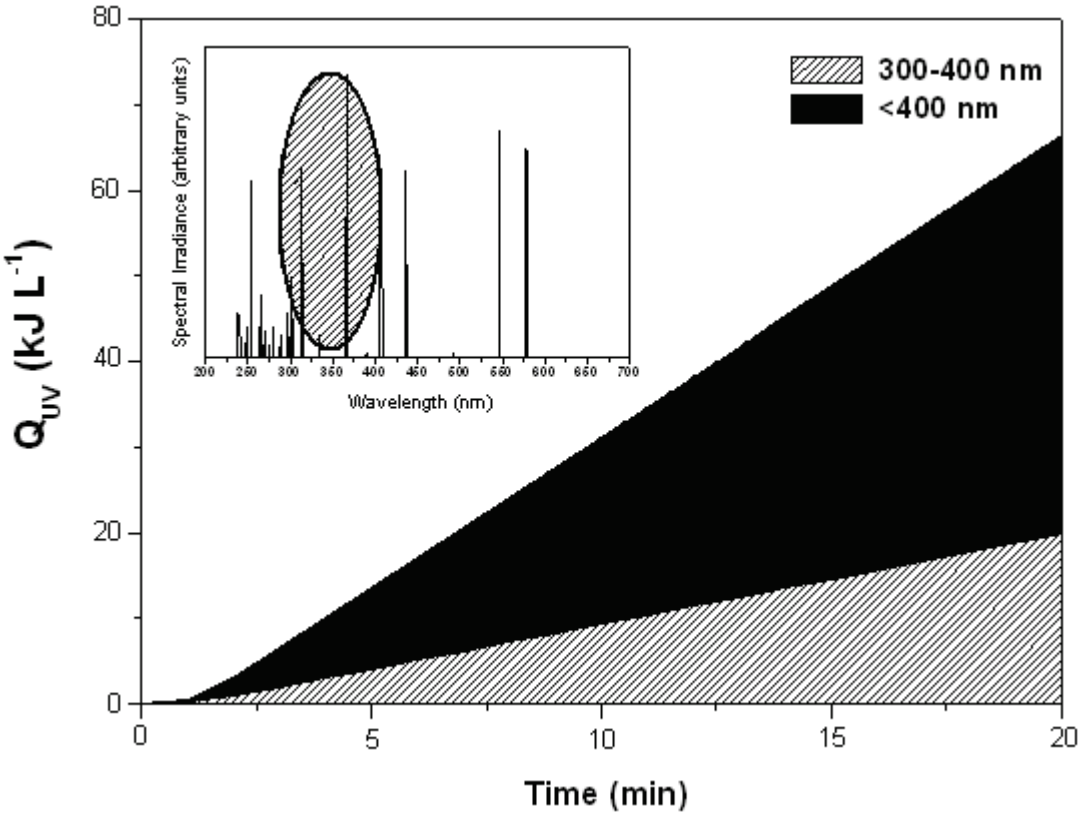

Fig. 4.10. Lab-scale prototype *LsTQUV*: graphic representation of the accumulated UV energy per litre of solution, as a function of the time after turning the UV lamp on [obs.: insert picture represents the emission spectrum of the medium pressure mercury lamp Heraeus TQ 150W].

Ultimately, some considerations shall be made regarding the CPC solar pilot plant. Besides the composition of the material used for its construction (the abovementioned borosilicate glass, with a cut-off at 280 nm), the design of solar photoreactors conceived for photochemical applications is of no less importance.

The original photochemical reactors were based on line-focus parabolic-trough concentrators (PTCs) (Fig. 4.11 (b)). PTCs made an efficient use of direct solar radiation and, as an additional advantage, the thermal energy collected from the concentrated radiation could simultaneously be used with other purposes. Nevertheless, they presented three main disadvantages: i) the use of only direct radiation; ii) the high cost; iii) the low optical and quantum efficiencies for TiO_2 applications (49). On the other hand, one-sun (non-concentrating) collectors (Fig. 4.11 (a)) were cheaper than PTCs, since they were

static and had no solar tracking devices. Moreover, they did not concentrate radiation, thus the efficiency of the process was not reduced by factors associated with concentration and solar tracking (50). Finally, compound parabolic concentrators (CPCs) (Fig. 4.11 (c)) appeared as an interesting cross between trough concentrators and one-sun systems. They are considered by many the choice for optimal possibilities (51). CPCs are static collectors with an involute reflective surface around a cylindrical reactor tube. The reflector design enables almost all UV radiation arriving at the CPC aperture to be collected and available for the photochemical process taking place inside the reactor. These static collectors provide the best optics for low concentration systems and present the advantages of both PTCs and one-sun collectors, since they can capture both direct and diffuse UV sunlight (Fig. 4.12) (52, 53).

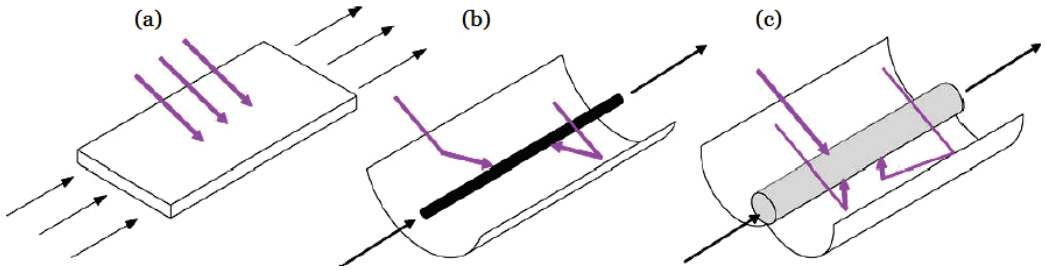

Fig. 4.11. Design of photocatalytic reactors used for water treatment: (a) one-sun system; (b) parabolic-trough concentrator (PTC); (c) compound parabolic collector (CPC).

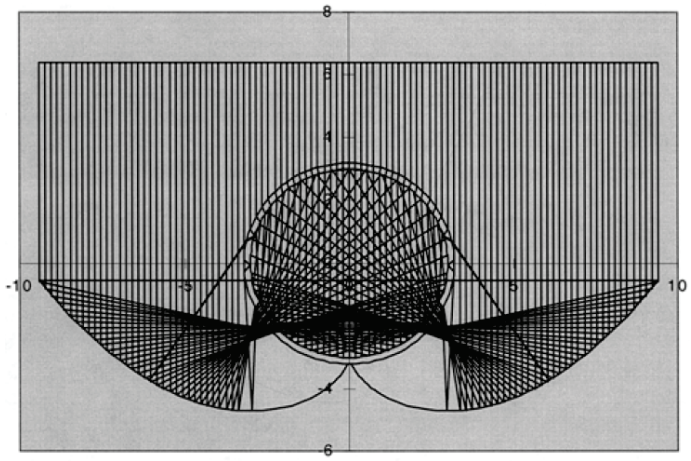

Fig. 4.12. Ray tracing example for a CPC collector (*from Ajona et al.* (51)).

The intensity of solar UV radiation is measured by radiometers. In our work, a global UV radiometer (ACADUS 85-PLS) was mounted on the pilot plant, tilted at the same angle (41º), providing data in terms of incident W_{UV} m^{-2}.

4.1.2.1.5. Analytical Determinations

In order to accompany the degradation of target and non-target emerging compounds over the phototreatment experiments (**Paper 3**, **Paper 4** and **Paper 5**), several analytical determinations were carried out, two of which deserve special attention: LC-tandem MS and the determination of dissolved organic carbon (DOC).

While the utilized LC-tandem MS methodology preceded by a cleanup/SPE step, in order to purify and concentrate the samples on target analytes, was part of our initial work (described in Chapter 2), the identification of transformation products (TPs) and the elucidation of the mechanisms involved in the attenuation of a particular xenobiotic – lorazepam, required a completely different approach. A lot of effort was devoted to this issue and the adopted strategy and achieved results are presented in the following chapter (Chapter 5, including **Paper 5**).

The mineralization state of a sample can be determined by its DOC content. Thus, DOC was also followed throughout photodegradation experiments, showing, as expected, a considerable decrease along the reactions.

Notwithstanding, one main conclusion which can be withdrawn from this work relates to the fact that both natural and artificial remediation processes are potential sources of TPs for the environment, should total mineralization be not attained.

Herein follow the two papers (**Paper 3** and **Paper 4**) encompassing the experimental work and achieved results in the field of AOPs applications for contaminated water photo-remediation. Another publication, involving the use of combined biological treatment and AOPs for the decontamination of pesticide-containing wastewaters, resulted from a collaboration work with the Faculty of Engineering of the University of Porto, and is included in the present thesis as Annex IV.

4.2. Paper 3 – Photolytic and TiO_2-Assisted Photocatalytic Oxidation of the Anxiolytic Drug Lorazepam (*Lorenin® pills*) under Artificial UV Light and Natural Sunlight: A Comparative and Comprehensive Study

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Photolytic and TiO_2-assisted photocatalytic oxidation of the anxiolytic drug lorazepam (Lorenin® pills) under artificial UV light and natural sunlight: A comparative and comprehensive study

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Abstract

Lorazepam is a recalcitrant drug, frequently quantified in WWTPs' effluents and surface waters, even though it has never been studied under accelerated phototransformation processes. Therefore, the main goal of the present work was to comparatively evaluate lorazepam's photolytic and photocatalytic degradation kinetics using two experimental systems: a lab-scale photochemical reactor provided with an UV medium pressure mercury lamp (*LsAUVP*) and a solar pilot plant with compound parabolic collectors (*SPP-CPCs*). Lorazepam was tested in its most commercialized dosage form in Portugal – Lorenin® pills, 1 mg (*Wyeth*), thus simulating a more realistic scenario.

Preliminary results showed that, using the *LsAUVP* apparatus, lorazepam's highest degradation yield was obtained through photolysis, while using the *SPP-CPCs* system, the best degradation performance was achieved by photocatalysis with a TiO_2 concentration of 200 mg L^{-1}. Furthermore, the degradation kinetic constant obtained for the optimized method when using the *SPP-CPCs* system ($k = 1.49 \pm 0.03$ L kJ^{-1}) was higher than that with the *LsAUVP* set-up ($k = 0.131 \pm 0.006$ L kJ^{-1}), which in terms of necessary accumulated UV energy for complete lorazepam degradation, considering the respective method's detection limit (MDL), corresponds to ca. 3 and 25 kJ L^{-1}, respectively.

Keywords: Lorazepam; Anxiolytic; Photolysis; TiO_2-photocatalysis; Artificial UV photochemical reactor; Solar pilot plant with CPCs

1. Introduction

Water contamination with emerging pollutants, including pharmaceuticals, personal care products, hormones and pesticides, is an issue receiving growing attention worldwide. Pharmaceuticals, such as antibiotics, anxiolytics, antidepressives, analgesics, anti-inflammatories and diuretics, among others, have been given special interest due to their potential adverse effects over non-target species, even at environmental trace levels (ng to μg L^{-1}) (Sousa et al., 2011).

These emerging pollutants are continuously released into the environment, reaching the aquatic compartment through pharmaceutical industries and hospitals' effluents,

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households, agriculture and fish farming, improper disposal or metabolic excretion (Mompelat et al., 2009). Hence, they have a widespread presence in the aquatic environment, being detected in hospital and municipal wastewaters, in surface waters such as rivers and lakes, in marine waters, as well as in soil matrices (Boyd et al., 2003; Castiglioni et al., 2005; Gros et al., 2006; Kasprzyk-Hordern et al., 2009; Kümmerer, 2001; Santos et al., 2009; Scheytt et al., 2006; Weigel et al., 2004; Zuehlke et al., 2007).

Conventional wastewater treatment plants (WWTPs) are not prepared to completely remove these micropollutants. Many of them are not easily degraded and tend to accumulate in aquatic or terrestrial organisms, through the food chain, causing long-term ecological and environmental effects (Miège et al., 2009; Okuda et al., 2008; Vilar et al., 2009). Total elimination of these hazardous compounds from contaminated streams is urgent and advanced tertiary/quaternary treatment processes are required, especially near problematic areas (Hayes et al., 2006; Herrmann, 1999; Khetan and Collins, 2007; Vilar et al., 2009).

Recently, there has been a research outbreak in the field of advanced water treatment technologies, designed to become part of the solution to that problem. They include the Advanced Oxidation Processes (AOPs), using the combination of strong oxidants, e.g. O_3 or H_2O_2, with high energy sources, such as ultraviolet (UV), ultrasound (US) or electron beam (EB), and catalysts, e.g. Fe^{2+}, or photocatalysts, e.g. TiO_2, membrane based technologies (microfiltration, ultrafiltration, nanofiltration and reverse osmosis) and adsorption/ion exchange processes (activated carbon, resins), each one presenting their own pros and cons (Malato et al., 2009). AOPs are particularly recommended when wastewater constituents, such as pharmaceuticals, have a high chemical stability and/or low biodegradability (Dalrymple et al., 2007; González et al., 2007; Malato et al., 2007; Pereira et al., 2011; Pérez-Estrada et al., 2007). They are considered an effective technology for the mineralization of many persistent organic pollutants (POPs), through the generation of reactive radical species, such as $^{\bullet}OH$ and $O_2^{\bullet-}$ (Blanco-Galvez et al., 2007; Fernández et al., 2005; Malato et al., 2007).

AOPs based on photocatalytic reactions lead to the most promising technologies for POPs elimination, due to their high efficiency and applicability under different energy sources (Scheytt et al., 2006). Titanium dioxide (TiO_2, BGE = 3.2 eV) is the most commonly used catalyst because of its high reactivity, non-toxicity, low price, chemical stability and, above all, its capability of using natural and renewable solar UV energy. TiO_2 has an appropriate energetic separation between its valence and conduction bands, which can be surpassed by the energy content of a solar photon, thus reducing the cost associated with UV radiation production (Galvez and Malato, 2003; Radjenović et al., 2009).

The TiO_2-assisted photocatalytic process involves several reactions, represented by Eqs. (1)–(6) (Choina et al., 2010):

$$TiO_2 + h\nu \rightarrow e^-_{cb} + h^+_{vb} \quad (1)$$

$$h^+_{vb} + H_2O \rightarrow {}^{\cdot}OH + H^+ \quad (2)$$

$$h^+_{vb} + OH^- \rightarrow {}^{\cdot}OH_{ad} \quad (3)$$

$$h^+_{vb} + R_{ads} \rightarrow R^+ \quad (4)$$

$$e^-_{cb} + O_2 \rightarrow O_2^{\cdot-} \quad (5)$$

$$TiO^*_{2(ecb^- + hvb^+)} \rightarrow TiO_2 \quad (6)$$

where "e" stands for electron, "h" for hole, "cb" for conduction band, "vb" for valence band and "R" for the pollutant.

According to the International Narcotics Control Board (2010), over the year of 2008 a total of 30 billion S-DDD (defined daily doses for statistical purposes) of benzodiazepines were manufactured, the highest amount registered up until today. Moreover, Portugal was considered one of the countries with the highest anxiolytics consumption rate. Amongst the most marketed anxiolytic drugs in Portugal, lorazepam highlights with a quite elevated sales value over the past 5 years, according to unpublished data provided by the Portuguese National Authority of Medicines and Health Products (INFARMED, IP). Furthermore, lorazepam has already been quantified in levels ranging from approximately 40 ng L^{-1} in river waters to ca. 200 ng L^{-1} in wastewater effluents (Gros et al., 2010; Sousa et al., 2011). However, to the authors' knowledge, never has this drug been studied under accelerated phototransformation processes before.

Therefore, the purpose of the present work was to assess, for the first time, the photolytic and photocatalytic degradation profiles and kinetics of lorazepam under artificial UV light and natural solar radiation in CPCs. Furthermore, we aimed to optimize the experimental procedure, to allow its future application to the advanced treatment of real WWTP effluents, contaminated with lorazepam. One of lorazepam's most commercialized dosage forms in Portugal – Lorenin® pills, 1 mg (*Wyeth*), was used with the purpose of resembling a more realistic scenario encompassing improper disposal of out-of-shelf life medicines and complex excreta. Lorazepam's removal rates and UV energy consumption were studied under photolytic and TiO_2-photocatalytic conditions, using two experimental apparatus: a lab-scale reactor with artificial UV radiation and a solar pilot plant with compound parabolic collectors (CPCs), thus taking advantage of solar renewable energy. A thorough comparative analysis was further carried out.

2. Experimental

2.1. Chemicals and materials

Lorazepam's structural formula and some chemical characteristics are presented in Fig. 1. A 1 mg mL^{-1} lorazepam solution in methanol was supplied by LGC Standards (Barcelona, Spain), while the 1 mg Lorenin® pills used were from *Wyeth*. Apart from the active substance lorazepam

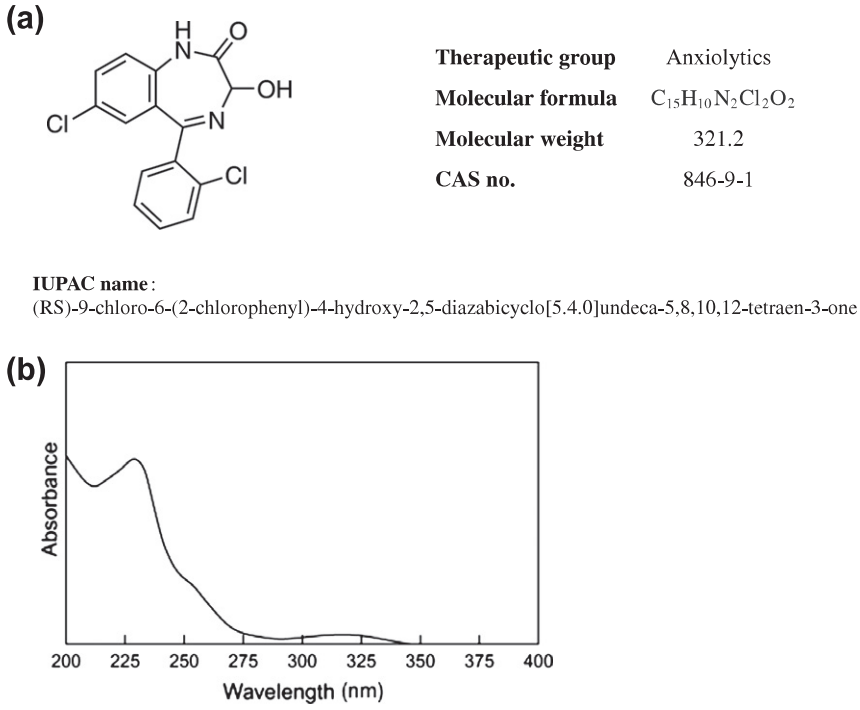

Fig. 1. (a) Lorazepam structural formula, IUPAC designation, pharmaceutical and chemical characteristics; (b) Lorazepam UV/Vis spectrum (adapted from http://mtnviewfarm.net/drugs-poisons-0958.html).

(1 mg per 100 mg pills), the pills are mainly composed by lactose monohydrate, microcrystalline cellulose, polacrilin potassium and magnesium stearate. Consequently, a more realistic scenario was simulated using the pills, and any possible interference due to the presence of the four excipients has not been disregarded.

TiO_2 catalyst (P25 Degussa, 80% anatase and 20% rutile) was purchased from Degussa Portuguesa, while Nylon syringe filters (0.2 μm) were provided by VWR International. High performance liquid chromatography coupled to mass spectrometry (LC–MS/MS) chemicals, materials and equipments were obtained as described by Sousa et al. (2011). Temperature and pH were measured using a pH meter HANNA HI8424.

2.2. Laboratory-scale Artificial UV Photoreactor (LsAUVP)

Photolytic and TiO_2-photocatalytic experiments were first conducted in a glass immersion photochemical reactor with a water column of 8 cm diameter and 16 cm height. The reactor can be loaded with 850 mL of solution (photolysis)/suspension (photocatalysis), with constant stirring for the total reaction period. It is equipped with a medium pressure mercury lamp Heraeus TQ 150 W, with a dominant emission line at 366 nm, placed axially and held in a quartz immersion tube with a surface contact area of ~233 cm^2. The system is refrigerated by a continuous tap water flow, which allowed the temperature to be properly controlled below 35 °C. Fig. 2 illustrates the apparatus used in these photo(cata)lytic experiments.

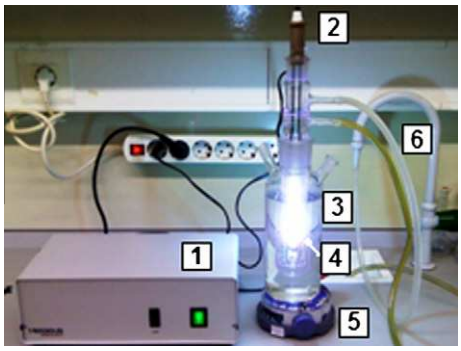

Fig. 2. Lab-scale photochemical reactor: 1 – electric transformer, 2 – UV medium pressure mercury lamp, 3 – glass reactor flask, 4 – quartz immersion tube, 5 – magnetic stirrer, 6 – refrigerating system.

2.3. Solar pilot plant with CPCs (SPP-CPCs)

Photolytic and TiO_2-photocatalytic experiments were also carried out, during sunny days, in a CPC solar pilot plant (Fig. 3) installed in the roof of the Chemical Engineering Department of the Faculty of Engineering, University of Porto (FEUP), Portugal. The CPC solar collector (0.91 m^2) is composed of four borosilicate tubes (Schott-Duran type 3.3, Germany, cut-off at 280 nm, external diameter 50 mm, length 1500 mm and thickness 1.8 mm) connected in series by polypropylene junctions with their

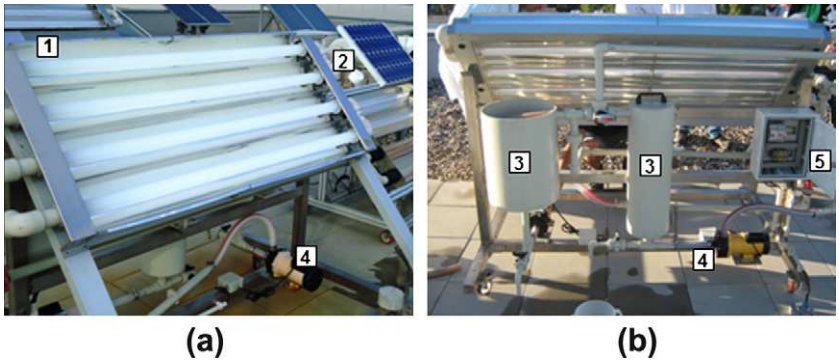

Fig. 3. Solar Pilot Plant with CPCs. (a) Front view; (b) Back view; 1 – photoreactor (CPC units), 2 – radiometer, 3 – recirculation tanks, 4 – recirculation pumps, 5 – electric board.

CPC mirrors in anodized aluminum, supported by an aluminum structure and tilted 41° (local latitude). The pilot plant has also two recirculation tanks (10 L and 20 L), two recirculation pumps (maximum 20 L min^{-1}), two rotameters, five polypropylene valves and an electric board for process control. The pilot plant operates in batch mode and can be used in two ways: using the total CPCs area (0.91 m^2) or using 0.455 m^2 of the CPCs area individually, giving the possibility to carry out two distinct experiments at the same time and at the same solar radiation conditions.

The intensity of the solar UV radiation is measured by a global radiometer (ACADUS 85-PLS), placed at the same inclination angle, which provides the data in terms of instantaneous UV radiation (W m^{-2}). Eq. (7) allows to calculate the accumulated UV energy ($Q_{UV,n}$, kJ L^{-1}) received on any surface in the same position with regard to the sun, per unit of water volume inside the reactor, in a given time interval Δt:

$$Q_{UV,n} = Q_{UV,n-1} + \Delta t_n \overline{UV}_{G,n} \frac{A_r}{V_t}; \quad \Delta t_n = t_n - t_{n-1} \tag{7}$$

where t_n is the time corresponding to n-water sample(s), $V_{t,n}$ is the total volume of wastewater at n-water sample (L), A_r is the illuminated collector surface area (m^2), and $\overline{UV}_{G,n}$ is the average solar UV radiation (W m^{-2}) measured in the time interval Δt_n (s).

2.4. Experimental procedures

2.4.1. Hydrolysis and TiO_2-adsorption (dark controls)

Prior to the photo(cata)lytic treatments, lorazepam's hydrolysis and TiO_2-adsorption tests were performed, in order to evaluate the possible contribution of these two factors to lorazepam's concentration decrease. Solutions containing 200 μg L^{-1} of lorazepam were simultaneously prepared using, on the one hand, lorazepam methanol solution (LMS) from LGC Standards and, on the other hand, Lorenin® pills (LLP) from *Wyeth*, both diluted/dissolved in distilled water, with no pH adjustment and at room temperature. Whenever lorazepam from LMS was used, methanol was previously evaporated to dryness and lorazepam recovered in an equal volume of MilliQ water.[1] TiO_2 was tested in the concentration of 200 mg L^{-1}. Four experiments were conducted in 500 mL Pyrex beakers, using both LMS and LLP lorazepam, kept in the dark, with constant stirring: in the absence of TiO_2 only hydrolysis effects were evaluated, while in the presence of the catalyst adsorption reactions were also assessed.

2.4.2. Phototreatment processes: *LsAUVP* and *SPP-CPCs*

For both experimental systems used to perform the phototreatment processes (*LsAUVP* and *SPP-CPCs*), lorazepam from Lorenin® pills was always used in the concentration of ca. 200 μg L^{-1}. TiO_2 catalyst was tested in different concentrations: 0 (corresponding to photolysis), 10, 25, 50, 100, 200 and 400 mg L^{-1}.

Before use, Lorenin® pills were properly ground using a porcelain pestle and mortar. After their complete dissolution in distilled water, for approximately 15 min. in the dark (*LsAUVP*: by stirring in 850 mL (100% illuminated volume, 0.02325 m^2 illuminated area); *SPP-CPCs*: by turbulent recirculation of 15 L (~0.3 min residence time, ~40% illuminated volume, 0.455 m^2 illuminated area)), a control sample was taken. Then, TiO_2 was added (for photocatalytic experiments) and after 30 min. another sample was taken to evaluate the contaminant's adsorption onto the catalyst surface. Finally, experiments began by turning on the UV light in the case of the *LsAUVP* and uncovering the CPCs. Samples were taken at pre-defined times along all photo(cata)lytic experiments. Temperature and pH were continuously monitored.

2.4.3. HPLC-tandem MS analysis and data processing

Previously to LC–MS/MS analysis, all sample aliquots containing TiO_2 were pre-filtered through 0.2 μm membrane filters. Afterwards, lorazepam was quantified by HPLC-tandem MS, as thoroughly detailed in Sousa et al. (2011). The used analytical column was a C18 Pursuit

[1] The importance of this procedure step will be later discussed in Section 3.1.

UPS column (2.1 mm i.d. × 50 mm, 2.4 μm) from Varian, the autosampler was fitted with a 10 μL sample loop and the LC flow rate was set at 300 μL min^{-1}, with mobile phases consisting of (A) 10 mM formic acid in ultrapure Milli-Q water and (B) Baker-analysed methanol. The detector was a Varian 500 MS ion trap with electrospray ionization mass spectrometer. Lorazepam was ionized in ESI(+) mode, presenting a precursor ion at 321 *m/z* and a MS^2 product ion at 303 *m/z*.

Finally, lorazepam's degradation kinetics were properly evaluated and compared between the two experimental procedures. A first-order kinetic model was fitted to the experimental data using a nonlinear regression method (Fig.P for Windows from BIOSOFT).

3. Results and discussion

3.1. Hydrolysis and TiO_2-adsorption (dark controls)

A lorazepam concentration of ca. 200 μg L^{-1} was adopted throughout the experiments as a compromise between a value close to real aquatic environmental conditions and the concentration required for a proper kinetic characterization, according to instrumental quantification limits ($IQL_{Lorazepam} = 6$ μg L^{-1} by LC–MS/MS (Sousa et al., 2011)).

The performed dark control experiments revealed no significant variations in lorazepam's concentration, over a 2-h period, in either of the tested conditions: possible deviations due to adsorption to the surface of the catalyst were inferior to 5%, using both LMS (2.8%) and LLP (4.6%), while potential hydrolysis effects only accounted for 6.4% deviations in the case of LMS and 4.8% for LLP experiments. Consequently, it was assumed that lorazepam does not undergo significant hydrolysis or adsorption onto the catalyst's surface, in the tested concentration. Nonetheless, it must be noticed that whenever lorazepam from LMS was used, all MeOH was carefully evaporated, as aforedescribed in the Experimental section. This procedure step was performed to prevent MeOH action as a radical scavenger, i.e., to avoid its competition with lorazepam for $^{\bullet}OH$ radicals, which would decrease the real lorazepam photodegradation rate.

It is important to add that a 48-h experiment was also performed, using the lab-scale UV photochemical reactor, with lorazepam from Lorenin® pills in a concentration of 200 μg L^{-1} and TiO_2 at 200 mg L^{-1}. Identically, no significant decrease of lorazepam's concentration was observed during this time period, much longer than those used on the photo(cata)lytic experiments (max. 20 min. for *LsAUVP* and 5–12-h for *SPP-CPCs*, depending on the weather conditions).

Finally, given that lorazepam's p*Ka* is 12.46 and that pH was always maintained within the interval]5.5–6.5[, conclusion is that lorazepam was mostly present in its positively charged ionic form. On the other hand, it is well known that TiO_2 surface is amphoteric and, consequently, its charge is pH-dependant. For TiO_2-P25, used in this work, the zero charge point (pH_{zcp}) is located between pH 5.6 and 6.4 (pH ≈ 6.3) (Pereira et al., 2011). Since the experimental pH was mostly kept under TiO_2 pH_{zcp}, the catalyst surface was globally positive and the ionic repulsion of also positively charged lorazepam ions could explain the low adsorption results.

3.2. Artificial UV light - laboratory-scale photochemical reactor

The presence of the already mentioned pills' excipients (lactose monohydrate, microcrystalline cellulose, polacrilin potassium and magnesium stearate), simulating a more realistic scenario, was not disregarded. Notwithstanding, among the four compounds, only lactose is significantly water soluble. Therefore, this compound may compete with lorazepam for the reactive radical species, while microcrystalline cellulose, polacrilin potassium and magnesium stearate can contribute to the increase of turbidity of the mixture. Either way, all four excipients are expected to result in a decay of lorazepam's photodegradation rate.

The UV lamp photonic flux was previously determined at different wavelengths with the use of the Hatchard–Parker actinometer (Kuhn et al., 2004; Silva and Faria, 2009), one of the most reliable and practical actinometers for UV and visible light up to 500 nm. Under light excitation the potassium ferrioxalate decomposes according to the following equations (Eqs. (8) and (9)):

$$Fe(C_2O_4)_3^{3-} \xrightarrow{h\nu} Fe^{2+} + C_2O_4^{\bullet-} + 2C_2O_4^{2-} \quad (8)$$

$$Fe(C_2O_4)_3^{3-} + C_2O_4^{\bullet-} \xrightarrow{\Delta} Fe^{2+} + 2CO_2 + 3C_2O_4^{2-} \quad (9)$$

The quantity of ferrous ions formed during the irradiation period is monitored by conversion to the colored tris-phenanthroline complex ($\varepsilon = 11{,}100$ L mol^{-1} cm^{-1} at $\lambda_{max} = 510$ nm). The original ferric ions are not appreciably complexed by phenanthroline and the complex does not absorb at 510 nm.

Afterwards, calculations regarding the accumulated UV energy ($Q_{UV,n}$, kJ L^{-1}) took into account the lab-scale photochemical reactor geometry and dimensions, the used solution/suspension volume and the time duration of each experiment. Results showed that, in the described experimental conditions, the UV mercury lamp provided a constant $Q_{UV(total)}$ dose of approximately 3.5 kJ L^{-1} min^{-1}. ($Q_{UV(300–400\ nm)} \approx 1$ kJ L^{-1} min^{-1}), except in the first ~2 min. of lamp warming up (Fig. 4a).

According to the experimental procedure previously described (refer to Sections 2.2 and 2.4), lorazepam's degradation kinetic was evaluated with different TiO_2 concentration levels in an UV photochemical reactor prototype, as represented in Fig. 4b. Results led to the conclusion that with the use of this artificial UV source, photolysis is the dominant process responsible for lorazepam's degradation and not photocatalysis. Departing from the initial concentration of 200 μg L^{-1}, lorazepam's complete removal was

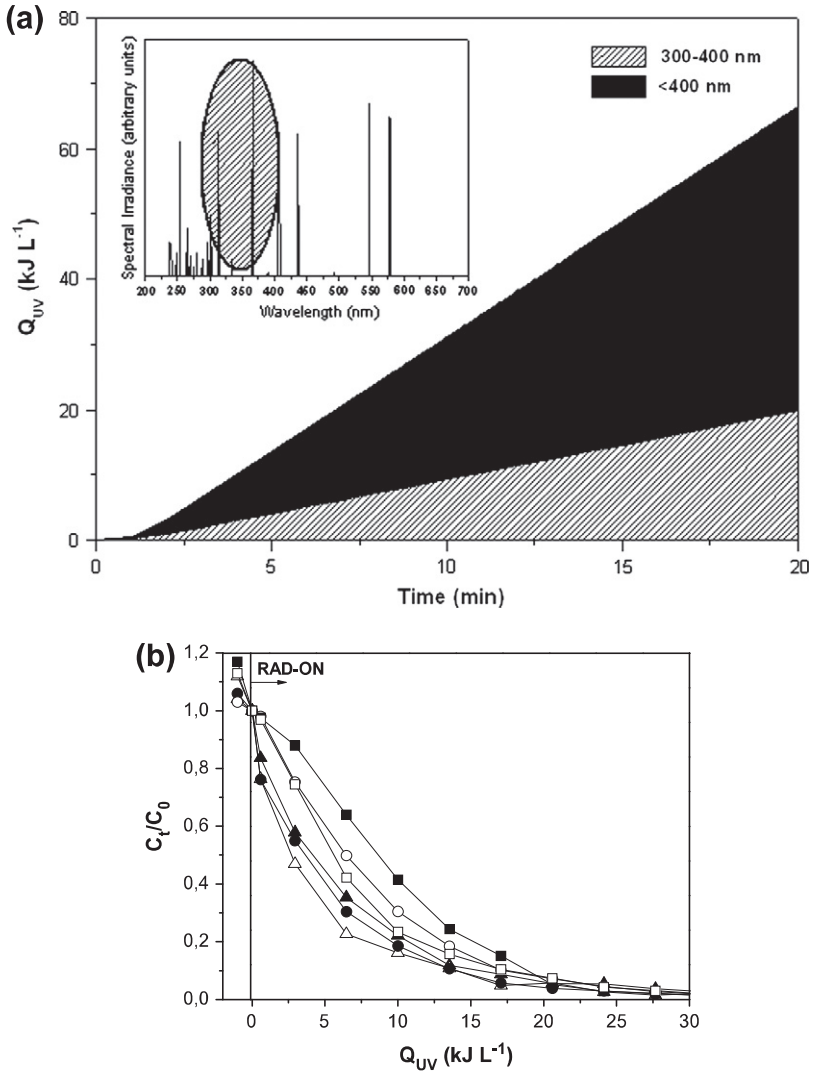

Fig. 4. Artificial UV Photochemical Experiments. (a) Graphic representation of the accumulated UV energy per liter of solution, as a function of the time after turning the UV lamp on (Obs.: Inlet picture represents the emission spectrum of the medium pressure mercury lamp Heraeus TQ 150 W, used in this work). (b) Graphic representation of lorazepam's concentration ratio C_I (concentration at time "t") over C_0 (initial concentration), as a function of the amount of accumulated UV energy per liter of solution (□ – photolysis)/ suspension (photocatalysis: ■ – 10 mg L^{-1} TiO_2, ○ – 25 mg L^{-1} TiO_2, ● – 50 mg L^{-1} TiO_2, Δ – 100 mg L^{-1} TiO_2, ▲ – 200 mg L^{-1} TiO_2).

achieved after ca. 25 kJ L^{-1} (corresponding roughly to 8 min. of irradiation), regardless of TiO_2 concentration (Fig. 4b). The wide TiO_2 concentration range tested did not reflect any significant kinetic difference, relatively to the photolysis experiment.

Through the analysis of Fig. 4a, and in view of the presented lamp's emission spectrum, one can comprehend that within the useful wavelength range for the TiO_2-photocatalytic reactions performed (i.e., $\lambda < 400$ nm), only less than one third of the total accumulated energy is due to radiation with $300 < \lambda < 400$ nm (white stripped portion of the total Q_{UV}). Besides, and according to the UV/Vis absorption spectrum present on Fig. 1b, lorazepam has a maximal absorption for $\lambda < 300$ nm, which thus corresponds to approximately two thirds of the radiation emitted by the UV lamp. It is therefore understandable that the photolytic process is faster. Besides, in the case of photocatalysis, there must occur the transference of lorazepam from the liquid phase to the surface of the catalyst particles, which constitutes itself the limiting step of the reaction. Furthermore, TiO_2 particles also act as light scatterings agents. Therefore, it is likely that a continuous raise in TiO_2 concentration induces a decrease in the degradation rate constant, due to the growing turbidity of the suspension caused by the catalyst.

Finally, owing to the refrigeration system, the maximum temperature reached in the mixture was ~31 °C, thus insufficient to induce lorazepam's thermolysis (Chauvet, 1995).

3.3. Solar UV light – pilot plant with CPCs

Following the methodology described in Sections 2.3 and 2.4, the same TiO_2 concentrations plus 400 mg L^{-1} were tested in order to optimize lorazepam's degradation

rate using the CPCs solar pilot plant experimental system. Results obtained are presented in Fig. 5a.

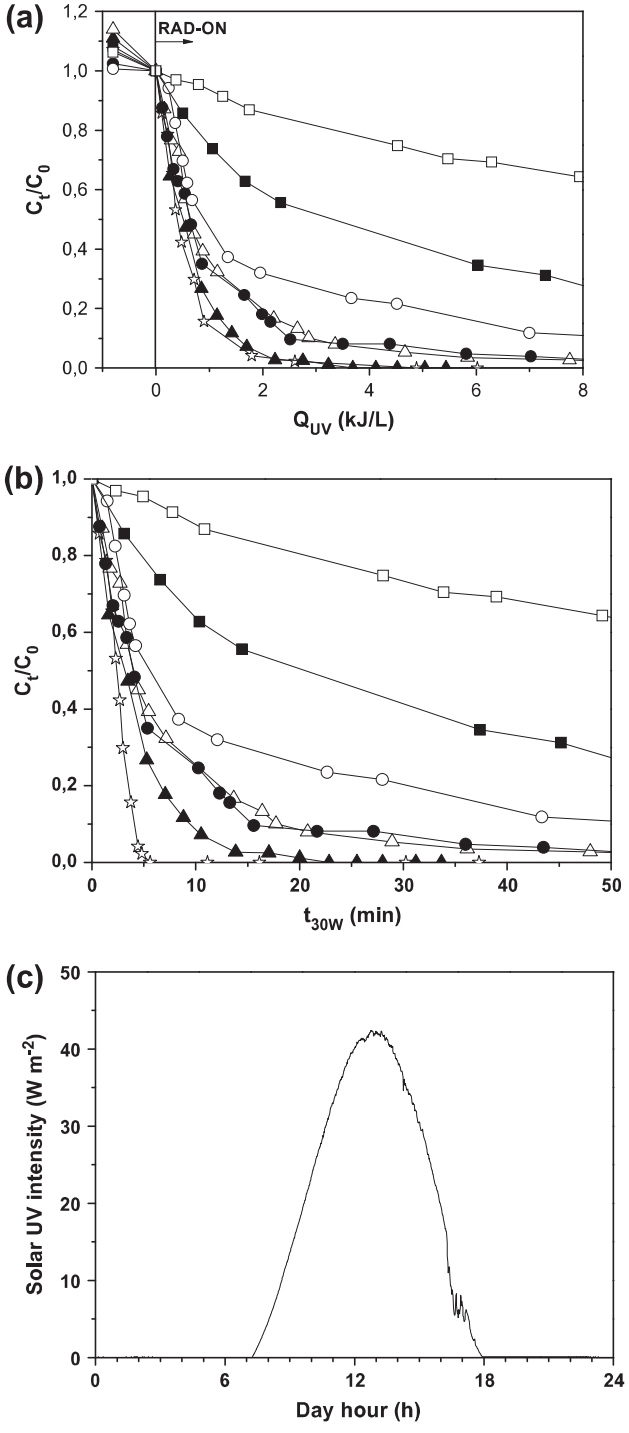

Fig. 5. CPCs Solar Pilot Plant. Graphic representation of lorazepam's concentration ratio C_t (concentration at time "t") over C_0 (initial concentration), as a function of (a) the amount of accumulated UV energy per liter of solution and (b) t_{30W} (□ – photolysis)/suspension (photocatalysis: ■ – 10 mg L^{-1} TiO_2, ○ – 25 mg L^{-1} TiO_2, ● – 50 mg L^{-1} TiO_2, Δ – 100 mg L^{-1} TiO_2, ▲ – 200 mg L^{-1} TiO_2, ★ – 400 mg L^{-1} TiO_2), using the Solar Pilot Plant with CPCs. (c) Typical within-day variation of solar $UV_{[300–400\ nm]}$ intensity during the month of March, at Porto (Portugal), where the experiments took place.

Contrary to what was observed when using the artificial UV lab-scale reactor, with the use of solar radiation we could detect a substantial increase of lorazepam's degradation rate constant by increasing the catalyst's concentration. pH and temperature were also monitored over the entire experiments, never falling outside the range of 5–6 and 11–22 °C (within-day natural variations), respectively.

Again, as observed in the UV/Vis absorption spectrum (Fig. 1b), lorazepam's maximal absorption occurs in the low UV wavelength range (between ca. 210 and 250 nm), though some radiation absorption still takes place up to ~330 nm. Since CPCs are made of a special borosilicate material, with a cut-off at 280 nm, UV radiation with a wavelength between 280 and 330 nm can still be responsible for the low degradation rate observed in the absence of TiO_2 – photolysis (Fig. 5a). However, in this experiment photocatalysis is, undoubtedly, the dominant process. Furthermore, TiO_2-photocatalytic reactions are mediated by the hydroxyl radicals generated in solution and are, therefore, [TiO_2] dependent.

Fig. 5b presents the experimental results in terms of t_{30W}, allowing similar conclusions. Through Eq. (10) it is possible to combine data from several days' experiments and compare them with other photo(cata)lytic experiments:

$$t_{30W,n} = t_{30W,n-1} + \Delta t_n \frac{\overline{UV}}{30}\frac{V_i}{V_T}; \quad \Delta t_n = t_n - t_{n-1} \tag{10}$$

where t_n is the experimental time for each sample, $\overline{UV}$ is the average UV radiation (W m^{-2}) measured in the time interval Δt_n (s) and $t_{30W,n}$ is a "normalized irradiation time". This is to say that, in this case, time refers to a constant solar UV power of 30 W m^{-2}, which corresponds to the typical solar UV power on a perfectly sunny day around noon (Gros et al., 2010).

Furthermore, Fig. 5c illustrates the typical within-day variation of the solar $UV_{[300–400\ nm]}$ intensity during the days in which the solar-driven photo(cata)lytic experiments were performed. At Porto (Portugal), during the month of March 2011, maximum $UV_{[300–400\ nm]}$ power could reach over 40 W m^{-2} around noon.

Lastly, and according to results previously reported by Rodríguez et al. (2004), [TiO_2] = 200 mg L^{-1} was considered to be the optimal concentration value when working with the CPC unit herein described (internal diameter 46.4 mm). These results have recently been supported by accurate modeling of radiation field in a CPC solar photoreactor by a six-flux absorption scattering model (SFM), showing that TiO_2 in the concentration of 200 mg L^{-1} is able to absorb 100% of the solar UV photons (Colina-Márquez et al., 2010). In the present work, for [TiO_2] = 200 mg L^{-1}, lorazepam's complete removal was achieved after ca. 3 kJ L^{-1} (corresponding to a t_{30W} ≈ 20 min.). The raise to 400 mg L^{-1}, besides increasing the expenses regarding the catalyst load, does not result in any substantial improvement of lorazepam's degradation kinetic constant.

Table 1
First order kinetic parameters determined for the Laboratory-scale Artificial UV Photoreactor (*LsAUVP*) and Solar Pilot Plant with CPCs (*SPP-CPCs*) photo(cata)lytic experiments, using Lorenin® pills.

$[TiO_2]$ (mg L^{-1})	C_0[a] (μg L^{-1})		k[b] (L kJ^{-1})		r_0[c] (μg kJ^{-1})		R^2[d]	
	LsAUVP (artificial UV)	*SPP-CPCs* (solar UV)	*LsAUVP* (artificial UV)	*SPP-CPCs* (solar UV)	*LsAUVP* (artificial UV)	*SPP-CPCs* (solar UV)	*LsAUVP* (artificial UV)	*SPP-CPCs* (solar UV)
0	195	213	0.131 ± 0.006	0.058 ± 0.001	26 ± 2	12.3 ± 0.3	0.996	0.991
10	215	226	0.096 ± 0.008	0.17 ± 0.01	21 ± 2	38 ± 3	0.981	0.972
25	187	219	0.117 ± 0.003	0.60 ± 0.07	22 ± 1	131 ± 15	0.998	0.960
50	206	188	0.19 ± 0.01	1.02 ± 0.05	39 ± 2	192 ± 9	0.992	0.990
100	203	215	0.23 ± 0.02	0.93 ± 0.04	46 ± 4	201 ± 9	0.990	0.988
200	193	220	0.160 ± 0.007	1.49 ± 0.03	31 ± 1	328 ± 8	0.997	0.997
400	n.d.[e]	194	n.d.	1.64 ± 0.09	n.d.	318 ± 17	n.d.	0.986

[a] C_0 – concentration after the addition of TiO_2.
[b] k – kinetic rate constant.
[c] r_0 – initial reaction rate.
[d] R^2 – determination coefficient.
[e] n.d. – not determined.

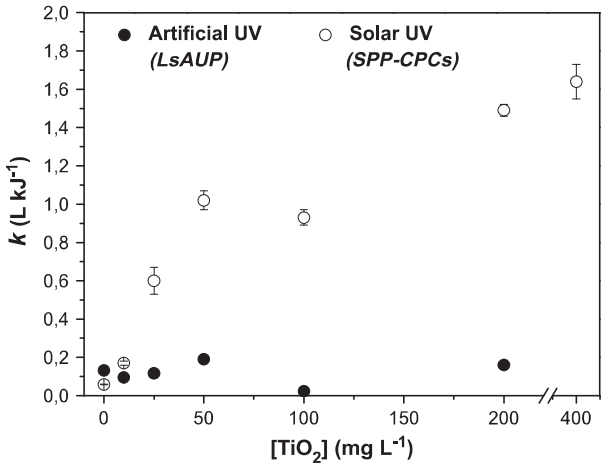

Fig. 6. Graphic representation of the kinetic rate constants obtained for lorazepam's degradation reactions, using artificial and solar UV experimental systems, as a function of TiO_2 concentration.

3.4. LsAUVP vs. SPP-CPCs

Table 1 summarizes all kinetic parameters calculated for the performed lorazepam's phototreatment processes, using both described experimental set-ups. The results of every photo(cata)lytic reaction proved to fit well a pseudo-first order kinetic model (all determination coefficient values (R^2) obtained were superior to 0.95). The determined kinetic rate constants corroborate the conclusions aforementioned: while with the use of the lab-scale reactor there is no clear increasing/decreasing trend regarding k values as a function of TiO_2 concentration, using the CPC pilot plant there is a notorious boost on the degradation rate constant with $[TiO_2]$ increase. A similar behavior can be observed when analyzing the corresponding initial reaction rate values (r_0).

In Fig. 6, the k values calculated for each experimental system were plotted against the corresponding tested $[TiO_2]$. Using the *LsAUVP*, there is no significant difference in the degradation reaction rate with the addition of TiO_2 until 25 mg L^{-1} comparatively to photolysis. The reaction constant then raises with a $[TiO_2]$ increase up to 50 mg L^{-1}, slightly decreasing for a $[TiO_2]$ of 100 mg L^{-1} and raises again for 200 mg L^{-1} of TiO_2. Nevertheless, all these consist of non-significant kinetic variations/improvements, since the turbidity caused by TiO_2, which hinders the photolytic process, counterbalances its activity as the reaction photocatalyst. On the other hand, with the use of the *SPP-CPCs* system, the reaction constant continuously raises with the increase of $[TiO_2]$ up to 200 mg L^{-1}; although slightly higher, the k value for a $[TiO_2] = 400$ - mg L^{-1} is not significantly different and, bearing also in mind the global cost balance, $[TiO_2] = 200$ mg L^{-1} was considered the optimal concentration value.

On the overall, and despite similar amounts of accumulated UV energy could be expected to result in fairly equal lorazepam degradation extents using both experimental set-ups, the *SPP-CPCs* system proved to enhance lorazepam's degradation performance relatively to the *LsAUVP* set-up. According to the hypothesis proposed by Pereira et al. (2011), this can probably be due to the fact that, in the case of the *LsAUVP* system, UV photons have a much shorter pathway length (only ca. 3 cm) to interact either with lorazepam (directly by photolysis) and/or TiO_2 (photocatalysis through the formation of $^{\bullet}OH$ radicals), thus resulting in a diminished final photoreaction yield. Since photolysis is, in this case, the main responsible process for lorazepam's depletion, the UV photons are not being as effectively absorbed by lorazepam molecules, therefore hampering the break-down reactions.

Hence, it is understandable that k values obtained with the *SPP-CPCs* apparatus are, in general, significantly higher than the respective ones when using the *LsAUVP* (Fig. 6). Furthermore, it must also be noticed that the useful wavelength ranges were slightly different for both experimental set-ups: in the case of solar UV experiments,

useful wavelengths ranged from ~300 nm (borosilicate cut-off) to 400 nm, while with the Heraeus TQ 150 W UV lamp a broader wavelength spectrum was used (refer to Fig. 4b), including more energetic radiation ($\lambda < 300$ nm). Had both experimental systems been performed using the same useful wavelength range and the difference between the respective k values should be even more significant.

3.5. Figures-of-merit: electric- vs. solar-energy-driven systems

Finally, and according to some figures-of-merit recommended by IUPAC (Bolton et al., 2001), further comparison has been performed between both experimental systems employed in this study. These figures-of-merit enable the rapid determination of the AOP system costs and efficiencies, regardless of the nature of the used radiation, i.e., electric- or solar-energy-driven AOP processes.

Given that the optimized photo(cata)lytic experiments for both batch systems (photolysis using *LsAUVP* and 200 mg L^{-1} TiO_2-photocatalysis for *SPP-CPCs*) proved to fit well a pseudo-first order kinetic model, the figures-of-merit to be used are:

$$E_{EO} = \frac{P \cdot t \cdot 1000}{V_t \cdot \log\left(\frac{C_0}{C}\right)}, \quad \text{for electric-energy-driven systems} \tag{11}$$

where E_{EO} (kW h m^{-3}-order) is the electric energy in kilowatt hours (kW h) required to degrade a contaminant (C) by one order of magnitude in a unit volume (e.g. 1 m^3) of contaminated water, P is the rated power (kW) of the system, t is the period of treatment (h), V_t the total reactor volume (L), and C_0 and C are the initial and final lorazepam concentrations (mM)

and:

$$A_{CO} = \frac{A_r \cdot \overline{UV_G} \cdot t}{E_s^0 \cdot t_0 \cdot V_t \cdot \log\left(\frac{C_0}{C}\right)}, \quad \text{for solar-energy-driven systems} \tag{12}$$

where A_{CO} (m^2 m^{-3}-order) is the collector area required to reduce the concentration of a contaminant (C) in an unit of volume of polluted water by one order of magnitude in a time ($t_0 = 1$ h) when the standardized incident solar irradiance (E_S^0) is 1000 W m^{-2}, A_r is the illuminated collector surface area (m^2), $\overline{UV_G}$ is the average solar ultraviolet irradiance (W m^{-2}) over the period t of treatment (h), V_t the total reactor volume (m^3), and C_0 and C are the initial and final lorazepam concentrations (mM).

The calculated E_{EO} and A_{CO} values were 18 kW h m^{-3}-order and 0.4 m^2 m^{-3}-order, respectively. Bearing in mind the cost of electricity for the *LsAUVP* system and of a m^2 of borosilicate glass for the *SPP-CPCs* set-up, it can be concluded that the *SPP-CPCs* prototype is approximately 100 times more expensive than the lab-scale apparatus. This is certainly one of the main reasons why, despite their high efficiency, CPCs solar pilot plants are still not commonly used.

4. Conclusions

The main finding of the present work relates to the fact that the anxiolytic drug lorazepam, never before studied throughout environmental phototransformation processes, can be totally eliminated using the experimental systems herein presented and optimized: with the *SPP-CPCs* set-up and a $[TiO_2] = 200$ mg L^{-1}, the necessary Q_{UV} is approximately 3 kJ L^{-1} (corresponding to a t_{30W} - ≈ 20 min.), while with the *LsAUVP* apparatus, the photolytic process itself is able to completely degrade lorazepam, but only after ca. 8 min. light on ($Q_{UV} \approx 25$ kJ L^{-1}). Hence, the photodegradation reactions' yield is higher when using the CPCs Solar Pilot Plant.

Furthermore, in the described experiments, lorazepam was tested in the dosage form of Lorenin® pills and was, therefore, "surrounded" by four other compounds, which constitute the majority of each pill. Despite some competition for the hydroxyl radicals could be predicted, the photocatalytic process showed to be quite efficient.

Thus, results are promising and they uphold the future application of the photocatalytic process using $[TiO_2] = 200$ mg L^{-1} and the Solar Pilot Plant with CPCs system for the complementary treatment of real WWTP effluents contaminated with lorazepam, since it requires the use of solar renewable energy. Nonetheless, it is obvious that this experimental system has its limitations, namely climatic (only applicable during sunny days). Hence, it will better succeed in countries/areas with high solar irradiation per year. Nevertheless, the complementary use of an artificial UV source (lamp) can also be considered as a viable option.

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4.3. Paper 4 – Suspended TiO_2-Assisted Photocatalytic Degradation of Emerging Contaminants in a Municipal WWTP Effluent using a solar Pilot Plant with CPCs

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Suspended TiO_2-assisted photocatalytic degradation of emerging contaminants in a municipal WWTP effluent using a solar pilot plant with CPCs

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HIGHLIGHTS

- Municipal WWTP effluent contaminated with pharmaceuticals and earthy-musty compounds.
- CPC pilot plant for TiO_2-solar photocatalysis as a complementary tertiary treatment.
- Complete removal of 19 out of 22 pharmaceuticals with ca. 32 kJ L^{-1} solar UV energy.
- Zahn–Wellens test indicates many pharmaceuticals are refractory to biotreatment.
- *V. fischeri* bioassay revealed no toxicity increase during photocatalytic treatment.

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ABSTRACT

Emerging pollutants, such as pharmaceuticals, are widely disseminated in the aquatic media. Though in low concentrations in the environment, they still pose concerns mainly over potential chronic toxicity effects. Consequently, this work reports on the successful attempt to develop a photocatalytic treatment method, using suspended TiO_2 in a concentration of 200 mg L^{-1} and solar UV radiation as the photon source, firstly designed for lorazepam (Lorenin® pills) degradation and further applied to the treatment of a real municipal WWTP effluent, containing several other emerging contaminants (ECs).

Initial effluent physicochemical characterization revealed the presence of 22 pharmaceutical compounds in moderate concentrations (maximum of 680 ng L^{-1}, except for diclofenac ~24 μg L^{-1} and hydrochlorothiazide ~3 μg L^{-1}) and a low dissolved organic carbon (DOC) content. Therefore, the main purpose of the work was not to increase the effluent's biodegradability, but to improve the removal rates of the several present ECs.

Pharmaceuticals' degradation kinetics, using a solar pilot plant with CPCs, were thoroughly studied. A pseudo-first order kinetic model was able to successfully predict the experimental data. The overall treatment was considered efficient, with a complete removal of the majority of these micropollutants, except for ciprofloxacin (35%), ketoprofen (61%) and bisoprolol (77%). Nevertheless, a small increase in the reaction time could easily accomplish their total degradation.

Zahn–Wellens biodegradability assay allowed withdrawing some conclusions about which pharmaceuticals could be degraded by means of biotreatment, thus avoiding the need to apply a photocatalytic treatment.

Finally, *Vibrio fischeri* acute toxicity test showed that the effluent itself presented no significant toxicity and that the intermediate oxidation compounds, possibly formed during phototreatment, did not reflect any significant increase of toxicity.

1. Introduction

Emerging pollutants are defined as compounds that are not currently covered by the existing legislation in the field of water quality, whose environmental impact is not yet sufficiently studied and which are thought to be potentially harmful to environmental ecosystems and human health. They encompass a wide range of products, including pharmaceuticals, personal care products, fragrances, detergents, plasticizers, flame retardants, pesticides and several other classes [1].

Concerns about the hazards associated with long-term exposure to pharmaceutically active compounds for non-target organisms and for

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human health are rising. One of the best known detrimental effects of these compounds in the environment is the disruption of the endocrine system in wild species, affecting their growth, physiology and reproduction. The selection of multi-resistant strains of pathogenic microorganisms is also a threat to humans and ecosystems, resulting from the uncontrolled use of antibiotics and deficient treatment of effluents from wastewater treatment plants (WWTPs) [2,3].

In fact, WWTPs' effluents are considered a major source of environmental pharmaceutical contamination, since they are not prepared for the complete removal or degradation of such micropollutants, as mentioned in several studies [4–6]. They can, therefore, reach the aquatic media, where they have been found in concentrations ranging from 0.1 to 20 $\mu g\ L^{-1}$, among treated wastewaters, surface and groundwater and even drinking water [7–11].

Recently, there has been a research outbreak in the field of advanced water treatment technologies, designed to become part of the solution to this problem. They include the advanced oxidation processes (AOPs) (using the combination of strong oxidants, e.g. O_3 or H_2O_2, with high energy sources, such as ultraviolet (UV), ultrasound (US) or electron beam (EB), and catalysts, e.g. Fe^{2+}, and photocatalysts, e.g. TiO_2, to produce hydroxyl radicals), membrane based technologies (microfiltration, ultrafiltration, nanofiltration and reverse osmosis) and adsorption/ion exchange processes (activated carbon, resins), each one presenting their own advantages and disadvantages [12].

Special attention has been given to AOPs, in which hydroxyl radicals (·OH) are the responsible agents for the oxidation and mineralization of almost any organic molecule, yielding CO_2 and inorganic ions as final products, due to their strong unselective oxidative power [13]. Among AOPs, the heterogeneous photocatalysis with UV/TiO_2 has shown to be a promising technique for wastewater detoxification [12]. In heterogeneous photocatalysis, dispersed solid particles absorb efficiently larger fractions of the UV spectrum and generate chemical oxidants *in situ* from dissolved oxygen or water. Regarding the catalyst choice, TiO_2 has generally been demonstrated to be the most active, whenever tested against other semiconductor materials, under comparable conditions [14]. When illuminated with light energy higher than their bandgap, semiconductors like TiO_2 (BGE = 3.2 eV) produce excited high-energy states of electron and hole pairs (e^-/h^+) [12]. TiO_2 is also technologically interesting due to its chemical and photocorrosion resistance, its safety and low cost and, specially, to its capability of using natural and renewable solar UV energy (since the energetic separation between its valence and conduction bands can be surpassed by the energy content of a solar photon) [14].

The purpose of this work was to study the application of heterogeneous photocatalysis with solar UV/TiO_2 to the degradation of an anxiolytic drug – lorazepam – which is frequently detected in WWTPs' effluents and surface waters [1]. To the authors' knowledge, never has this drug been studied under accelerated phototransformation processes (photo-induced structural alterations). To do so, a solar pilot plant with compound parabolic collectors (CPCs) designed for solar photo(cata)lytic applications was used, taking profit of solar renewable energy. Besides, lorazepam was tested in one of its most commercialized dosage forms in Portugal – Lorenin® pills, 1 mg (*Wyeth*) – in order to simulate a more realistic scenario. The optimized treatment process was further applied to a real WWTP effluent containing, apart from lorazepam, several other emerging contaminants.

2. Experimental

2.1. Contaminated water samples

TiO_2 (P25 Degussa, 80% anatase and 20% rutile) concentration optimization was performed using a ca. 200 $\mu g\ L^{-1}$ lorazepam solution, obtained by dissolving 1 mg Lorenin® pills (*Wyeth*) in distilled water [15]. Lorazepam's photodegradation (photo-induced structural simplification) was furthermore evaluated on a real wastewater effluent, where several other emerging contaminants were as well present.

Table 1 presents the main characteristics of the effluent sample collected at Febros WWTP. This WWTP discharges its treated effluent into Febros River, a southern bank small tributary of Douro River, located in the north of Portugal. Moreover, a 2 $mg\ L^{-1}$ lorazepam spiked effluent sample was also submitted to photocatalytic treatment. This concentration was obtained by dissolving 40 Lorenin® pills (1 mg of lorazepam each) in 20 L of the real effluent sample. Besides assuring a proper kinetic characterization and possible future identification of some lorazepam's degradation products (LDPs) formed during photodegradation, this procedure also simulates a more realistic scenario, where lorazepam is among several other compounds, also present in pharmaceutical formulations (numerous excipients, including titanium dioxide itself).

2.2. Analytical determinations

pH, temperature and conductivity were measured using a pH meter HANNA HI8424 and a conductivity meter HANNA HI4522; turbidity was determined in a Merck® Turbiquant 3000 R; dissolved oxygen was measured using a multi-parametric YSI probe; oxidability was determined according to ISO 8467:1993 [16] and total suspended solids (TSSs) and volatile suspended solids (VSSs) were determined according to the standard methods book [17]. Nitrate, nitrite, bromide, chloride, fluoride and sulphate were quantified by ion chromatography (Dionex DX-120), using a Dionex Ionpac AS9-HC 4 × 250 mm column. The program for anions determination comprises a 20 min run using 9 mM Na_2CO_3 as eluent at a flow rate of 1.0 $mL\ min^{-1}$. Ammonium and phosphate were measured with Merck® Spectroquant kits. Metals were analyzed and quantified using an ICP-MS (X-Series, Thermo Elemental). Dissolved organic carbon (DOC) was measured in a TC-TOC-TN analyzer (Shimadzu, model TOC-V_{CSN}) provided with a NDIR detector and calibrated with standard solutions of potassium phthalate. Dissolved nitrogen was measured by thermal decomposition and NO detection by a chemiluminescence method in the same TC-TOC-TN analyzer coupled with a TNM-1 unit (Shimadzu, model TOC-V_{CSN}) calibrated with standard solutions of potassium nitrate. Absorbance at 254 nm was determined using an Unicam spectrophotometer (model Super Sipper), in order to indirectly evaluate the content in aromatic compounds. Finally, the concentration profile of several pharmaceutical compounds was obtained by solid-phase extraction followed by liquid chromatography coupled to mass spectrometry (SPE-LC-tandem MS), according to the procedure thoroughly described by Sousa et al. [1], while some fragrances and earthy–musty compounds were also quantified by gas chromatography–mass spectrometry (scan or MS/MS mode), as detailed in Machado et al. [18].

During the photocatalytic experiment, pH, temperature, absorbance, total nitrogen (TN) and DOC were followed, giving indications about the mineralization state. Finally, all different emerging contaminants detected in the original effluent sample were also continuously followed through the entire photo-treatment process.

2.3. Solar CPC pilot plant

The solar TiO_2-photocatalytic experiments were performed in a pilot plant with compound parabolic collectors (CPCs) installed in the roof of the Chemical Engineering Department of the Faculty of Engineering, University of Porto (FEUP), Portugal (Fig. 1) [19]. The plant is composed by one CPC unit (0.91 m^2) of four borosilicate tubes (Schott-Duran type 3.3, Germany, cut-off at 280 nm, external diameter 50 mm, length 1500 mm and thickness

Table 1
Physicochemical characterization of the effluent sample collected at Febros WWTP and used for the solar photocatalytic experiment.

Parameter (units)	Febros WWTP effluent (18/03/2011)	ELV[a] Dec. Lei no. 236/98, de 1 de Agosto 1998 (portuguese legislation)
Color	Pale yellow; n.d[b] at dil.1:20	n.d. For dilution 1:20
Odor	n.d. (dil.1:20)	n.d. For dilution 1:20
pH	7.3	6.0–9.0
Temperature (°C)	20.0	3 °C Increase[c]
Turbidity (NTU)	115	–
Conductivity (μS cm^{-1})	555	–
Dissolved oxygen (mg L^{-1})	2.2	–
Oxidability (mg L^{-1})	56.2	–
Total dissolved carbon (mg L^{-1})	37.5	–
Inorganic carbon (mg L^{-1})	25.4	–
Dissolved organic carbon (mg L^{-1})	12.1	–
Absorbance at 254 nm (AU)	0.07	–
Total suspended solids (mg L^{-1})	363	60
Volatile suspended solids (mg L^{-1})	223	–
Ammonium – NH_4^+ (mg L^{-1})	1.2	10
Nitrate – $NO)_3^-$ (mg L^{-1})	23.3	50
Nitrite – NO_2^- (mg L^{-1})	<0.5	–
Bromide – Br^- (mg L^{-1})	<0.5	–
Chloride – Cl^- (mg L^{-1})	78.5	–
Fluoride – F^- (mg L^{-1})	<0.5	–
Phosphate – PO_4^{3-} (mg L^{-1})	7	–
Sulphate – SO_4^{2-} (mg L^{-1})	59.8	2000
Phosphorus – ^{31}P (mg L^{-1})	3.0	10,000
Metals		
Sodium – ^{23}Na (mg L^{-1})	78.8	–
Potassium – ^{39}K (mg L^{-1})	21.1	–
Calcium – ^{44}Ca (mg L^{-1})	274.8	–

[a] ELV – Emission limit value.
[b] n.d. – Not detected.
[c] Comparatively to the receptor medium.

1.8 mm) connected in series by polypropylene junctions with their CPC mirrors in anodized aluminum, supported by an aluminum structure and tilted 41° (local latitude). The pilot plant has also two recirculation tanks (10 L and 20 L), two recirculation pumps (maximum 20 L min^{-1}), two flow rate meters, five polypropylene valves and an electric board for process control. The pilot plant is operated in batch mode and can be used in two ways: using the total CPCs area (0.91 m^2) or using 0.455 m^2 of the CPCs area individually, giving the possibility to carry out two distinct experiments at the same time and at the same solar radiation conditions.

The intensity of the solar UV radiation is measured by a global radiometer (ACADUS 85-PLS), placed at the same inclination angle, which provides the data in terms of instantaneous UV radiation (W m^{-2}). According to Eq. (1), one is able to calculate the accumulated UV energy ($Q_{UV,n}$, kJ L^{-1}) received on any surface in the same position with regard to the sun, per unit of water volume inside the reactor, in a given time interval Δt:

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

where t_n is the time corresponding to n-water sample (s), $V_{t,n}$ is the total volume of wastewater at n-water sample (L), A_r is the illuminated collector surface (m^2), and $\overline{UV}_{G,n}$ is the average solar UV radiation (W m^{-2}) measured in the time interval Δt_n.

Fig. 1. Solar Pilot Plant with CPCs where the TiO_2-photocatalytic treatment of the WWTP effluent was performed.

2.4. Experimental set-up

Photocatalytic processes were conducted during sunny days, using the solar CPC pilot plant described in Section 2.3.

Experiments using the WWTP effluent started with the addition of 20 L to one recirculation tank and 25 L to the other recirculation tank of the CPC units, followed by homogenization by turbulent recirculation during 15 min, in the darkness (a first control sample was taken for characterization). The goal was to perform two parallel experiments, one with the real effluent and another with lorazepam spiked effluent, under the same solar irradiation conditions, taking profit of the two independent CPC units (see Section 2.3.). Then, TiO_2 was added to both reservoirs, up to a final concentration of 0.2 g L^{-1} – optimal determined concentration [15,20]. Next, Lorenin® pills were dissolved only in the reservoir supplying the CPC unit containing the 20 L of WWTP effluent, in order to achieve a lorazepam spiking concentration of 2 mg L^{-1}. After 15 min of turbulent recirculation in the dark, another control sample was taken from each tank to evaluate the contaminants' adsorption onto the TiO_2 surface and, finally, the solar collectors were uncovered and the experiments began.

Samples were taken at pre-defined times, from each recirculation tank, with several purposes: degradation kinetics evaluation (by SPE-LC-MS/MS [1] and GC–MS [18]) of the different emerging

contaminants, including lorazepam, DOC, TN and absorbance (254 nm) measurements, biodegradability and toxicity assays.

2.5. Biodegradability test

A 28 days Zahn–Wellens biodegradability test was carried out, according to the OECD standards [21], for biodegradability evaluation of all samples, collected at different phototreatment times, from the experiment with 2 ppm lorazepam spiked effluent. 240 mL of each sample were added to an open glass flask, along with some mineral nutrients (KH_2PO_4, K_2HPO_4, Na_2HPO_4, NH_4Cl, $CaCl_2$, $MgSO_4$ and $FeCl_3$) and activated sludge from Freixo WWTP (Porto, Portugal), previously centrifuged and added in proportion to the DOC of the sample. A control and a blank experiment were performed in parallel, using glucose as carbon source (highly biodegradable) and distilled water, respectively. All vessels were kept in the dark, inside a thermostatic chamber at 25 °C, under continuous magnetic stirring, for a 28 days period.The percentage of biodegradation (D_t) can be determined by Eq. (2):

$$D_t = \left[1 - \frac{(C_t - C_B)}{(C_A - C_{BA})}\right] \times 100 \quad (2)$$

where C_A and C_{BA} are the DOC (mg L^{-1}) in the mixture and in the blank experiment, respectively, measured 3 h after the beginning of the experiment, C_t and C_B are the DOC (mg L^{-1}) in the mixture and in the blank experiment, measured at the sampling time t. Samples are considered biodegradable only when D_t is higher than 70%. After the 28 days experiment, the levels of lorazepam, as well as of all other pharmaceuticals previously detected, were determined by SPE-LC-MS/MS [1] and compared to those obtained prior to the biodegradability assay.

2.6. Toxicity assessment

The toxicity test herein used for acute toxicity measurements is based on the bioluminescence inhibition of *Vibrio fischeri* NRRL B-111 77 by means of the ToxAlert 100 system from Merck. It is a well-established test, extensively described in literature [21–24] and similar in principle to the better-known Microtox® test.

It is based on the fact that toxic substances will cause changes either in cell structures and/or metabolic pathways of the marine bacteria *V. fischeri*, consequently reflected in a decrease of bioluminescence. This inhibition, caused by the toxic effect of the sample, can be calculated against the response given by a saline control solution, which accounts for the natural decrease in light emission. Data were collected after 15 and 30 min of time exposure of the bacteria to all non-spiked effluent samples, collected over the photocatalytic treatment, after a tonicity adjustment corresponding to 2% NaCl. Phenol (20 mg L^{-1}) and potassium dichromate (40 mg L^{-1}) were used as positive controls.

3. Results and discussion

3.1. Lorazepam and TiO_2 concentrations

Experimental variables were previously optimized in distilled water, including lorazepam/catalyst's concentrations and UV light source [15], in order to achieve a maximal removal efficiency at the least reagents/energy expenses, so that the process becomes economically viable and potentially applicable at a larger scale.

Different criteria were taken into account for decision making regarding which lorazepam's concentration ought to be used. The concentration of ca. 200 μg L^{-1} (obtained by dissolving 1 mg Lorenin® pills) was adopted, bearing in mind a compromise between a concentration value simulating real conditions in WWTP effluents and the concentration required for a proper kinetic characterization, according to instrumental quantification limits (LC–MS). Under these conditions, and using the Solar Pilot Plant with CPCs described in Section 2.3., the required TiO_2 concentration for optimal performance was ca. 200 mg L^{-1} [15].

3.2. WWTP effluent physicochemical characterization

The performance of the optimized photo-oxidation treatment process was further assessed using a real municipal WWTP effluent. Some physicochemical properties of this grab effluent sample are summarized in Table 1.

Results of the analyzed parameters were compared to the legal emission limit values (ELVs) of wastewaters' discharge into the aquatic environment, as stated in the Law Decree 236/98 [25]. However, these discharge values are only established for monthly averages, considering daily average values of representative 24 h composite samples, collected and analyzed over the respective month.

Nevertheless, all analyzed parameters were within/below the respective legal interval/limit value, with the exception of TSS – 363 mg L^{-1}, far above the legally imposed 60 mg L^{-1}. With regard to the DOC content, it was low (12.1 mg L^{-1}), as expected, since we are dealing with a municipal WWTP effluent. A low DOC indicates that some recalcitrant organic compounds that might still exist, namely pharmaceuticals (see Table 2), shall be in low concentrations.

Regarding metals' composition, apart from those presented in Table 1 (which are below the respective ELV), several others were investigated (including arsenic, lead, mercury, cadmium, iron and aluminum), but all showed to be below the respective method quantification limit (MQL), already in agreement with legal limit values.

Therefore, AOPs' application, and in this particular case TiO_2-photocatalysis, may be seen as an alternative or complementary method to the already existing/applied technologies, in order to improve the removal rates of some refractory compounds, such as pharmaceuticals. These compounds are usually present at low levels, though which may be high enough to induce several chronic toxicity effects.

3.3. Heterogeneous TiO_2-photocatalytic treatment

In this work, the photon source used for the phototreatment of the municipal WWTP effluent was the sunlight radiation, a renewable energy source and, therefore, more economically attractive and sustainable.

Samples collected from both experiments, at pre-defined times, were analyzed with several purposes. From DOC analysis (Fig. 2a), it can be deduced that the significant difference between the spiked and non-spiked effluent samples is mainly due to lactose, the only water-soluble excipient present in Lorenin® formulation. The excipients constitute the major portion of each Lorenin® pill (100 mg), in which there is only 1 mg of lorazepam. Fig. 2a also shows that a considerable DOC reduction (∼37% and ∼41% for spiked and non-spiked effluents, respectively) took place still in the dark phase of the experiment, due to adsorption on the catalyst surface, as described in literature [26]. Total mineralization percentages achieved at the end of the photocatalytic reaction were approximately 50% for spiked and 59% for non-spiked effluents. These results allowed deriving two main conclusions: on the one hand, and regarding the non-spiked effluent, there was still a significant amount of organic compounds not totally degraded by the phototreatment – though chemical toxicants, including pharmaceuticals, may have ceased to exist, possible oxidation intermediates still remained; on the other hand, regarding the spiked effluent, the major components of the pills (excipients) are also refractory to complete mineralization (i.e. leading to H_2O and CO_2 as sole final products), since the final DOC value was still very distant from the initial DOC of the non-spiked effluent sample.

Table 2
Initial concentration values (C_i) and first order degradation kinetic parameters ($C_t = C_0 \times e^{-kt}$) determined for all emerging contaminants (pharmaceuticals, fragrances and earthy–musty compounds) quantified in the WWTP effluent sample (value ± standard error).

Emerging Pollutant		MQL[a] (ng L^{-1})	C_i[b] (ng L^{-1})	C_0[c] (ng L^{-1})	k[d] (L kJ^{-1})	r_0[e] (ng L^{-1})	r^2[f]	S_r^2[g] (ng L^{-1})
Pharmaceutical compounds	Fluoxetine	9	24	29	1.2 ± 0.3	34 ± 8	0.8873	14
	Paroxetine	3	29	38	0.8 ± 0.02	29 ± 9	0.7331	58
	Diclofenac	79	24,256	10,473	0.48 ± 0.03	4991 ± 320	0.9888	217570
	Clotrimazole	7	12	16	0.40 ± 0.08	6 ± 1	0.8176	4
	Azithromycin	5	631	680	0.37 ± 0.03	250 ± 19	0.9834	1402
	Lorazepam	6	682	640	0.33 ± 0.05	208 ± 35	0.9143	6465
	Propanolol	17	52	30	0.26 ± 0.05	8 ± 1	0.9371	4
	Furosemide	35	492	448	0.26 ± 0.03	117 ± 16	0.9331	1539
	Hydrochlorothiazide	58	3051	2740	0.256 ± 0.033	701 ± 92	0.9434	80,508
	Carbamazepine	7	417	399	0.21 ± 0.02	82 ± 6	0.9888	642
	Bisoprolol	2	132	119	0.17 ± 0.01	20 ± 2	0.9730	59
	Fenofibrate	7	53	39	0.16 ± 0.02	6.3 ± 0.9	0.9091	20
	Ofloxacin	33	101	90	0.14 ± 0.01	13 ± 1	0.9430	29
	Losartan	6	149	78	0.117 ± 0.008	9.1 ± 0.6	0.9598	19
	Ketoprofen	7	410	317	0.10 ± 0.01	33 ± 3	0.9569	519
	Norfloxacin	33	138	112	0.10 ± 0.01	11 ± 2	0.8364	167
	Lorazepam spk[h]	6	1.21×10^6	1.08×10^6	0.094 ± 0.003	$1.0 \times 10^5 \pm 0.4 \times 10^5$	0.9912	1×10^9
	Carvedilol	7	95	78	0.06 ± 0.01	4.5 ± 0.8	0.8933	75
	Fluconazole	25	110	82	0.049 ± 0.003	4.0 ± 0.2	0.9658	3
	Ciprofloxacin	4	254	229	0.040 ± 0.002	9.2 ± 0.4	0.9660	33
	Gemfibrozil	36	215	223	0.035 ± 0.002	7.8 ± 0.4	0.9675	26
	Alprazolam	4	244	89	0.026 ± 0.004	2.3 ± 0.4	0.8917	34
	Terbinafine	15	37	34	–	–	–	–
Fragrances & earthy–musty compounds	2,4,6-Trichloroanisole	7	226	170	0.56 ± 0.02	95 ± 4	0.9958	19
	Galaxolide	7	3604	206	0.24 ± 0.04	49 ± 9	0.9245	548
	Musk ketone	10	134	33	0.15 ± 0.01	5.0 ± 0.4	0.9536	2
	Tonalide	13	703	55	0.12 ± 0.02	6 ± 1	0.8285	63

[a] MQL – Method quantification limit.
[b] C_i – Concentration before the addition of TiO_2.
[c] C_0 – Concentration after the addition of TiO_2.
[d] k – Reaction rate constant.
[e] r_0 – Initial reaction rate.
[f] r^2 – Determination coefficient.
[g] S_r^2 – Estimation of variance.
[h] spk – Spiked.

Concerning total nitrogen content, it did not vary significantly during the photocatalytic process for both experiments.

Temperature and pH variations were also accompanied over the phototreatment experiments (Fig. 2b). Firstly, it should be remarked that the photocatalytic experiments took place mainly during one day – only the last sample (at ca. 32 and 42 kJ L^{-1} of accumulated UV energy for non-spiked and spiked effluent samples, respectively) was collected at the end of the following day. Bearing in mind that temperature usually rises from morning start-up (15–20 °C) to an almost constant value for several hours until 2 pm, decreasing again over the afternoon, depending on the sunlight intensity, the maximum temperature achieved during the phototreatment was approximately 28 °C, what discards the hypothesis of any thermo-mediated degradation [27]. Since pH is "temperature-dependent", pH variations accompanied temperature fluctuations over the day. Nevertheless, pH values were in compliance with the Law Decree 236/98 (between 6.0 and 9.0) during the entire experiment.

Finally, the aromatic content followed a similar trend to the DOC, decreasing 94% for the non-spiked effluent, while for the spiked sample it only diminished ca. 64%. Additionally, special attention was given to the degradation kinetics, over the phototreatment, of each emerging contaminant (EC) detected in the initial WWTP effluent sample.

As already pointed out in the Introduction section, conventional WWTPs are not specifically prepared for the complete removal[1] of such micropollutants as pharmaceuticals. These can still remain in the effluents due to their low tendency to adsorb onto the activated sludge or because their microbial degradation is not fast enough to be completed within the respective WWTP hydraulic retention time [6]. Therefore, this photocatalytic approach was a promising attempt to increase pharmaceuticals' removal rates.

Table 2 presents the initial concentration values (C_i) obtained for the 22 pharmaceuticals, 3 fragrances and 1 earthy–musty compound (2,4,6-Trichloroanisole – 2,4,6-TCA) detected in the effluent sample, prior to phototreatment. As previously mentioned, these C_i values may differ from the concentrations measured at $Q_{UV} = 0$ (C_0) due to adsorption to the catalyst surface (in the dark). Apart from some exceptions (fluoxetine, paroxetine, clotrimazole, norfloxacin, carvedilol, alprazolam and tonalide), the majority of the degradation profiles of the detected ECs follows well a first order kinetic model, with r^2 above 0.90. Table 2 also compiles the kinetic constant (k) and the initial reaction rate (r_0) estimated for each compound, when submitted to the described TiO_2-photocatalytic reaction. In Table 2, all pharmaceutical compounds are ordered from the highest to the lowest rate constant value (k). Interestingly, if we compare the k value of native lorazepam (naturally present in the effluent sample) with that of total lorazepam, achieved after spiking with Lorenin® pills, we can observe that the first one is higher ($0.33 \pm 0.05 > 0.094 \pm 0.003$). Since the only difference relates to the presence of the pills' excipients in the spiked sample, it can be concluded that they might be competing for the hydroxyl radicals, leading to a lower lorazepam degradation rate. Please note that the DOC content in the spiked sample was much higher than the unspiked one.

[1] The term "removal" is always used in this article referring to the conversion of the pollutant to compounds other than the parent compound.

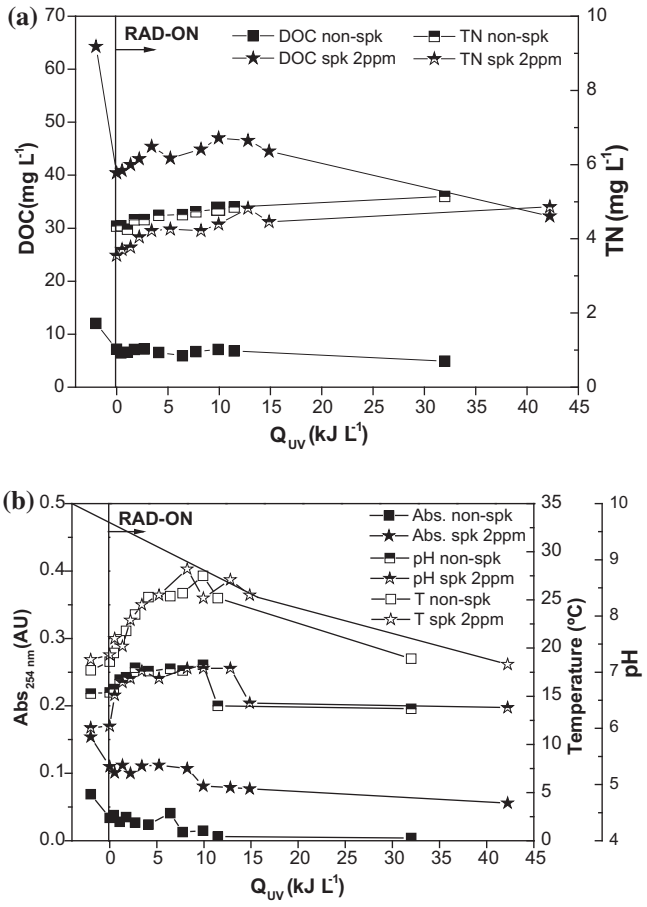

Fig. 2. Physicochemical parameters followed throughout the photocatalytic reaction: (a) DOC (dissolved organic carbon) and TN (total nitrogen) and (b) absorbance at 254 nm, temperature and pH variation represented as a function of the amount of accumulated UV energy per liter of effluent.

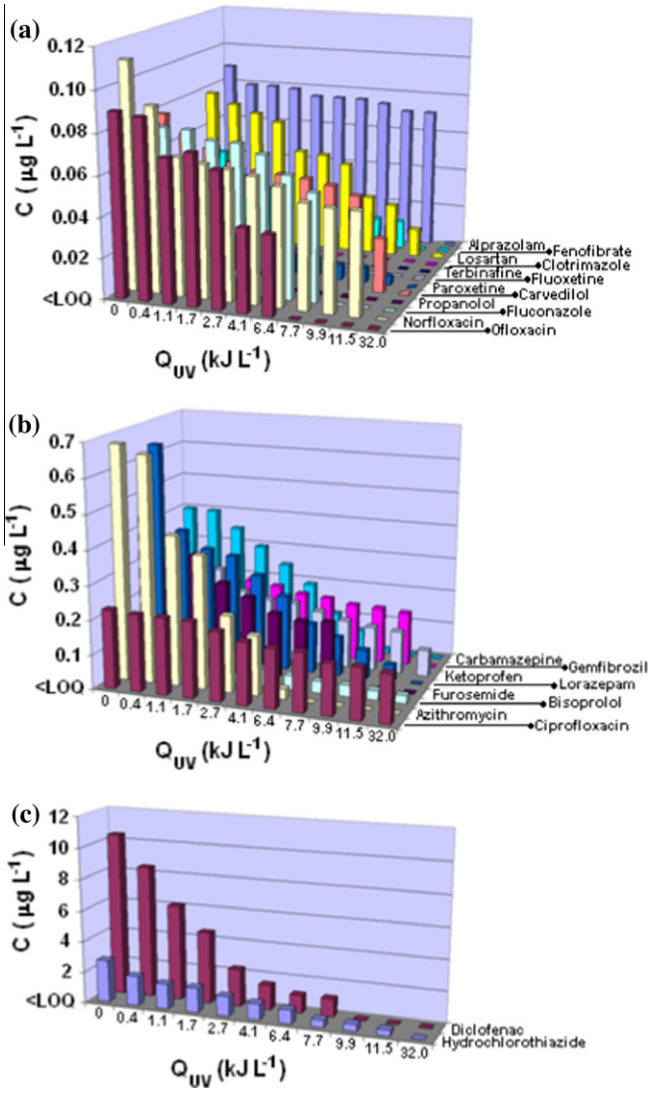

Fig. 3. Graphic representation of the concentration evolution of the 22 pharmaceutical compounds present in the WWTP effluent, over the phototreatment process, as a function of the amount of accumulated UV energy per liter of effluent.

Some problems could be anticipated when applying the optimized photocatalytic method to the treatment of a real WWTP effluent, since wastewaters are more complex mixtures. Interference of other oxidizable compounds and scavengers such as carbonates, which typically occur in high concentrations in wastewaters [28,29], could influence the method's efficiency. Nevertheless, except for ciprofloxacin and a small amount of ketoprofen and bisoprolol, all pharmaceuticals initially quantified in the effluent sample decreased to concentration levels below the respective MQLs, attesting therefore the method's practical applicability (Fig. 3). Even lorazepam, a recalcitrant compound whose removal improvement has never been tested by means of accelerated oxidation processes, showed a complete degradation.

Nonetheless, and still regarding the three remaining pharmaceuticals, they presented already much lower concentrations at the end of the phototreatment (corresponding to a total accumulated UV energy of approximately 32 kJ L^{-1}). A small increase in the UV irradiation exposure (time/intensity) could easily allow their complete removal (up to 38 kJ L^{-1}, except for ciprofloxacin – 101 kJ L^{-1} necessary). Otherwise, and depending on the local weather conditions, a proper management of the combination with a complementary artificial UV [30] source could also be an economically viable solution.

Furthermore, and according to Gros et al. [6], the anti-epileptic carbamazepine usually presents a low removal percentage, or sometimes even a concentration increase, after leaving the WWTP, due to conversion of carbamazepine glucuronides and other conjugated metabolites to the parent compound by enzymatic reactions occurring over the treatment processes. With the applied photocatalytic treatment, its removal was complete, which is in agreement with previous results obtained by Miranda-García et al. [31] and Rizzo et al. [32]. The same occurred with the moderately removed antibiotics norfloxacin and ofloxacin, the diuretic furosemide and the lipid regulators bezafibrate and gemfibrozil [1,6].

3.4. Biodegradability assay

Zahn–Wellens biodegradability test is considered to be especially accurate, as it involves a long contact period (28 days) between the effluent sample and the sludge, in order to allow some adaptation of the microorganisms.

This assay was performed at different stages of the photocatalytic treatment of the Lorenin® spiked effluent, with the purpose of determining the optimal phototreatment time to reach a biodegradable effluent (D_t > 70%). It is however important to highlight that it would not be reasonable to perform such a test over the non-spiked effluent, since it was collected at the exit of the WWTP (itself including the treatment by conventional activated sludge)

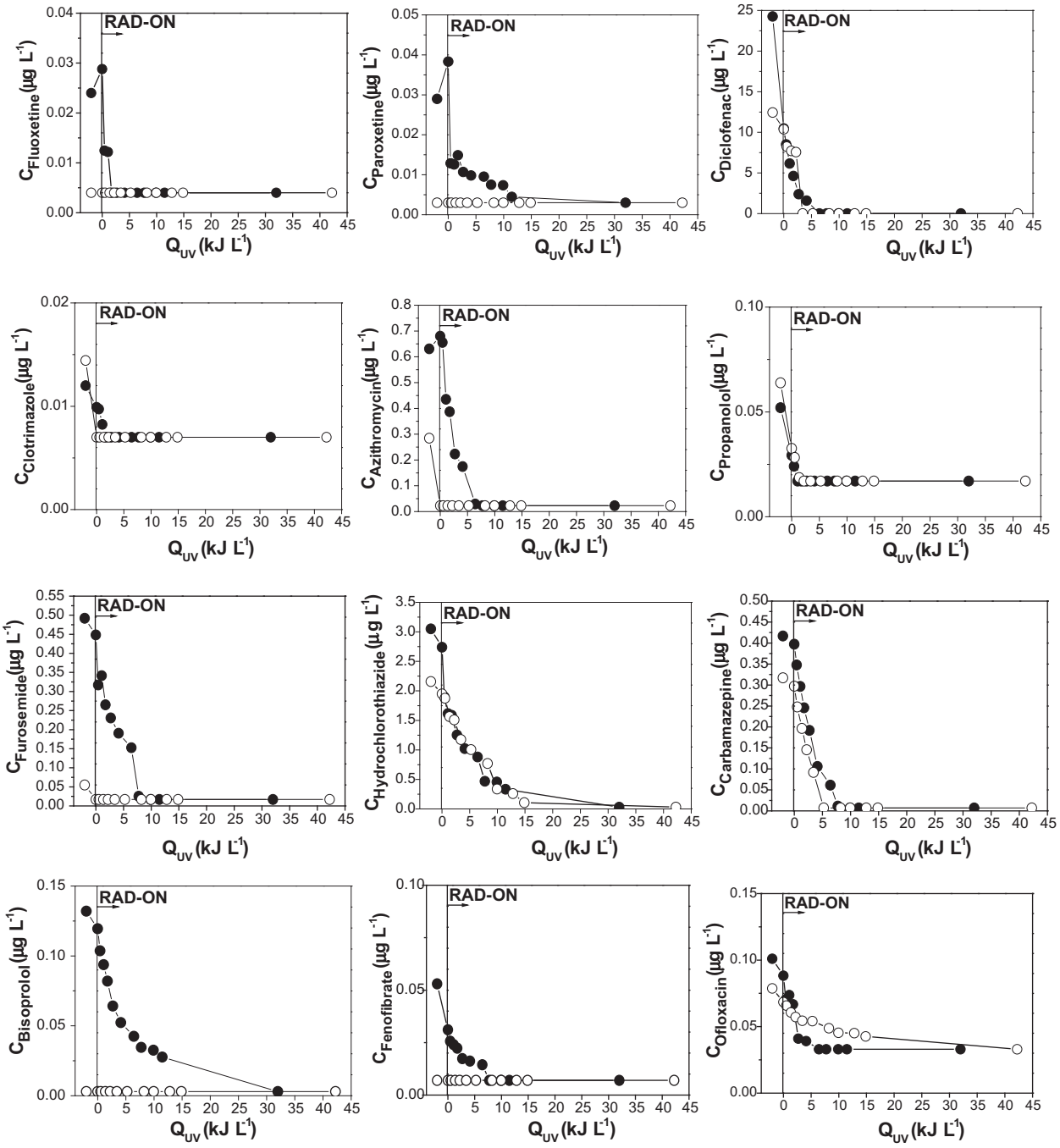

Fig. 4. Representation of the concentration evolution of all pharmaceutical compounds (except for terbinafine) present in the spiked WWTP effluent, over the phototreatment process, as a function of the amount of accumulated UV energy per liter of effluent: ● – before Zahn–Wellens test; ○ - after Zahn–Wellens test; ★ – lorazepam already present in the effluent sample, before spiking with Lorenin® pills.

presenting an already low DOC of ca. 12 mg L^{-1}, which moderately decreased to ca. 5 mg L^{-1} at the end of the phototreatment (refer to Section 3.2.).

Zahn–Wellens' results showed that none of the spiked effluent samples collected over the photocatalytic process presented a D_t value >70%, therefore the phototreatment did not significantly improve the effluent's biodegradability. Nevertheless, all samples were again analyzed in terms of pharmaceutical content, with the aim of understanding which pharmaceutical compounds would already be biodegradable in the first place. This aspect is particularly important considering that biological treatments are usually more interesting than phototreatments (when efficient), due to economic reasons.

Fig. 4 demonstrates that the majority of the analyzed pharmaceuticals seems refractory to biological treatment, since the concentrations before and after Zahn–Wellens test are quite similar. Nonetheless, some pharmaceuticals such as fluoxetine, paroxetine, azithromycin, furosemide, bisoprolol, fenofibrate, losartan, ketoprofen, norfloxacin, carvedilol and gemfibrozil look susceptible to the microorganisms present in the sludge, as their concentrations significantly decreased after the Zahn–Wellens biodegradability test. After thorough analysis of the removal rates of several pharmaceuticals and personal care products by different biological wastewater treatment processes, Sui et al. [33] reported that in the case of gemfibrozil, conventional activated sludge treatment could reach an 80% removal efficiency, which is in agreement with

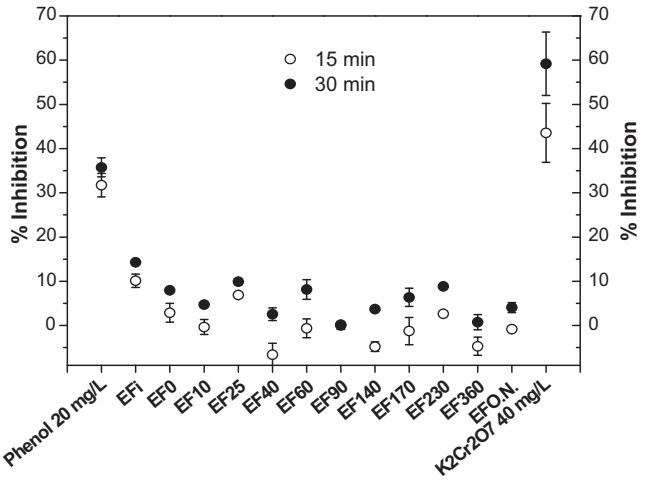

Fig. 5. *Vibrio fischeri* inhibition percentage, after 15 and 30 min, for two positive controls (phenol 20 mg L^{-1} and potassium dichromate 40 mg L^{-1}) and all non-spiked effluent samples collected over the phototreatment process; EF – effluent; *i* – initial (before TiO_2 addition); 0, 10, 25, 40, 60, 90, 140, 170, 230, 360 and O.N. (overnight) correspond to the sunlight exposure time (following TiO_2 addition) after which each sample was collected, during the photocatalytic reaction.

the results herein obtained. Also Laera et al. [34] observed that the refractory compound carbamazepine, despite not being biodegradable, presented a ca. 95% removal rate after photocatalytic treatment with TiO_2, which again reinforces our results/conclusions.

3.5. Toxicity outcome

According to Rizzo [35], despite the fact that AOPs are being widely used in wastewater treatment for the removal of both organic and inorganic contaminants, as well as to increase effluents' biodegradability, a partial oxidation may result in the formation of intermediates more toxic than parent compounds.

Therefore, and to prevent this potential drawback in cases where complete mineralization is not achieved, AOPs are expected to be carefully monitored and, consequently, toxicity tests must be used to evaluate whether effluents' detoxification is accomplished.

In this work, the acute toxicity of the non-spiked effluent sample was followed over the photocatalytic treatment, by means of % inhibition of *V. fischeri*'s bioluminescence, after 15 and 30 min. Two positive controls (phenol and potassium dichromate) were used to assure bacteria viability.

Results given in Fig. 5 show that inhibition percentages were already low (<15%) from the beginning of the experiment, therefore indicating that the effluent presented no significant toxicity. Nevertheless, these results are still of great importance to attest that no toxicity increase occurred over the phototreatment process, contrarily to the examples reported by Klamerth et al. [36].

Although acute toxicity tests give a fast and preliminary information on the hazard of the sample, these tests may not always be the most suitable to evaluate the ecotoxicological hazard of micropollutants such as pharmaceuticals, due to their usually low environmental concentrations [37]. Thus, studies on chronic effects should be used in environmental studies, but due to their duration they are not appropriate to be used as toxicity checks in photocatalytic experiments.

4. Conclusions

The optimized TiO_2-photocatalytic treatment, using a solar pilot plant with CPCs, proved to fit its purpose of removal/degradation of several ECs, among which are lorazepam and other pharmaceuticals, present in a real WWTP effluent. With a total accumulated UV energy of approximately 32 kJ L^{-1}, 19 out of the 22 pharmaceutical compounds present in the effluent sample were completely removed. Regarding the three remaining pharmaceuticals, ciprofloxacin, ketoprofen and bisoprolol, their removal percentages were 35%, 61% and 77%, respectively.

In addition to the almost complete pharmaceuticals' removal through phototreatment, a Zahn–Wellens biodegradability test allowed to distinguish, among the present pharmaceutical compounds, between the ones that could be removed through biological treatment and those probably refractory to biotreatment, therefore requesting the photocatalytic treatment.

The acute toxicity assay using the marine bacteria *V. fischeri* revealed that, despite the initial effluent itself presented no significant toxicity, it did not increase over the photocatalytic process, thus discarding the possibility of formation of more toxic intermediate oxidized compounds.

In conclusion, this photocatalytic treatment proved to be a promising tool with practical future applicability, since it requires the use of solar renewable energy.

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Chapter 5

Phototreatment Mechanisms and By-Products Structural Elucidation

Identifying pollutants' degradation products in environmental matrices is a very complex task faced by researchers. However, it is also fundamental to know the identity of these compounds, in order to further assess their environmental impact and potential individual/mixture toxicity.

In this context, the present chapter includes the study of the photodegradation treatment of lorazepam, a recalcitrant anxiolytic drug frequently detected in environmental water samples. To the best of our knowledge, this work was never undertaken before.

Herein follows **Paper 5**, which presents the work carried out with the aim to elucidate lorazepam photodegradation mechanism. The phototreatment was performed via different processes, including solar UV radiation vs. artificial UV radiation sources, as well as photolytic vs. photocatalytic pathways. Moreover, the identification of the main photoproducts was carried out by ultra performance liquid chromatography–quadrupole-time-of-flight-mass spectrometry (UPLC/QqToF-MS), as previously described in chapter 2.

5.1. Paper 5 – Lorazepam Photofate under Photolysis and TiO_2-assisted Photocatalysis: By-products' Identification and Evolution Profiles during Phototreatment of a Contaminated WWTP Effluent

Scientific Paper:

Sousa, M.A., Lacina, O., Hrádková, P., Pulkrabová, J., Vilar, V.J.P., Gonçalves, C., Boaventura, R.A.R., Hajšlová, J. and Alpendurada, M.F., *Lorazepam Photofate under Photolysis and TiO_2-assisted Photocatalysis: By-products' Identification and Evolution Profiles during Phototreatment of a Contaminated WWTP Effluent*. Submitted for publication in Environmental Science and Technology, September 25, 2012.

Lorazepam Photofate under Photolysis and TiO_2-assisted Photocatalysis: By-products' Identification and Evolution Profiles during Phototreatment of a Contaminated WWTP Effluent

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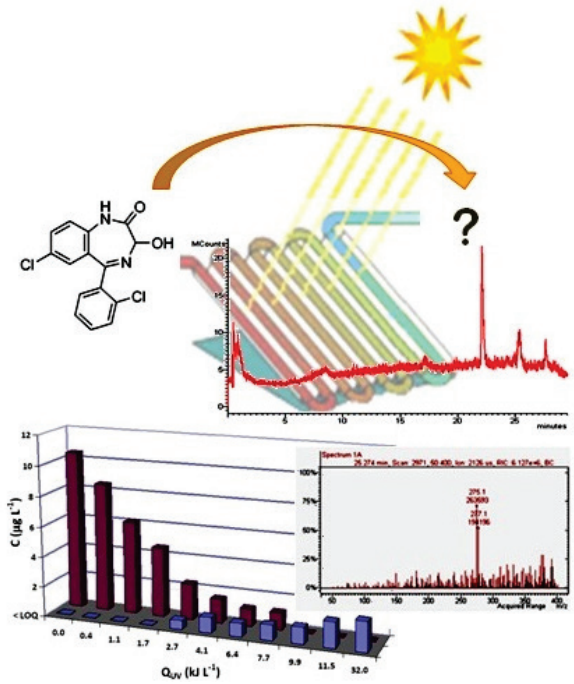
ABSTRACT: This manuscript reports on the study of Lorazepam (LZP) phototransformation pathways under artificial UV and natural solar irradiation, through photolytic and TiO_2-assisted photocatalytic processes. Three experimental set-ups were employed: two lab-scale photoreactors, each provided with an UV lamp (one medium pressure mercury lamp and one blacklight blue lamp), and a pilot-scale Solar Plant with Compound Parabolic Collectors (CPCs). Samples collected along the different phototreatment experiments were analyzed by ultra-high performance liquid chromatography–quadrupole-time-of-flight–mass spectrometry (UHPLC/QqToF-MS). The key assumption of the analytical approach was that related compounds (LZP and its by-products (LBPs)) provide identical "diagnostic fragment ions". Identification was also based on the chlorine atoms specific isotopic pattern, as well as accurate masses. Six major LBPs were identified and elucidated, with nominal $[M+H]^+$ masses of 337, 303, 319, 275, 291 and 293 Da. The proposed LZP photodegradation mechanism included the initial opening of the diazepinone seven-membered ring, followed by a rearrangement into a highly stabilized six-membered aromatic ring and subsequent cleavage and/or hydroxylation reactions. LBPs' evolution profiles were described for each of the three experimental prototypes and the CPCs Solar Pilot Plant proved to be the most efficient one. Finally, LZP photocatalytic degradation was further assessed on a contaminated municipal effluent, where the photoproducts generated showed to be more persistent than LZP itself.

Abstract Art



INTRODUCTION

Pharmaceuticals have become prominent emerging contaminants due to their demonstrated presence and persistence in environmental waters, as well as for the potential toxicological effects they may induce on non-target organisms. Some major concerns have been raised regarding their possible estrogenic effects, not to mention the development of different bacterial resistances (creation of "Super Bugs"). It is currently estimated that approximately 3000 different compounds are used as pharmaceutically active substances, including analgesics, antibiotics, anxiolytics, antidepressants, β-blockers, lipid regulators, antidiabetics, neuroleptics, contraceptives and impotence drugs. Nevertheless, still only a rather small subset of these compounds has been investigated in environmental studies.[1]

Due to the growing competitiveness installed in modern society, the consumption of anxiolytic drugs has recently shown a remarkable increase. Within these drugs are benzodiazepines (BDZ), a family of compounds which enhance the effect of the neurotransmitter gamma-aminobutyric acid (GABA), thus resulting in anxiolytic (anti-anxiety) and sedative activities, as well as hypnotic (sleep-inducing), anticonvulsant, muscle relaxant and amnesic effects.[2] According to the International Narcotics Control Board,[3] in 2008 a total of 30 billion S-DDD (defined daily doses for statistical purposes) of BDZ were manufactured, the highest amount registered up until today.

Moreover, Portugal was considered one of the countries with the highest anxiolytics consumption rate.[3] Amongst the most marketed anxiolytic drugs in Portugal, Lorazepam (LZP) highlights with a quite elevated sales value over the past 5 years, according to unpublished data provided by the Portuguese National Authority of Medicines and Health Products, IP (INFARMED).

Pharmaceutical compounds in general reach the aquatic media, where they have been found in concentrations ranging from < 0.1 to ca. 20 μg L^{-1}, among treated wastewaters,

surface and groundwater and even drinking water.[4-8] LZP, in particular, has already been quantified in levels ranging from approximately 0.040 μg L^{-1} in river waters to ca. 0.200 μg L^{-1} in effluent wastewaters.[9-11] The environmental aquatic pollution by such persistent organic pollutants is commonly attributed either to point-source contaminations or following the incomplete removal in wastewater treatment plants (WWTPs). In fact, WWTPs are not prepared for the complete degradation of such micropollutants, as mentioned in several studies.[12-14]

The limitations of conventional wastewater treatments for the remediation of emerging pollutants require the development and application of advanced tertiary/quaternary treatment processes. In this field, special attention has been given to Advanced Oxidation Processes (AOPs), in which hydroxyl radicals ($^{\bullet}OH$) are the responsible agents for the oxidation and mineralization of almost any organic molecule, due to their strong unselective oxidative power, yielding CO_2 and inorganic ions as final products.[15] Common techniques already reported in the literature include the use of UV alone, UV/TiO_2, UV/H_2O_2, UV/Fe^{3+}, UV/H_2O_2/Fe^{3+}, UV/$S_2O_8^{2-}$, UV/chlorine and UV in combination with other photocatalysts.[16,17] Among them, the heterogeneous photocatalysis with UV/TiO_2 showed to be a promising technique for wastewater detoxification.[18] TiO_2 has generally been considered the most active catalyst, whenever tested against other semiconductor materials, under comparable conditions.[19] However, despite the fact that AOPs are being widely used in wastewater treatment for the removal of both organic and inorganic contaminants, as well as to increase effluents' biodegradability, a partial oxidation may result in the formation of intermediates more toxic than parent compounds.[20] For this reason, it becomes indispensable to identify the resulting photoproducts, in order to allow the study of their individual/mixture toxicity.

The work herein described aims the elucidation of the photodegradation mechanism of

Lorazepam. LZP phototreatment was performed via different processes, including solar UV light *vs.* artificial UV radiation sources, as well as photolytic *vs.* photocatalytic pathways. The identification of the main photoproducts was carried out by ultra performance liquid chromatography–quadrupole-time-of-flight–mass spectrometry (UHPLC/QqToF-MS) and their formation was followed up concomitantly with the degradation of the parent compound. To the best of the authors' knowledge, this work has never been undertaken before. Furthermore, LZP photodegradation products were also analyzed over the phototreatment of a real contaminated municipal WWTP effluent.

EXPERIMENTAL

Chemicals and Materials.

LZP structural formula and some chemical characteristics are presented in Fig. S1 (Supporting Information). LZP used in the experiments was in the form of standard solution 1 mg mL^{-1} supplied by LGC Standards (Barcelona, Spain) and Lorenin® pills (1 mg) were from *Wyeth*. The pills were also composed of lactose monohydrate, microcrystalline cellulose, polacrilin potassium and magnesium stearate (ca. 100 mg). Consequently, a more realistic scenario was simulated using the pills, and any possible interference due to the presence of the 4 excipients has not been disregarded. TiO_2 catalyst (P25 Degussa, 80% anatase and 20% rutile) was purchased from Degussa Portuguesa. Demineralized and ultrapure water, used in the phototreatment experiments, were obtained with a reverse osmosis system (Panice®) and a Millipore® system (Direct-Q model), respectively. Methanol (LiChrosolv®) for UHPLC/QqToF-MS analyses was LC gradient grade purchased from Merck. Ultrapure water was obtained using a Milli-Q system (Millipore Corporation, Bedford, MA, USA). Formic acid (~98% purity) and ammonium acetate (>99.9% purity) were both purchased from Sigma-Aldrich (Germany). Nylon

syringe filters (0.2 μm) were provided by VWR International. Temperature and pH data were collected using a pH meter HANNA HI8424.

Photodegradation Systems

In this work, three distinct experimental set-ups were used to induce LZP photodegradation. A first lab-scale experimental apparatus consisted of a glass immersion photochemical reactor with a water column of 8 cm diameter and 16 cm height. The reactor was loaded with 850 mL of solution, with constant stirring for the total reaction period. It was equipped with a medium pressure mercury lamp Heraeus TQ 150W, with a dominant emission line at 366 nm, placed axially and held in a quartz immersion tube with a surface contact area of ~233 cm^2 - *LsTQUV* (Lab-scale TQ150W-UV prototype) (Fig. S2a). This system was refrigerated by a continuous tap water flow, which allowed temperature to be properly controlled below 30 ºC. The second employed lab-scale prototype was composed by a 5 L beaker, loaded with 4.5 L of solution constantly stirred, and an immersed pyrex glass cylinder placed axially, holding a LYNX S 11W blacklight blue lamp with maximal emission at 365 nm. The surface contact area between the pyrex cylinder and the solution was approximately 456 cm^2. The water column was 23.5 cm high with 11 cm diameter - *LsBLBUV* (Lab-scale Blacklight Blue-UV system) (Fig. S2b).

Furthermore, photocatalytic experiments were also performed, during sunny days, in a Solar Pilot Plant with Compound Parabolic Collectors (CPCs) installed in the roof of the Chemical Engineering Department of the Faculty of Engineering, University of Porto (FEUP), Portugal. This CPC solar collector (0.91 m^2) was composed of four borosilicate tubes (Schott-Duran type 3.3, Germany, cut-off at 280 nm, external diameter 50 mm, length 1500 mm and thickness 1.8 mm) connected in series by polypropylene junctions with their CPC mirrors in anodized aluminum, supported by an aluminum structure and

tilted 41º (local latitude) - *SPP-CPCs* (Solar Pilot Plant with CPCs) (Fig. S2c).

More detailed information regarding the characteristics of the used UV lamps and the design/functioning of the described phototreatment experimental set-ups can be found in previous publications.[21,22]

UHPLC/QqToF-MS Analysis.

Ultra-high performance liquid chromatography analyses, carried out to resolve LZP and its phototransformation products, were performed by Dionex UltiMate 3000 RS UHPLC system (Thermo Fisher Scientific, Waltham, USA), equipped with a 100 mm × 2.1mm i.d., 1.8 μm particle size Acquity UPLC® HSS T3 column (Waters, Milford, MA, USA), maintained at 40 ºC. The mobile phases consisted of (A) 0.010 M formic acid in Milli-Q water for ESI(+) mode and 0.005 M ammonium acetate in Milli-Q water for ESI(–) analyses, and (B) methanol, with the following multi-step elution gradient: 0 min (98% A; 0.30 mL min^{-1}), 8.0 min (0% A; 0.45 mL min^{-1}), 10.0 min (0% A; 0.45 mL min^{-1}), 10.1 min (98% A; 0.40 mL min^{-1}) and a short column reconditioning period up to 12.0 min (98% A; 0.40 mL min^{-1}). Nevertheless, this gradient was later adjusted to an initial methanol concentration of 20%, in order to better separate the many co-eluted peaks between ca. 5 – 8 min. The sample injection volume was 10 μL, in all experiments, and the autosampler temperature was fixed at 5 ºC.

For the identification of LZP photoproducts, high-resolution MS analyses were performed using an ABSCIEX TripleTOF® 5600 (Toronto, ON, Canada), provided with a Duo Spray™ ion source, operated in positive mode. The ion source was set at the following conditions: ion-spray voltage floating (ISVF) 4 kV, temperature (TEM) 600 ºC, ion source gases (GS1 and GS2) 60 psi, curtain gas (CUR) 35 psi and a declustering potential (DP) of 60 V.

LZP By-products Elucidation Strategy

The initial screening method consisted of two TOF MS (+) scan simultaneous experiments, the second with induced fragmentation (collision energy (CE) of 35 V). Acquired *m/z* values ranged between 50 and 650 Da, with an accumulation time of 0.25 s and a total acquisition time of ca. 12 min. The chosen samples for these primary analyses were selected from an intermediate phototreatment time, so that they were likely to contain a significant amount of some LZP degradation products. Afterwards, with the attained information regarding LZP characteristic/diagnostic fragment ions, associated to exact mass results, it was possible to determine a great variety of molecular ions. Subsequently, information dependent acquisition (IDA) method was employed to collect full scan MS and MS/MS information simultaneously and allow their following quantitative analysis. The method consisted of a survey TOF MS (+) experiment from *m/z* 100 to *m/z* 650 and product ion (PI) spectra for the eight most intense ions of the survey spectra throughout the chromatographic run. Dynamic background subtraction was activated to automatically acquire PI spectra of co-eluting compounds. PI spectra were collected only ions from *m/z* 150 to *m/z* 400, moreover the isotopes within 1 Da and the former precursor ion were excluded for 5 s (mass tolerance of 30 mDa) and totally excluded after three occurrences. This setting allowed acquisition of thousands of unique *m/z* / t_R combinations. The collision energy of 35 V with a collision energy spread of ±15 V was used for the PI spectra. The collision energy spread resulted in more characteristic MS/MS spectra since both low and high energy fragment ions were present in a single spectrum. Since the total cycle (survey scan and 8 PI spectra) took only 0.55 s, at least 12 points per peak were always achieved for accurate quantitation.

An automatic *m/z* calibration was performed every ten samples with the positive APCI calibration solution, using the calibration delivery system (CDS), and each set of samples

was preceded by two blank controls: Milli-Q water and methanol. In the end, the same MS approach was conducted in ESI(–) mode, though the resulting chromatograms presented no relevant additional information. The MS detector was used, in both ESI(+) and ESI(–) modes, the resolving power was > 31,000 (*m/z* 321.0192) full width at half maximum (FWHM). Since the PI spectra were measured in high sensitivity mode, half resolution was obtained.

Instrument control and data acquisition were carried out with the AnalystV1.5.1TF software (ABSciex) and the qualitative analysis was performed using PeakView, also equipped with the XIC Manager and Formula Finder tools. All identified LZP degradation products were further quantitatively processed using MultiQuantV2.1 software.

Photodegradation Experiments and Procedure.

Irradiation experiments of LZP were performed using the three described systems: *LsTQUV, LsBLBUV* and *SPP-CPCs* (refer to section 2.2.). Prior to use, Lorenin® pills were ground using a porcelain pestle and mortar.

For the *LsTQUV* experiment, 5 Lorenin® pills were dissolved in 100 mL Milli-Q water. LZP was first extracted with 100 mL ethyl acetate (solubility of 30 mg mL^{-1} in ethyl acetate and 0.08 mg mL^{-1} in water),[23] using a separatory funnel, ethyl acetate was then completely evaporated and LZP redissolved in Milli-Q water, up to a final volume of 850 mL ($[LZP]_{final} \sim 6$ mg L^{-1}). After constant stirring of the solution, in the dark (lamp-off) for approximately 15 min, using the described *LsTQUV* system, a first sample was collected (time 0); following, the photolytic process began by turning the UV light on and different samples were taken, every minute during 20 min. Moreover, a similar experiment was performed with the total Lorenin® pills (LZP plus excipients). In this case, a dark control experiment under analogous experimental conditions was also conducted. In the case of the

LsBLBUV experiment, 22 Lorenin® pills were dissolved in 4.5 L of Milli-Q water ($[LZP]_{final}$ ~ 5 mg L^{-1}) and further submitted to a photocatalytic treatment with 200 mg L^{-1} of TiO_2 (in suspension). Likewise, the suspension was continuously stirred during the whole experiment and several samples were collected, at different time intervals, after turning the UV light on. According to previous results,[24] LZP undergoes no significant hydrolysis nor adsorption onto the catalyst surface, thus in the present work no control sample was collected after the catalyst addition and prior to the beginning of irradiation. Lastly, in the case of the *SPP-CPCs* system, 30 Lorenin® pills were dissolved in 15 L of distilled water ($[LZP]_{final}$ ~ 2 mg L^{-1}) and photocatalytic experiments were performed with 200 mg L^{-1} of TiO_2 (experimentally determined optimal TiO_2 concentration).[24] The suspension was constantly mixed by turbulent recirculation (~0.3 min residence time, ~40% illuminated volume). Finally, experiments began by uncovering the CPCs and different aliquots were collected at pre-defined times. Afterwards, a similar experiment was performed over a real municipal WWTP effluent sample, further spiked with Lorenin® pills in order to attain a final LZP concentration of ca. 2 mg L^{-1}. No pH adjustments were performed in any of the photodegradation processes. Temperature and pH were continuously monitored along all experiments.

Previous to LC–MS/MS analysis, all sample aliquots containing TiO_2 were pre-filtered through 0.2 μm membrane filters. Afterwards, LZP and its photoproducts were analyzed using the UHPLC/QqToF-MS system at the established settings described in section 2.3.

RESULTS AND DISCUSSION

According to previous results described by Sousa et al.,[24] using the *LsTQUV* apparatus, LZP highest degradation yield is obtained through photolysis, while with the *SPP-CPCs* system the best degradation performance is achieved through a photocatalytic process,

using a TiO_2 concentration of 200 mg L^{-1}. Besides, the degradation kinetic constant determined for the optimized method using the *SPP-CPCs* system (k = 1.49±0.03 L kJ^{-1}) was higher than the one calculated for the *LsTQUV* set-up (k = 0.131±0.006 L kJ^{-1}). In the case of the *LsBLBUV* apparatus, photocatalysis with a TiO_2 concentration of 200 mg L^{-1} was elected as well, since LZP photolytic degradation was still quite lengthy (data not shown). Building on these grounds, the purpose of the present work was to clarify LZP photodegradation pathway(s) and accurately identify the different resulting photoproducts, rather than to characterize its degradation kinetics. In order to achieve so, the tested LZP concentrations were in the low ppm range (2 - 6 mg L^{-1}), despite significantly higher than the already reported aquatic environmental values (ca. 40 - 200 ng L^{-1}).

As aforementioned, the employed non-target screening approach initially consisted of two TOF MS (+) scan simultaneous experiments, one with and one without in-source fragmentation. This interesting strategy, based upon the concept of "diagnostic fragment ions", was first described by Ferrer and Thurman.[25] Applied to the present case-study, the key assumption is that LZP photodegradation products will still present a basic structure analogous to that of the parent compound and, consequently, a similar in-source fragmentation pattern. The resulting diagnostic fragment ions were then selected bearing in mind the presence of the chlorine atom specific isotopic pattern (Fig. S1), as well as accurate masses. Subsequently, the corresponding molecular ions were identified in the respective non-fragmented sample chromatogram, using background subtraction at the corresponding retention times (t_R) and taking into account peaks with identical shape.[26] Afterwards, MS/MS spectra of every $[M+H]^+$ ions were acquired by IDA product ion (+) MS analysis, as previously mentioned in section 2.3. Finally, with the help of the Formula Finder tool, from the PeakView software, the structural enlightening of several LZP photoproducts was possible, taking into account LZP structural formula and respective

tandem MS spectra, as well as some of the software proposed fragments with corresponding spectra fitting probabilities.

Structural Elucidation of LZP By-products and Mechanism Proposal.

As previously mentioned, the samples selected for preliminary UHPLC/QqToF-MS analyses were chosen from an intermediate phototreatment time, in order to potentially contain a higher amount of most LZP photoproducts, and the selected system was the *LsTQUV*, since it allowed to start with higher LZP concentrations. Nevertheless, all compounds herein identified were later confirmed in most samples obtained in the remaining photocatalytic systems (*LsBLBUV* and *SPP-CPCs*), bearing in mind the same exact masses and t_R, in addition to comparable MS^2 spectra.

Initially, several molecular ions were included in the group of potential LZP by-products (LBPs), though some of them were later identified as corresponding to either $^{35}Cl/^{37}Cl$ isotopes or Na^+/K^+ adducts of other $[M+H]^+$ ions. Such examples were the cases of LZP itself (*m/z* 321.0196 (^{35}Cl) / 323.0167 (^{37}Cl)), presenting a sodium adduct at *m/z* 343.0014 / 344.9984 and a potassium adduct at *m/z* 358.9749 / 360.9723, as well as LBP291 (*m/z* 291.0088 / 293.0058), with a sodium adduct at *m/z* 312.9906 / 314.9877, or LBP319 (*m/z* 319.0034 / 321.0007), with a sodium adduct at *m/z* 340.9862 / 342.9828 (Fig. S3). For these preliminary identifications, besides taking into account the two chlorine atoms typical isotopic pattern, both isotopes exact masses (^{35}Cl isotope – 34.9689 and ^{37}Cl isotope – 36.9659) were also considered. Thus, the selected ions were those corresponding to an isotope *m/z* difference slightly inferior to 2 Da. Furthermore, chromatographic data was also found to be an indispensable tool to enable the distinction between true LZP photoproducts and some potentially formed LZP in-source fragments. For instance, at first

sight, the 303.0085 *m/z* fragment could be considered a LBP, since it fitted all MS requisites imposed by the adopted analytical strategy. However, when comparing its chromatographic profile with LZP's, the obtained peaks were overlapping (i.e., similar shapes and t_R), leading to the conclusion that this compound was merely the result of in-source fragmentation and not of the phototreatment process. Moreover, it was later confirmed that there was actually a LBP with a nominal mass of 303 Da, but its exact monoisotopic mass was still significantly higher (303.0560 *m/z*).

Finally, fourteen compounds (excluding structural isomers) were identified as presumable LBPs and none of the corresponding peaks was detected in either of the used control blanks. All TOF MS scan extracted ion chromatograms (XICs) are presented in Fig. S4. Herein, one can observe that these putative LBPs were mostly eluted in the narrow range between 4-7 min, even after some preliminary improvements in the chromatographic elution gradient (refer to section 2.3.). Nonetheless, the QToF still afforded sufficient selectivity to record good quality product ion spectra for the majority of these compounds: LBP257, LBP266, LBP275, LBP282, LBP291, LBP293, LBP303, LBP319 and LBP337. Their respective MS^2 XICs and mass spectra are displayed, along with LZP's, in Fig. S5. Other less abundant LBPs included LBP156, LBP223, LBP230, LBP239 and LBP273. Fig. S5 illustrates some examples of the use of diagnostic ions for the identification of non-target by-products obtained after phototreatment of the (targeted) parent compound LZP. One example was the case of the fragment ion 275.0171 *m/z*, identified in the mass spectrum of LBP319. Other fragment ions such as 229.0547 *m/z* and 163.0064 *m/z* were identified as well in the mass spectra of LBP303 and LBP257/LBP275/LBP291, respectively. However, despite constituting a rather useful tool for finding structurally related compounds, namely degradation products, the applicability of the diagnostic fragment ions concept is slightly limited and many exceptions exist.[26] Therefore, other

important pieces of information such as chlorine isotopic pattern, exact masses, cleavage/oxidation and energetically favored reactions were considered when trying to establish some LBPs' structures.

In Fig. S5, the detected ion with a nominal mass of 319 Da was rationalized as being the product of the opening of the diazepinone seven-membered ring, followed by a rearrangement into a highly stabilized six-membered aromatic ring (LBP319).[27] The resulting exposed carboxylic function is then likely to be cleaved, yielding an ion with 275 Da (LBP275) – neutral loss of 44 Da, corresponding to the carboxylic group. It should be highlighted that this photoproduct with monoisotopic mass of ca. 275.0175 *m/z* corresponds also to a LZP in-source fragment, as one can observe on LZP PI spectrum and corroborated by the presence of the small peak at 5.76 min (LZP t_R) in LBP275 chromatogram. This aspect stresses, once again, the importance of good resolution of the parent compound/photoproducts.

Concerning the remaining proposed LBPs, their structures were put forward based on the common reactions taking place in photocatalysis, namely oxidative processes mediated by $^{\bullet}OH$ species. Consequently, from a mechanistic point of view, different hydroxylation reactions could be predicted. Regarding LBP337 and LBP303, they were considered side products of LZP phototreatment and interpreted as the result of the addition of a hydroxyl group to one of LZP's benzene rings and the substitution of one chlorine atom by the hydroxyl group, respectively. However, these hydroxylation reactions could take place at different carbons of LZP skeleton, leading to the formation of distinct isomeric hydroxyl derivates and, thus, different chromatographic peaks.[26] As one can observe in Fig. S5, in the case of LBP337, it was possible to distinguish between two potential structural isomers, each one derived from hydroxylation of a different aromatic ring, leading to the identification of ions at *m/z* 154.0078 and 179.0040 and corresponding putative structures.

Still, the exact position of the –OH substituent in the benzene ring remains undetermined. On the other hand, in the case of LBP303, it was possible to pinpoint exactly which chlorine atom was being substituted by the hydroxyl group, thanks to the structures tentatively attributed to ions at *m/z* 145.0388 and 153.0204 These ions were separately identified in the spectrum of each of the two most intense peaks, located at t_R 3.65 and 5.92 min. With reference to the probable LBP291, also shown in Fig. S5, the three observed peaks were interpreted as three different structural isomers: the most intense peak at 6.92 min, most likely corresponding to the hydroxyl addition in the middle ring (LBP291(b)) according to the proposed structure for the 179.0009 Da ion, and two others at 6.05 and 6.46 min (LBP291(a)), matching the hydroxylation on each of the two additional aromatic rings. Nevertheless, the latter two structures were again indistinguishable. According to some results reported by Calisto et al.,[27] LBP266 was also identified in the present work, showing some common MS^2 fragments with another proposed LZP photoproduct - LBP282 (ions with the nominal mass of 154 and 126 Da) (Fig. S5). Thus, it was our belief that LBP282 may result from the hydroxylation of LBP266 in one of the aromatic rings. Finally, the structure proposed for the putative LBP257 differs significantly from that previously presented by Calisto et al.[27], consisting of a fused tricyclic structure, while our suggestion is more coherent with earlier stage photoproducts. Besides, Calisto et al.[27] have only simulated the environmental degradation of BDZ, including LZP, in the laboratory i.e., they disregarded accelerated photodegradation processes such as TiO_2-driven photocatalysis, as well as any mechanistic elucidation.

Considering the importance of having an holistic overview of LZP photofate in the environment, in the present work a photodegradation pathway is proposed (Scheme 1), based on the results discussed so far. As it can be observed, the suggested scheme is divided in three main stages: a first one, leading to the formation of key LBP275, through

the intermediate LBP319, including as well some LBP275 side products resulting from the hydroxylation of the central diamino-ring; a following stage, comprising LBP291, LBP257 and LBP273, corresponding to –OH addition, –Cl substitution and both, respectively; a third stage encompassing different photoproducts resulting from probable cleavage and/or hydroxylation reactions (LBP266, LBP282, LBP239, LBP230, LBP223 and LBP156).

In conclusion, the unambiguous identification and confirmation of some LZP photoproducts was enabled taking into account the parent compound structural formula and performing an accurate mass analysis of the protonated molecules, together with that of additional characteristic fragment ion(s), including characteristic isotopic signals and retention times.

Comparison Between LsTQUV, LsBLBUV and SPP-CPCs Experiments.

The evolution profiles of the above enumerated LZP photoproducts and whether or not they were produced using the three employed experimental systems was then investigated. Since authentic standards are not commercially available for these LBPs, their concentrations in the several collected samples could not be accurately calculated. Thus, in Fig. 1 we illustrated their evolution in terms of peak area (i.e., ratio $A_t/A_{max.}$) in relation to the accumulated UV energy (Q_{UV}), calculated for each experimental set-up. All data used for the determination of peak area values are given in Table S1. The normalization attained with the Q_{UV} parameter allowed the comparison of the different LBPs increase/decrease profiles between the three experimental systems. On the overall, it is also worth noting that since in most cases the different isomers could not be distinguished, their respective peak areas were presented as a global value. Exception noted for LBP291, for which isomeric structures (a) and (b) were displayed separately.

As preliminary conclusions two main aspects must be highlighted: first, the *SPP-CPCs*

photocatalytic system seemed to be the most efficient one amongst the three tested, since a faster LZP decrease was observed and the increase/decrease variations of several LBPs appeared at an earlier stage (Fig. 1); second, using this experimental system, some compounds such as LBP291(a), LBP257, LBP266, LBP282 and LBP230 were never detected. Remains to be clarified if this was due to the fact that the initial LZP concentration used in these experiments was lower than in the other two systems (2 ppm for *SPP-CPCs vs.* 6 and 5 ppm for *LsTQUV* photolysis and *LsBLBUV* photocatalysis, respectively), or if the efficient degradation of LZP didn't allow isolating some intermediates.

Lastly, the obtained evolution profiles for LBP337, LBP303, LBP319 and LBP275, regarding all three experimental set-ups, showed a noticeable increase as soon as light exposure began, which further reinforces the aforeproposed LZP photodegradation pathway. Moreover, their respective peak areas (data not shown) corresponded to the highest initial absolute values among all proposed LBPs' structures. Though we are perfectly aware that such comparison is not flawless, since different compounds will most likely present different MS responses, it is likely that the signal intensities should fall in a rather similar range, given that the basic structure is still maintained.

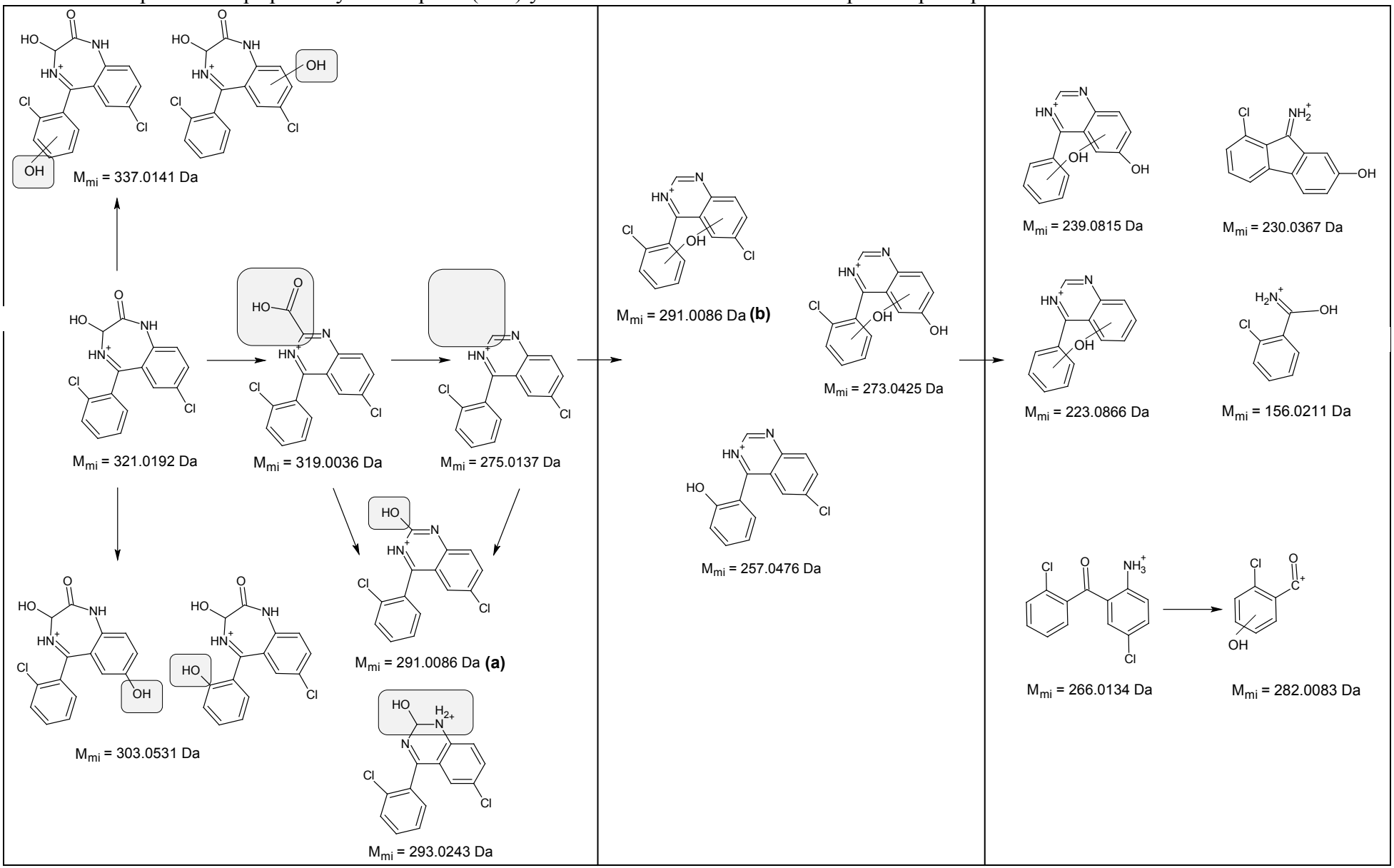
LZP Photodegradation in a Real Contaminated WWTP Effluent

The experimentally confirmed most effective LZP photodegradation system – 200 mg L^{-1} TiO_2-assisted photocatalysis using the Solar Pilot Plant with Compound Parabolic Collectors (*SPP-CPCs*) – was finally used to envisage LZP photofate in a real effluent sample. A preliminary physicochemical characterization (including some emerging pollutants) of this sample was performed and the results are displayed in Table S2. The effluent was further spiked with 2 mg L^{-1} LZP, in order to facilitate the identification of

potential LZP photoproducts. pH and temperature were constantly measured over the experiment, never falling outside the range of 6.35-7.10 and 18.5-28.5 ºC (normal within-day variation), respectively.

The evolution profiles of the most abundant LBPs were then monitored and the charts obtained are presented in Fig. 2. It is well-known that nitrates, humic and fulvic acids present in wastewaters can act as sensitizers, giving rise to highly reactive species such as hydroxyl-radicals, singlet oxygen or H_2O_2 and thus promoting indirect photolysis.[28] On the other hand, humic acids can also exert an optical filter effect, thereby attenuating the direct photolysis process. These two competing mechanisms are widely recognized and the overall effect is considered to be dependent on the analyte being investigated.[29,30] However, even taking these aspects into consideration, no quantitative comparison with the results obtained for distilled water (Fig. 1) was possible. This holds true mainly due to the fact that the dissolved organic matter, present in the effluent sample, can render the analytes prone to different matrix effects. Nevertheless, from an overall analysis of Fig. 2, it can be observed that the photoproducts generated seem to be more persistent than LZP itself, requiring higher Q_{UV} amounts to achieve their photodegradation. Consequently, some ecotoxicity tests would be quite relevant to further assess their environmental impact.

Scheme 1. Proposed 3-steps pathway for the photo(cata)lytic conversion of LZP into the respective photoproducts



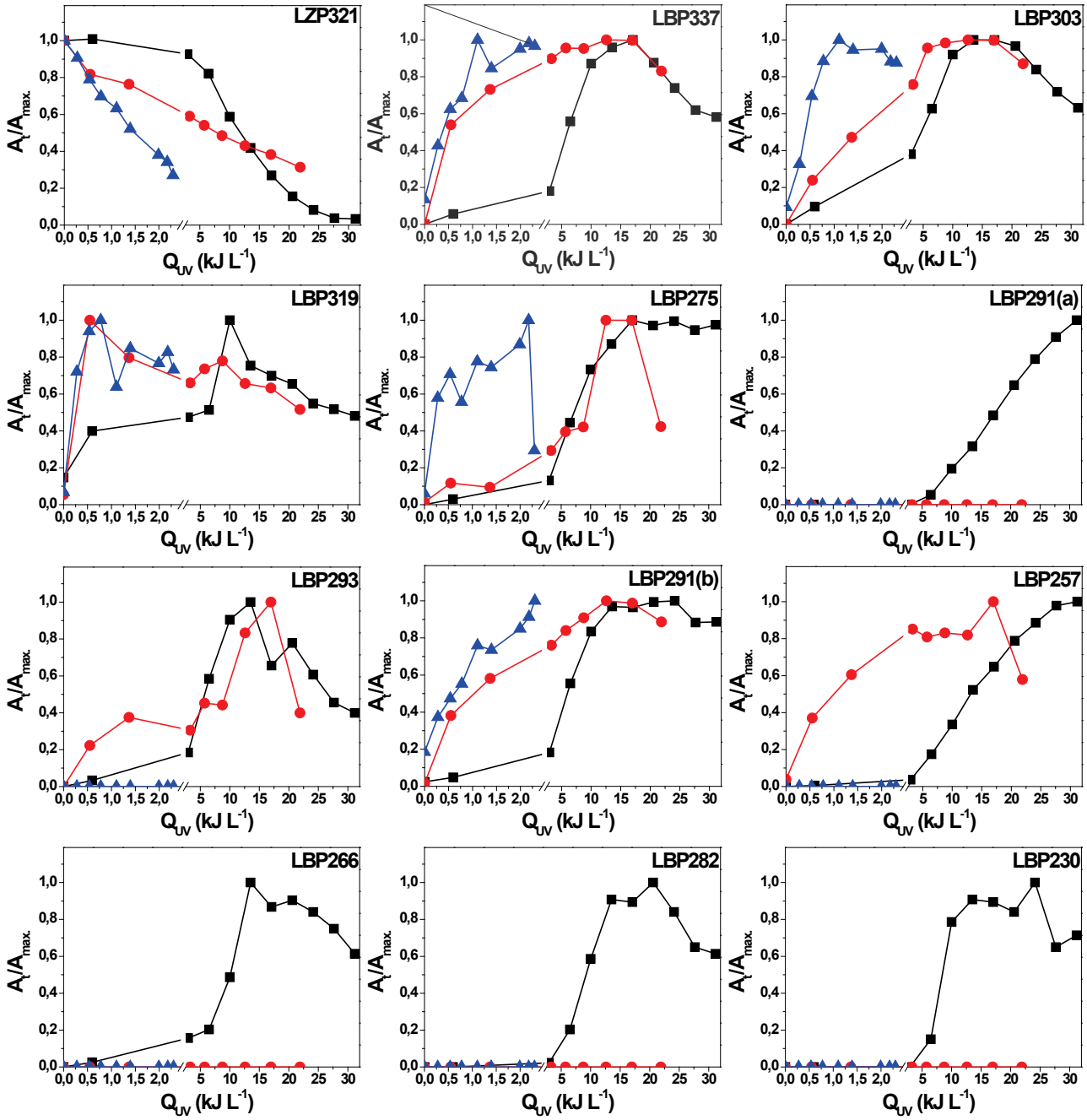


Figure 1. Graphical representation of LZP and respective LBPs evolution profiles, over the photo-treatment process, in terms of ratio $A_t/A_{max.}$ (peak area at time *t* / maximal peak area) as a function of the amount of accumulated UV energy per liter of sample: ■ - Lab-scale TQ150W-UV prototype (*LsTQUV*); ● - Lab-scale Blacklight Blue-UV system (*LsBLBUV*); ▲ - Solar Pilot Plant with CPCs (*SPP-CPCs*).

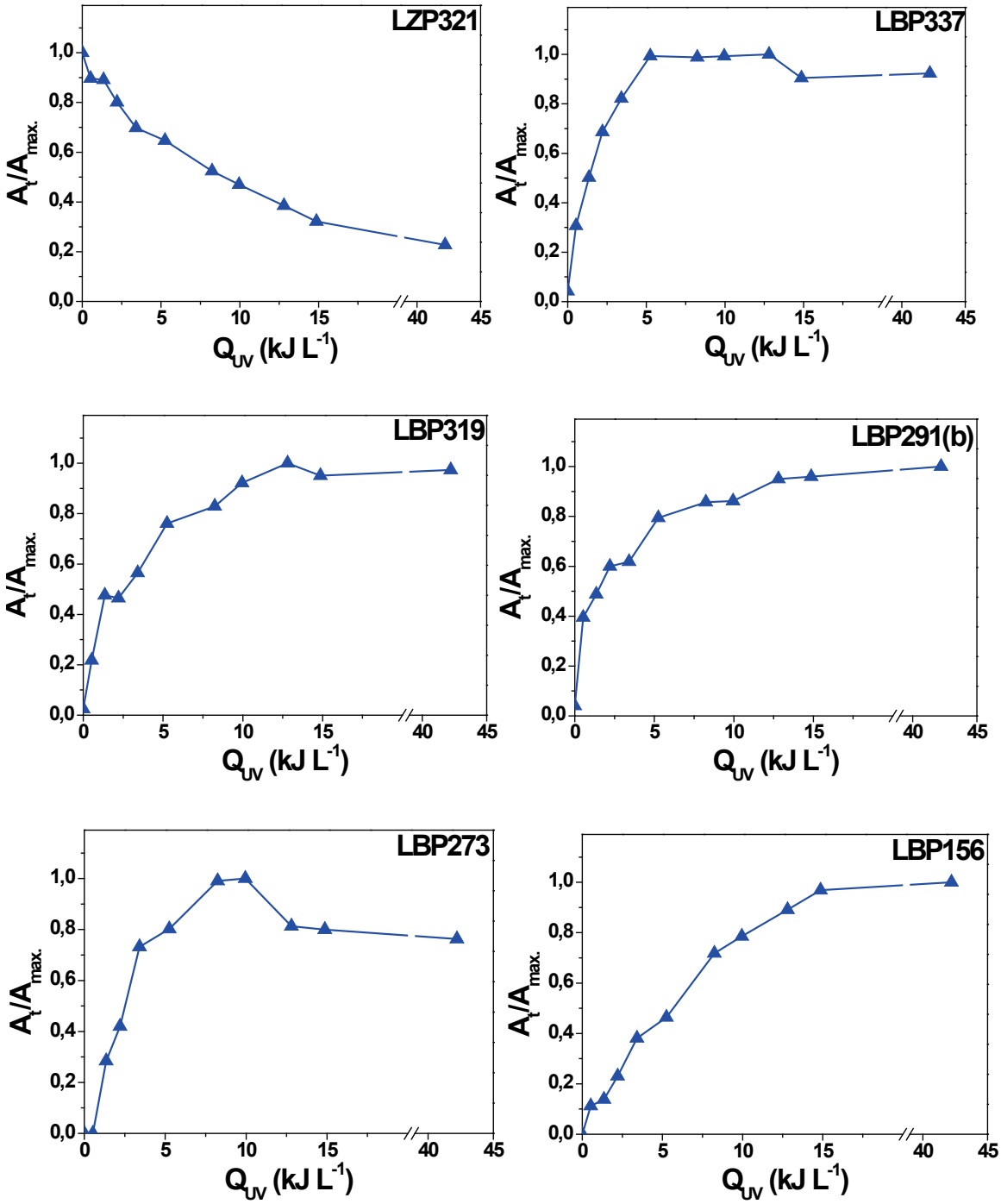


Figure 2. Graphical representation of LZP and respective LBPs evolution profiles, over the photo-treatment using the *SPP-CPCs* set-up, in terms of ratio $A_t/A_{max.}$ (peak area at time *t* / maximal peak area) as a function of the amount of accumulated UV energy per liter of effluent.

ASSOCIATED CONTENT

Supporting Information. Figures S1 to S5 and Tables S1 to S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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SUPPORTING INFORMATION

Lorazepam Photofate under Photolysis and TiO_2-assisted Photocatalysis: By-products' Identification and Evolution Profiles during Phototreatment of a Contaminated WWTP Effluent

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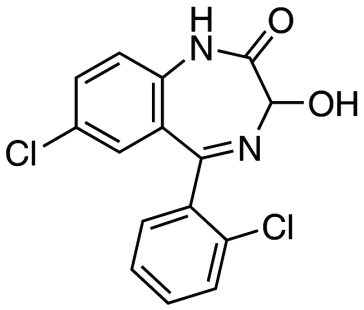
(10 pages, 5 figures and 2 tables)

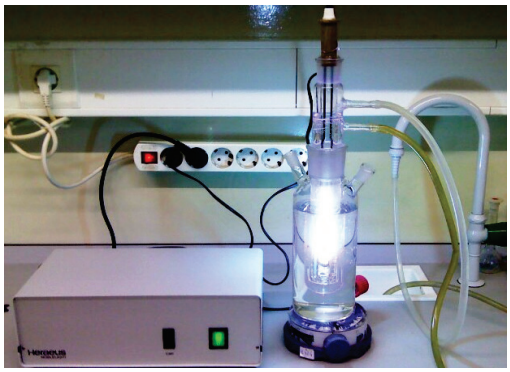
IUPAC name:
(RS)-9-chloro-6-(2-chlorophenyl)-4-hydroxy-2,5-diazabicyclo[5.4.0]undeca-5,8,10,12-tetraen-3-one



Therapeutic group	Anxiolytics
Molecular formula	$C_{15}H_{10}N_2Cl_2O_2$
Molecular weight	321.2
CAS no.	846-9-1

Figure S1. LZP structural formula, IUPAC designation, pharmaceutical and chemical characteristics (from Rang, H. P.; Dale, M. M.; Ritter, J. M.; Moore, P. K. *Pharmacology*; Bath Press: U.K., 2003).

(a)



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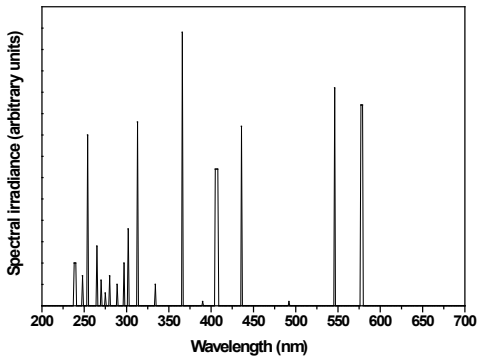
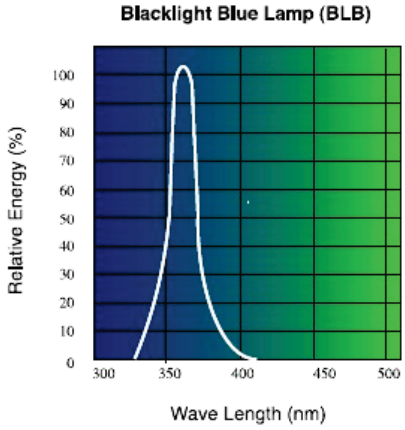


(2)

(b)

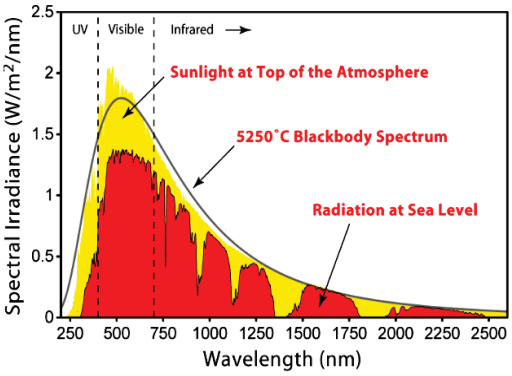
(1)



(2)

(c)

(1)



(2)

Figure S2. Experimental systems used for LZP phototreatment tests: **(a)** Laboratory-scale Photoreactor with UV medium pressure mercury lamp **(1)** and emission spectrum of the lamp Heraeus TQ 150W **(2)**; **(b)** Laboratory-scale Photoreactor with blacklight blue lamp **(1)** and LYNX S 11W lamp emission spectrum **(2)** (http://www.intertronics.co.uk/products/iuv026.htm, last accessed September 20, 2012); **(c)** Solar Pilot Plant with CPCs **(1)** and solar radiation spectrum **(2)** (http://org.ntnu.no/solarcells/pages/Chap.2.php?part=1, last accessed September 20, 2012).

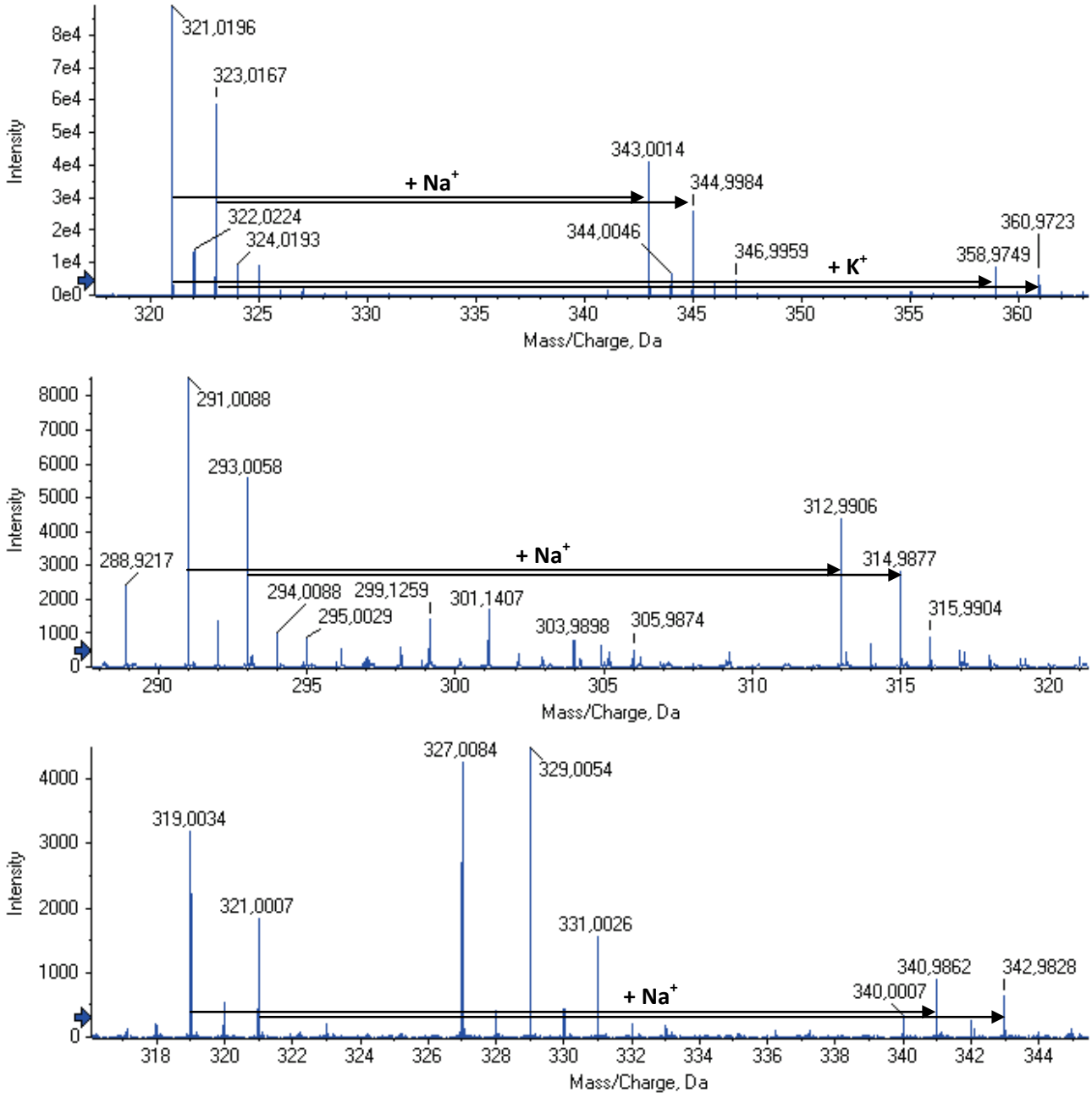


Figure S3. Illustration of some examples of sodium and potassium adducts: LZP321 and Na^+/K^+-adducts; LBP291 and LBP319 and respective Na^+-adducts.

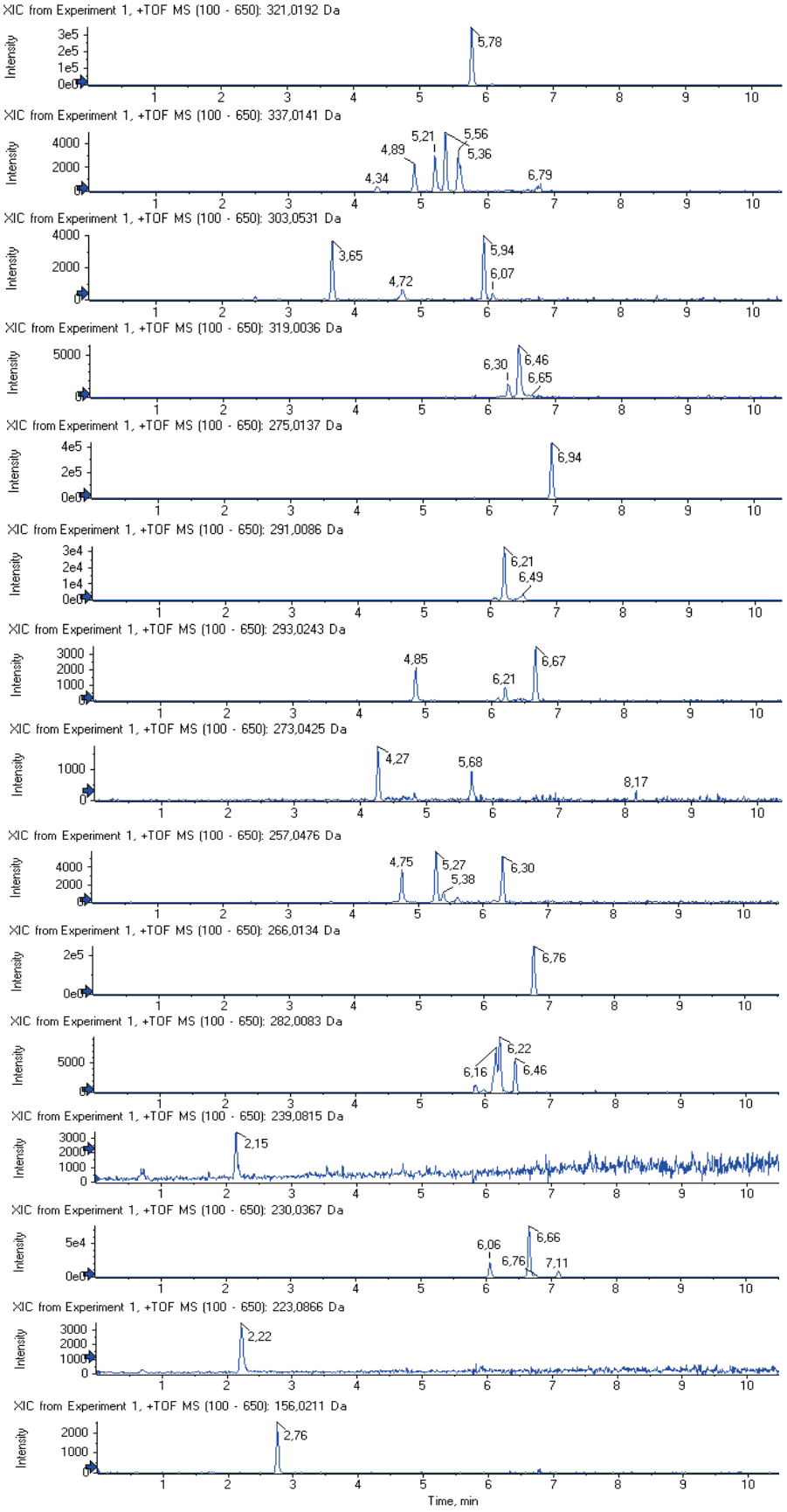


Figure S4. UHPLC/QqToF extracted ion chromatograms obtained for the sample corresponding to Q_{UV} = 13.5 kJ L^{-1} / *LsTQUV* prototype.

Figure S5

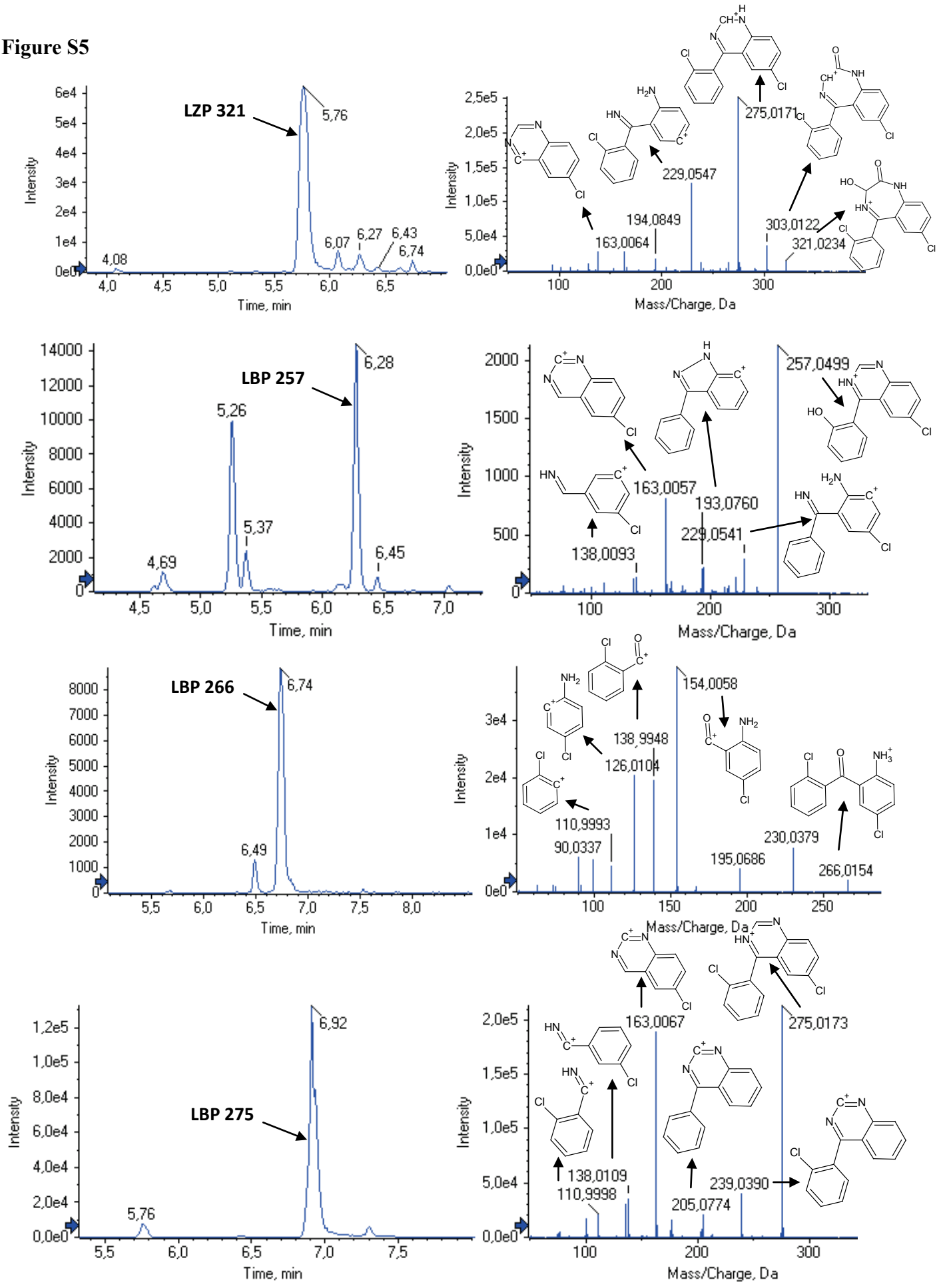
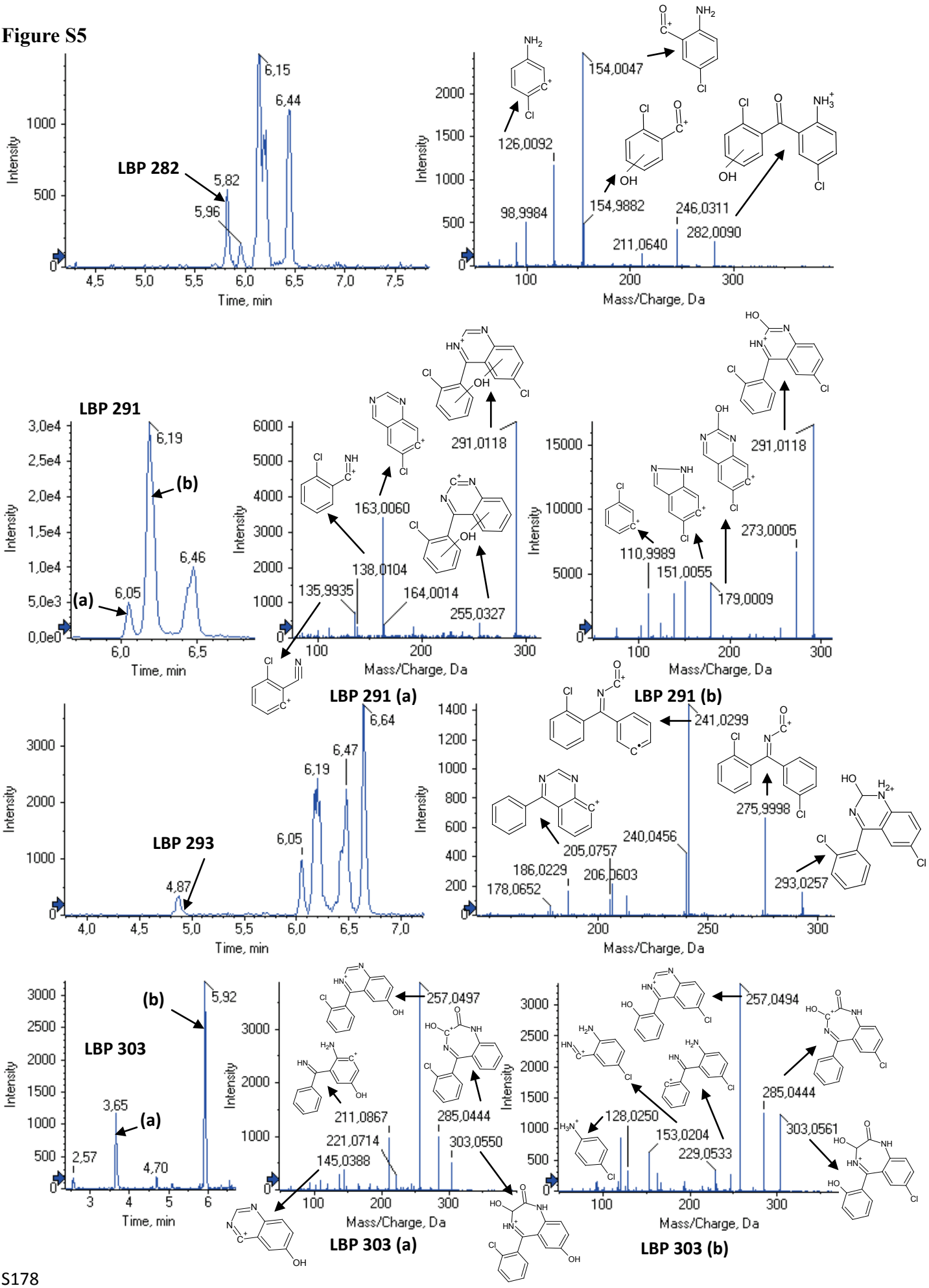

LZP 321
5,76
4,08
6,07
6,27
6,43
6,74
Intensity
Time, min
275,0171
229,0547
194,0849
163,0064
303,0122
321,0234
Mass/Charge, Da
LBP 257
6,28
5,26
5,37
4,69
6,45
Intensity
Time, min
257,0499
163,0057
193,0760
229,0541
138,0093
Mass/Charge, Da
LBP 266
6,74
6,49
Intensity
Time, min
154,0058
138,9948
126,0104
110,9993
90,0337
230,0379
195,0686
266,0154
Mass/Charge, Da
LBP 275
6,92
5,76
Intensity
Time, min
163,0067
275,0173
138,0109
110,9998
239,0390
205,0774
Mass/Charge, Da

Figure S5


LBP 282
6,15
6,44
5,82
5,96
Intensity
Time, min
154,0047
126,0092
98,9984
154,9882
246,0311
211,0640
282,0090
Mass/Charge, Da
LBP 291
6,19
(b)
6,46
6,05
(a)
163,0060
138,0104
135,9935
164,0014
255,0327
291,0118
110,9989
151,0055
179,0009
273,0005
LBP 291 (a)
LBP 291 (b)
LBP 293
6,64
6,47
6,19
6,05
4,87
241,0299
275,9998
205,0757
240,0456
186,0229
206,0603
178,0652
293,0257
LBP 303
(b)
5,92
3,65
(a)
2,57
4,70
257,0497
211,0867
285,0444
221,0714
303,0550
145,0388
257,0494
285,0444
128,0250
153,0204
229,0533
303,0561
LBP 303 (a)
LBP 303 (b)

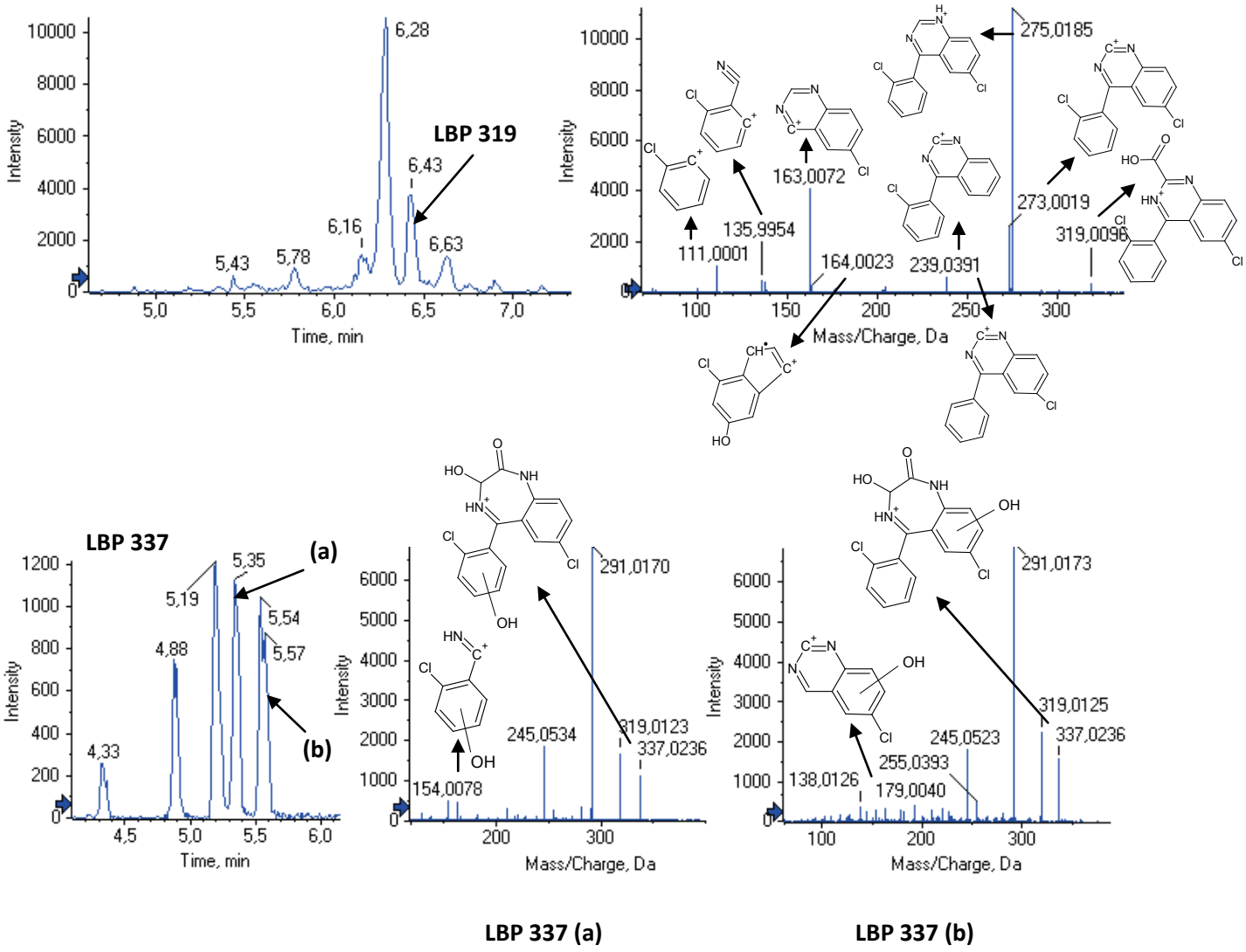


Figure S5. (+)ESI-MS^2 extracted ion chromatograms and mass spectra of LZP and its photoproducts, obtained with sample Q_{UV} = 13.5 kJ L^{-1} / *LsTQUV* prototype. For each MS spectrum, some putative structures are presented for the higher intensity signals.

Table S1. Calculated, extracted and observed mass, mass error, expected and found RT (*Retention Time*) and intensities determined for the predicted formulas of the identified LBPs, using the sample corresponding to Q_{UV} = 13.5 kJ L^{-1} and the *LsTQUV* prototype - the designation of the photoproducts is in accordance with Fig. 1

Name	Formula	Mass (Da)	Adduct / Modifications	Extraction Mass (Da)	Width (Da)	Found at Mass (Da)	Error (ppm)	Expected RT (min)	RT Width (min)	Found at RT (min)	Intensity
LBP156_2.76	C7H6ClNO	155.01380	+H	156.02107	0.01	156.02050	-3.7	2.76	0.5	2.76	8944
LBP230_6.06	C13H8ClNO	229.02944	+H	230.03672	0.02	230.03641	-1.3	6.06	0.5	6.06	102238
LBP230_6.66	C13H8ClNO	229.02944	+H	230.03672	0.02	230.03617	-2.4	6.66	0.5	6.66	336100
LBP230_6.76	C13H8ClNO	229.02944	+H	230.03672	0.02	230.03617	-2.4	6.76	0.5	6.76	336100
LBP230_7.11	C13H8ClNO	229.02944	+H	230.03672	0.02	230.03638	-1.5	7.11	0.5	7.11	44250
LBP257_4.75	C14H9ClN2O	256.04034	+H	257.04762	0.01	257.04722	-1.5	4.75	0.5	4.75	14055
LBP257_5.27	C14H9ClN2O	256.04034	+H	257.04762	0.01	257.04755	-0.2	5.27	0.5	5.27	23726
LBP257_6.30	C14H9ClN2O	256.04034	+H	257.04762	0.01	257.04762	0	6.30	0.5	6.30	20081
LBP266_6.76	C13H9Cl2NO	265.00612	+H	266.01340	0.02	266.01352	0.5	6.76	0.5	6.76	1090835
LBP273_4.27	C14H9ClN2O2	272.03526	+H	273.04253	0.01	273.04190	-2.3	4.27	0.5	4.27	6379
LBP275_6.94	C14H8Cl2N2	274.00645	+H	275.01373	0.01	275.01403	1.1	6.94	0.5	6.94	1638425
LBP275_7.32	C14H8Cl2N2	274.00645	+H	275.01373	0.01	275.01394	0.8	7.32	0.5	7.32	11583
LBP282_5.84	C13H9Cl2NO2	281.00103	+H	282.00831	0.01	282.00769	-2.2	5.84	0.5	5.84	5781
LBP282_5.98	C13H9Cl2NO2	281.00103	+H	282.00831	0.01	282.00832	0	5.98	0.5	5.98	38613
LBP282_6.16	C13H9Cl2NO2	281.00103	+H	282.00831	0.01	282.00821	-0.4	6.16	0.5	6.16	38613
LBP282_6.22	C13H9Cl2NO2	281.00103	+H	282.00831	0.01	282.00821	-0.4	6.22	0.5	6.22	38613
LBP282_6.45	C13H9Cl2NO2	281.00103	+H	282.00831	0.01	282.00821	-0.4	6.45	0.5	6.45	38613
LBP291_6.07	C14H8Cl2N2O	290.00137	+H	291.00864	0.01	291.00861	-0.1	6.07	0.5	6.07	126219
LBP291_6.21	C14H8Cl2N2O	290.00137	+H	291.00864	0.01	291.00861	-0.1	6.21	0.5	6.21	126219
LBP291_6.49	C14H8Cl2N2O	290.00137	+H	291.00864	0.01	291.00852	-0.4	6.49	0.5	6.49	15167
LBP293_4.85	C14H10Cl2N2O	292.01702	+H	293.02429	0.01	293.02441	0.4	4.85	0.5	4.85	7944
LBP293_6.66	C14H10Cl2N2O	292.01702	+H	293.02429	0.01	293.02403	-0.9	6.66	0.5	6.66	13170
LBP303_3.66	C15H11ClN2O3	302.04580	+H	303.05310	0.02	303.05276	-1.1	3.66	1	3.66	16585
LBP303_4.71	C15H11ClN2O3	302.04582	+H	303.05310	0.02	303.05238	-2.4	4.71	1	4.71	3256
LBP303_5.94	C15H11ClN2O3	302.04582	+H	303.05310	0.02	303.05307	-0.1	5.94	1	5.94	17491
LBP319_6.30	C15H8Cl2N2O2	317.99628	+H	319.00356	0.01	319.00359	0.1	6.30	0.5	6.30	24727
LBP319_6.46	C15H8Cl2N2O2	317.99628	+H	319.00356	0.01	319.00359	0.1	6.46	0.5	6.46	24727
LBP337_4.90	C15H10Cl2N2O3	336.00685	+H	337.01412	0.01	337.01400	-0.4	4.90	0.5	4.90	8765
LBP337_5.21	C15H10Cl2N2O3	336.00685	+H	337.01412	0.01	337.01427	0.4	5.21	0.5	5.21	18793
LBP337_5.36	C15H10Cl2N2O3	336.00685	+H	337.01412	0.01	337.01427	0.4	5.36	0.5	5.36	18793
LBP337_5.56	C15H10Cl2N2O3	336.00685	+H	337.01412	0.01	337.01409	-0.1	5.56	0.5	5.56	18793
LZP321_4.09	C15H10Cl2N2O2	320.01193	+H	321.01921	0.01	321.01909	-0.4	4.09	0.5	4.09	1939
LZP321_5.78	C15H10Cl2N2O2	320.01193	+H	321.01921	0.01	321.01934	0.4	5.78	0.5	5.78	1253349
LZP321_6.09	C15H10Cl2N2O2	320.01193	+H	321.01921	0.01	321.01935	0.4	6.09	0.5	6.09	21202
LZP321_6.28	C15H10Cl2N2O2	320.01193	+H	321.01921	0.01	321.01935	0.4	6.28	0.5	6.28	21202
LZP321_6.76	C15H10Cl2N2O2	320.01193	+H	321.01921	0.01	321.01913	-0.2	6.76	0.5	6.76	8162

Table S2. Physicochemical characterization of the WWTP effluent sample used for the solar photocatalytic experiment and concentration of every emerging pollutant therein quantified

Parameter (units)	WWTP Effluent	ELV [a] Dec. Lei nº236/98, 1 de Agosto 1998 (Portuguese Legislation)
Color	pale yellow; n.d.[b] at dil.1:20	n.d. for dilution 1:20
Odor	n.d. (dil.1:20)	n.d. for dilution 1:20
pH	7.3	6.0-9.0
Temperature (ºC)	20.0	3 ºC increase [c]
Turbidity (NTU)	115	-
Conductivity (μS cm^{-1})	555	-
Dissolved Oxygen (mg L^{-1})	2.2	-
Oxidability (mg L^{-1})	56.2	-
Total Dissolved Carbon (mg L^{-1})	37.5	-
Inorganic Carbon (mg L^{-1})	25.4	-
Dissolved Organic Carbon (mg L^{-1})	12.1	-
Absorbance at 254 nm (AU)	0.07	-
Total Suspended Solids (mg L^{-1})	363	60
Volatile Suspended Solids (mg L^{-1})	223	-
Ammonium - NH_4^+ (mg L^{-1})	1.2	10
Nitrate - NO_3^- (mg L^{-1})	23.3	50
Nitrite - NO_2^- (mg L^{-1})	<0.5	-
Bromide - Br^- (mg L^{-1})	<0.5	-
Chloride - Cl^- (mg L^{-1})	78.5	-
Fluoride - F^- (mg L^{-1})	<0.5	-
Phosphate - PO_4^{3-} (mg L^{-1})	7	-
Sulphate - SO_4^{2-} (mg L^{-1})	59.8	2,000
Phosphorus - ^{31}P (mg L^{-1})	3.0	10,000
Sodium - ^{23}Na (mg L^{-1})	78.8	-
Potassium - ^{39}K (mg L^{-1})	21.1	-
Calcium - ^{44}Ca (mg L^{-1})	274.8	-
Emerging Pollutants (ng L^{-1})		
Alprazolam	24	-
Azythromycin	29	-
Bisoprolol	24,256	-
Carbamazepine	12	-
Carvedilol	631	-
Ciprofloxacin	682	-
Clotrimazole	52	-
Diclofenac	492	-
Fenofibrate	3,051	-
Fluconazole	417	-
Fluoxetine	132	-
Furosemide	53	-
Gemfibrozil	101	-
Hydrochlorothiazide	149	-
Ketoprofen	410	-
Lorazepam	138	-
Losartan	95	-
Norfloxacin	110	-
Ofloxacin	254	-
Paroxetine	215	-
Propanolol	244	-
Terbinafine	37	-

[a] ELV – Emission Limit Value. [b] n.d. – not detected. [c] Comparatively to the receptor medium.

Chapter 6

Occurrence of Emerging Pollutants' By-Products in Water Resources

This chapter represents a résumé of the following book chapter: C. Gonçalves, M.A. Sousa and M.F. Alpendurada: ***Occurrence of Transformation Products of Emerging Contaminants in Water Resources***. In: Transformation Products of Emerging Contaminants in the Environment: Analysis, Processes, Occurrence, Effects and Risks. Book to be edited by Dimitra A. Lambropoulou and Leo M.L. Nollet, John Wiley & Sons Inc. (2013).

This chapter will focus on the occurrence of transformation products (TPs) of different classes of emerging compounds (e.g., pharmaceuticals, hormones, personal care products, pesticides, alkylphenols, etc.) in environmental matrices (surface waters, groundwaters, drinking waters, sea waters, wastewaters and sewage sludge) from a geographical perspective, seeking to present an integrated account of the characteristics, impacts and activities across Europe.

By-products are often detected in the environment in the absence of the parent compounds and may be more noxious and less biodegradable than their counterparts, therefore they must be taken into account in risk assessment studies. As stated in the previous chapter, using lorazepam as example, the identification of other emerging pollutants TPs, which will now be reviewed, is only the first step towards the further assessment of their potential toxicity effects and consequent environmental impact.

6.1. Brief Introduction on the Sources of Transformation Products of Emerging Contaminants

The first step on the transformation of chemicals used for therapeutic purposes can soon take place inside living organisms, giving rise to metabolites. Parent substances and metabolites then undergo further transformations in the environment, through biotic and abiotic processes, and the cascade of transformation products (TPs) is exponentially magnified until total mineralization is achieved. The identification of a huge number of unknown TPs in environmental matrices is complicated by their diverse physico-chemical properties, which make their determination difficult with one analytical procedure, and the absence, in most cases, of analytical standards to confirm their chemical structure. Nevertheless, a lot of progress has been made in recent years with the use of sophisticated analytical instrumentation, predictive tools and simulated laboratory assays (1).

Biochemical transformations of organic toxicants are governed by specific enzyme-mediated pathways, thus reasonably predictable and limited. On the other hand, abiotic processes are extremely diverse and non-specific (e.g., catalyzed by radicals), resulting in myriads of products with different predominance (2). Abiotic transformations comprise chemical hydrolysis, oxidation/reduction, isomerisation, photolysis, etc., influenced by chemical and physical factors such as temperature, pH, salinity, chemical sensitizers that make part of the water and soil composition, and sunlight radiation with different intensity and wavelength, depending on the latitude, altitude and season of the year (2-4). Different TPs with diverse environmental behaviour and ecotoxicological profile can be formed depending on the predominant chemical or biochemical process taking place, as well as the surrounding media where it occurs (surface water, groundwater, soil, sediment, wastewater effluents, etc.) (4, 5).

Fatta-Kassinos et al. (2) carried out a review on degradation products resulting from advanced oxidation processes (AOPs) used for water and wastewater treatment, aiming at the destruction of recalcitrant organic substances but which usually fail to achieve total mineralization. The disappearance of the original substance does not guarantee the complete efficacy of the process and the intermediates formed may preserve the mode of action of the parent compound or even be biologically more active (3).

As long as the identification of all TPs is excessively challenging, attempts can be made to assess the final biological activity, but the assays must be relevant for human or

environmental toxicology. Following this strategy, we enter another field of prospect since a large battery of assays has to be used to arrive to valid conclusions. Valid assays should tackle both real targets and real concentration levels, which is not always the case (6).

A review published by Farré et al. (1) is a good background work for this chapter and gives an account of the levels of several emerging pollutants and respective TPs in wastewaters (influent and effluent) and surface waters, until 2008. Nevertheless, it is discernible from the present literature review that a lot of progress has been made on this subject in these last 4 years.

6.2. Transformation Products in Natural Waters: from Contamination Sources to Drinking Water Production

Natural waters are the most relevant environmental compartment as regards the impact of pollution by emerging contaminants and their TPs, being the first to be endangered. Indeed, natural waters are the habitat of numerous species, give support to important human activities and are source of environmental services (as the supply of fish and raw water for the production of drinking water), which ought to be protected and preserved.

Horvat et al. (5) have studied the occurrence and fate of anthelmintics and their TPs in the environment based on their high applicability in animal husbandry and aquaculture. Benzimidazoles, the most popular group of anthelmintics, may undergo sulfide oxidation in the environment as well as in human metabolism. As a result, albendazole is converted into albendazole sulfoxide, also known as ricobendazole, the major active metabolite responsible for albendazole efficacy. Some of the sulfoxide is further transformed to albendazole sulfone which does not appear to have any anthelmintic activity. Similarly, flubendazole is transformed into active fenbendazole sulfoxide, known as oxfendazole. Benzimidazoles undergo ester-group demethylation followed by decarboxylation of the carbamic group giving rise to the amine-derivative, which has been reported to be the main photodegradation product and also the major metabolite of these drugs (7). Ricobendazole amine has been identified.

The information available on the levels of anthelmintics in the environment is very limited and even scarcer for metabolites and TPs. Although levels of flubendazole and

thiabendazole were measured in wastewaters (in the range 19.9–89.7 μg L^{-1} for influent and 55.0–671 ng L^{-1} for effluent) and surface waters (3.9 to 27.3 ng L^{-1}), respectively, no metabolites are reported. On the other hand, 22,23-dihydroavermectin B1a was found in sediments under a fish farm where ivermectin has been administered, at the mean concentration of 5.0 ng g^{-1} in the top 3 cm (8).

Gonçalves et al. (9) have studied the behaviour of oseltamivir carboxylate (OC), the active metabolite of oseltamivir (OE, Tamiflu), in the context of the past predicted pandemia of influenza virus H1N1. The levels measured in the Ebro River basin during fall/winter 2009 reached maximum concentrations in Sástago, downstream the city of Zaragoza (OC 50 and OE 100 ng L^{-1}), Miranda de Ebro (OC 46 and OE 83 ng L^{-1}) and El Bocal (OC 42 and OE 83 ng L^{-1}). Photolysis TPs of OE identified as TP330 (photo-induced hydration) and TP312 (photo-isomerisation) were found in the above sites at vestigial levels, which could not be quantitated due the absence of pure standards. Also some tributaries presented levels of antivirals: River Huerva contained a concentration of OC of 46 and OE of 73 ng L^{-1} while the concentrations in River Segre amounted to OC 22 and OE 35 ng L^{-1} (9).

Jongh et al. (10) studied the fate and levels of pharmaceuticals and degradation products along the water cycle, from source to finished drinking water. Concentrations in pre-treated surface waters by such simple treatments as fast sand filtration, infiltration in dunes or sole storage in large reservoirs generally reduced the concentrations by one order of magnitude. This was observed for tramadol, venlafaxine and carbamazepine and its TPs, but other compounds kept similar concentrations. Notably, the river bank filtrate concentrations of phenazone and 1-acetyl-1-methyl-2-phenylhydrazide (AMPH) significantly exceeded the concentrations in surface waters by almost one order of magnitude. This finding was explained by the authors as possibly historical contamination due to phenazone and dimethylaminophenazone high usage rates in the River Meuse and Rhine catchments some decades ago (10). The concentrations of degradation products rarely exceed 100 ng L^{-1}, with average values of several tens of ng L^{-1}. The concentrations of O-desmethyltramadol in the surface water samples ranged between 27 to 73% of its parent compound tramadol, which corresponds to the ratio of the excreted human metabolite via urine. In a different way, the concentrations of carbamazepine-10,11-epoxide were also below the parent compound carbamazepine (about 13% to 37%) despite the main human metabolite being carbamazepine-10,11-diol, to which the epoxide is transformed. Nevertheless, a study on wastewater showed that the rations of carbamazepine-10,11-epoxide to carbamazepine formed are preserved in surface waters

(12-13%) (10). Conversely, the concentrations of O-desmethylvenlafaxine were higher than its parent venlafaxine in the surface water samples (between 128 and 208%) (10).

Pesticides TPs in the environment are a particularly concerning group of substances because of their potential acute toxicity. Hernandez et al. (11) carried out the entire job of elucidation, identification and quantitation of several of these substances in surface and groundwaters of Valencia, Spain, an agricultural area much affected by the use of pesticides. TPs were detected in higher number than the parent substances and at concentration levels reaching some μg L^{-1}, which exceed the respective parent compounds. These concentrations surpasse the 0.1 μg L^{-1} limit generally established for natural waters, which would pass uncovered if the degradation products were not monitored.

The environmental contamination with polybrominated diphenyl ethers (PBDEs) poses a singular scenario. Although less brominated PBDEs have been phase-out, it has been shown that heavier ones, like deca-BDE, degrade by photolytic cleavage into lighter ones (debromination), including penta-BDE. This last substance is not only more toxic but also more stable and more easily transported in the atmosphere crossing long distances to remote regions. Schenker et al. (12) have estimated that about 13% of the penta-BDE and about 2% of the tetra-BDE homologues found in the environment arise from the degradation of deca-BDE. Although, this study focused on the air compartment, deposition in rainfall leads to contamination of water bodies.

Qu et al. (13) carried out an extensive protocol aiming the identification of neurotoxic brominated flame retardants (BFRs) and their by-products in environmental samples near a BFR-manufacturing plant, in China. Tetrabromobisphenol A diallyl ether was identified as a key developmental neurotoxicant in the most potent fraction of sediments where it was also measured in huge concentrations (around 10 mg Kg^{-1}). This compound partitions to the water phase in the ng L^{-1} concentration range and it was detected 3.1 km farther downstream the BFR manufacturing plant (13).

Alkylphenol ethoxylates (APEOs) constitute one of the main non-ionic surfactant groups with particular environmental relevance, since some of their possible metabolites (nonylphenol (NP), A_9PEO_1 and A_9PEO_2) have shown to be endocrine disrupting compounds. Nonylphenol polyethoxylates are large volume production chemicals used as detergents, emulsifiers and dispersing agents during the last 4 decades, both for domestic and industrial purposes (1, 14-16). The whole chain of degradation products is found in surface waters with concentrations ranging from hundreds of ng L^{-1} to several μg L^{-1} and

different toxic burden (14). Because all of these compounds exist together as mixture in the environment a risk assessment of the overall mixture is required.

Despite a reasonable amount of data is currently available concerning the occurrence of APEOs in fresh waters, the knowledge about their subsequent fate in saline waters is still limited. Maximum estuarine and sea concentrations reported in literature are around 1 μg L^{-1}, with some exceptions such as 10 μg L^{-1} in Spain and 25 μg L^{-1} in Israel. These values are found to be roughly one order of magnitude lower than those in fresh waters (17, 18). Jonkers et al. (19) elaborated a field study on surface waters, sediments and suspended particulate matter, with the goal to determine the sources and levels of A_9PEOs, NP and the carboxylated metabolites (A_9PECs) on the Dutch coastal zone, as well as their fate. According to different sampling campaigns, $A_9PEO_{1,2}$ and $A_9PEO_{>2}$ maximum concentrations varied between 0.14-0.73 and 0.27-35 μg L^{-1}, respectively, while NP and A_9PEC maximum levels ranged from 0.031-1.7 and 0.11-0.63 μg L^{-1}, respectively. On the other hand, on the fresh water side of the locks at the mouth of the North Sea canal, slightly higher concentrations of metabolite compounds were obtained: 0.042 μg L^{-1} for NP and 0.31 μg L^{-1} in the case of A_9PEC.

Poly- and perfluoroalkyl substances (PFAs) comprise a diverse range of high production volume chemicals that have been used in industrial and consumer products for the last 5 decades (in metal plating, fire-fighting foams and oil/water repellents). Chemicals like perfluorooctanosulfonates (PFOS) and perfluoroalkylcarboxylates (PFCA) are themselves better well-known than their chemical precursors of industrial origin (ammonium perfluorooctanoate (A-PFO); and fluorotelomer alcohols (FTOHs) and perfluoroalkane sulfonamidoethanols (PFASEs), respectively) (20). These compounds are so persistent and ubiquitous that could be found in sea waters of remote regions, most surface waters and also in wildlife (20, 21). Maclachlan et al. (22) conducted a survey of perfluorinated carboxylates (PFCA) in 14 major rivers including the Rhine, Danube, Elbe, Oder, Seine, Loire, and Po. The highest concentrations measured were 200 ng L^{-1} for perfluorooctanoate (PFOA) in the Po River followed by 32 ng L^{-1} of perfluorohexanoic acid (PFHxA) in Thames River. In most other cases the levels of PFAs substances remain below 10 ng L^{-1}. The Po River accounted for two-thirds of the total PFOA discharge in the studied rivers, which the authors attributed to a fluoropolymer manufacturing facilities in the Po watershed (22). In recent years attention has been expanded from environmental monitoring of acidic PFAs to include neutral compounds such as fluorotelomers (olefins, alcohols, acrylates), fluoroalkylsulfonamides, and fluoroalkylsulfonamidoethanols (23).

6.3. Wastewaters as Major Source of Transformation Products

Fatta-Kassinos et al. (2) stress that the knowledge on the biodegradation products of pharmaceuticals, i.e., TPs formed during biological wastewater treatment is currently only little, therefore studies focusing on this topic should be undertaken so that the possible hazards related to the biotransformation products released in the environment can be also assessed. One should bear in mind that effluents from municipal wastewater treatment plants are a major source of emerging contaminants (e.g., hormones, pharmaceuticals and personal care products) and their degradation products, which affect particularly small water streams in dry seasons.

Applying a systematic approach named "fragmentation-degradation relationship", Gomez-Ramos et al. (3) identified eight TPs in wastewaters out of 147 precursor compounds that were established in the database. The most frequent TPs in eight samples collected from four WWTPs in Spain were erythromycin anhydride, cleaved azithromycin and 4-aminophenol. Erythromycin anhydride (*m/z* 716) quantified in effluent samples in the range from 27 to 159 ng L^{-1}, using a commercial authentic standard. A hydrolysis product of erythromycin, erythralosamine (*m/z* 540), was also identified. The authors recall particular attention to the presence of 4-aminophenol in wastewaters, as degradation product of acetaminophen. Levels of this TP in the μg L^{-1} range were quantified, raising ecotoxicological concerns as 4-aminophenol has been described as very toxic to aquatic organisms, causing long term adverse effects (3).

TPs of carbamazepine, an ubiquitous, persistent and abundant drug in the aquatic environment were also detected: the monohydroxylated product with *m/z* 255 was assigned as 10,11-dihydro-10,11-hydroxycarbamazepine, while the dihydroxylated product was also evidenced with *m/z* 271, corresponding to 10,11-dihydro-10-dihydroxycarbamazepine, corroborating a previous report (24). Carbamazepine 10,11-epoxide, which is said to be frequently detected in wastewater samples, was also identified.

Ghosh et al. (25) have measured oseltamivir carboxylate at a maximum concentration of 293 ng L^{-1} in effluents from conventional activated sludge-based WWTPs, but the concentration decreased to 37.9 ng L^{-1} when an advanced WWTP with ozonation as a tertiary treatment was used. The levels of oseltamivir carboxylate measured by Prasse et al. (26) in two German WWTPs were 42.7 and 17.3 ng L^{-1} in the influent and effluent of

WWTP1 and 29.4 and 12.2 ng L^{-1} in WWTP2, respectively, while the levels of oseltamivir were constantly inferior to the metabolite, in agreement with the human excretion rate of about 80% in the carboxylate form.

A TP of amoxicillin, a commonly used antibiotic belonging to the group of penicillins, was recognized as being (5R) amoxicillin diketopiperazine-2´.5´ (*m/z* 366), recently reported in both wastewater and surface waters (27, 28).

Jia et al. (29) investigated the occurrence of six tetracyclines (TCs) and ten of their degradation products wastewaters and surface waters in China. They elicit ecotoxicity on fresh water species such as the cyanobacteria *Micocystis aeruginosa* and phytotoxicity on aquatic higher plants as *Lemna gibba* (30, 31). Tetracycline abiotical degradation is known to occur depending on pH, redox and light conditions, through epimerization, dehydration and proton transfer reaction pathways (32). 4-Epi-TCs can be formed in the aquatic medium under mildly acidic conditions (pH 2-6) and reversed back to their active form in specific alkaline conditions and in the presence of a complexing metal. Such are the cases of 4-epitetracycline (ETC) and 4-epioxytetracycline (EOTC), originated from TC and oxytetracycline (OTC), respectively. On the other hand, anhydro-TCs such as anhydrotetracycline (ATC) and anhydrochlortetracycline (ACTC) are formed under strongly acidic conditions (pH < 2). These compounds can also epimerize to form epi-analogues (29).

Jia et al. (29) then applied the developed method to the analysis of wastewaters and surface waters in China, using commercial standards for the clear identification of every compound. Apart from TC and OTC, the main analyte quantified in an influent sample at 72.5 ng L^{-1}, also several degradation products were detected at low nanogram per litre levels. Such were the examples of ETC, EOTC, ATC, isochlortetracycline (ICTC) and 4-epianhydrochlortetracycline (EACTC), mostly eliminated through the WWTP treatment; besides TC and OTC, only ICTC and EACTC were quantified in effluent samples ranging from 1.9 to 7.6 ng L^{-1}.

Metformin is a highly consumed antidiabetic drug worldwide, which partially justifies its release in significant amounts (low μg L^{-1} range) to environmental recipient waters. On the other hand, this compound is not metabolized in humans (33). Measured concentrations of metformin in Belgian WWTP influents went up to 94 μg L^{-1} (34), while Scheurer et al. (35) published values for German WWTP influents as high as 129 μg L^{-1}. It is also known that metformin is biologically degraded to guanylurea in WWTPs. Due to high metformin

concentrations in influents and its high yet incomplete removal during the treatment process, both compounds reach surface waters in considerable amounts of up to several tens of μg L^{-1} (36).

Although a study from Trawtwein et al. (37) indicated guanylurea as the only recalcitrant, aerobic, bacterial degradation product of metformin, the detected concentrations of guanylurea in the effluent samples did not completely account for the corresponding removed fraction of metformin. Information is also scarce concerning the ecotoxicological relevance of both these compounds. Therefore, Scheurer et al. (36) recently published a systematic study aiming to clarify the direct transformation of metformin into guanylurea in WWTPs, evaluating the effectiveness of different treatment techniques applied in waterworks. In the five WWTPs comprised in the study, guanylurea ranged from undetectable levels to 3 μg L^{-1} in influent samples, reaching a concentration of 99 μg L^{-1} in an effluent sample corresponding to an influent with 105 μg L^{-1} of metformin. This observed widespread occurrence of guanylurea reinforces the environmental relevance of metabolites.

To better assess the impact of emerging pollutants and their TPs in the environment and human health, there is the need to better understand the chemical and physical transformations occurring at the treatment plants, in order to determine their capacity to remove the contaminants or turn them into more persistent and toxic compounds. Gagnon et al. (38) reported on the influence of different wastewater treatment processes on the fate of some pharmaceutical compounds. During this study, 2-hydroxy-ibuprofen was identified as a metabolite of ibuprofen. This TP was quantified in effluent samples in concentrations ranging from ca. 300 to 3,500 ng L^{-1}, depending on the WWTP where the sample was collected and the treatment technique therein employed. The relatively high concentrations of 2-hydroxy-ibuprofen helped justifying the low concentration of ibuprofen after treatment (38).

Iodinated contrast media TPs were also detected in a municipal WWTP effluent with concentrations as high as 660 ng L^{-1} for iomeprol TP791. While the concentration levels measured were the highest among all environmental matrices analysed, less TPs (only 10) were detected (39).

In another work, Bueno et al. (40) described the development of an enhanced liquid chromatography-mass spectrometry (LC-MS) strategy for the analysis of a selected group of 56 organic pollutants in wastewaters composed by 38 pharmaceuticals and 10 of their

most frequent metabolites, 6 pesticides and 2 disinfectants. Since target analysis based on LC-MS provides a great performance for quantitative analysis, but fails in the determination of compounds not initially included in the multiresidue methods (non-target compounds), the latest trends to increase methods scope include the combination of two complementary LC-MS techniques or the use of hybrid systems with different analyzer designs. Therefore, Bueno and co-workers adopted a LC-MS methodology based in the use of a hybrid triple-quadrupole linear ion trap mass spectrometer (QTRAP), for accurate quantification, in combination with time-of-flight mass spectrometry (TOF-MS), providing unequivocal confirmation. The developed methodology was then successfully applied to a monitoring study of six WWTPs, in Spain. Herein, a group of pharmaceutical compounds metabolites were detected and quantified: carbamazepine-10,11-epoxide (13-110 ng L^{-1}), 1,7-dimethylxanthine (or paraxanthine) (131-80,875 ng L^{-1}), clofibric acid (67-81 ng L^{-1}), fenofibric acid (14-349 ng L^{-1}), and the metabolites of the antipyretic drug dipyrone, 4-methylaminoantipyrine (4-MAA, 9-9,253 ng L^{-1}), *N*-acetyl-4-aminoantipiryne (4-AAA, 2,109-25,030 ng L^{-1}), *N*-formyl-4-aminoantipiryne (4-FAA, 40-10,114 ng L^{-1}), 4-dimethylaminoantipiryne (4-DAA, 122 ng L^{-1}), 4-amino-antipiryne (4-AA, 131-9,286 ng L^{-1}) and antipyrine (17-2,760 ng L^{-1}) (40). The authors also recalled particular attention to the fact that paraxanthine and 4-AAA were present at the highest levels in the effluent samples collected in the WWTP of Almeria, possibly due to the proximity of a hospital. Moreover, the group of metabolites of dipyrenone was also detected at quite high concentrations (40).

Concerning the group of sulphonamides, widely used synthetic antibiotics, García-Galán et al. (41) studied the behaviour of sulfapyridine (SPY), typically used in human therapies, and the veterinary sulphonamide sulfamethazine (SMZ), as well as their acetylated metabolites, AcSPY and AcSMZ, in wastewater matrices under artificial irradiation conditions. Compounds as SPY and SMZ are amongst the sulphonamides most frequently detected in effluent wastewaters and surface waters, respectively. Results showed that the photolysis of SPY produced a total of 10 different TPs, 4 major compounds (M) and 6 other minor products (m), upon irradiation for 30 h: the hydroxylated metabolite ($C_{11}H_{12}N_3O_3S$), *m/z* 266.0599 (M); the SO_2 extrusion product, N-(pyridin-2-yl)benzene-1,4-diamine ($C_{11}H_{12}N_3$), *m/z* 186.1031 (M); the hydroxylated moiety of this desulfonated product, N-hydroxy-N-(pyridin-2-yl)benzene-1,4-diamine ($C_{11}H_{12}N_3O$), *m/z* 202.0980 (m); a compound resulting from the reduction of the hydroxylamine in the N^4 position of the hydroxylated product ($C_{11}H_{10}N_3O_3S$), *m/z* 264.0443 (M); pyridine-2-amine ($C_5H_7N_2$), *m/z* 95.0609 (M); hydroquinone (m); aniline (m); sulphanilamide (m); sulfanilic acid (m);

aminosulfonic acid (pyridin-2-ylsulfamic acid) ($C_5H_7N_2O_3S$), which resulted from the cleavage of the bond between the aniline ring and the sulfonic group, followed by an -OH addition to the SO_2 group, *m/z* 175.0177 (m). On the other hand, the photodegradation of N^4-acetylsulfapyridine yielded a new molecule with a predicted elemental composition $C_{13}H_{14}N_3O$ (*m/z* 228.1137), through the loss of the sulfonate group. Regarding SMZ, eight different TPs were identified: similarly to what happened with SPY, a desulfonated product of SMZ was detected, presenting two different signals corresponding to the same mass (*m/z* 215.1297) and yielding the same mass spectra – stereoisomers with the elemental composition $C_{12}H_{15}N_4$; its hydroxylated moiety $C_{12}H_{15}N_4O$, *m/z* 231.1246, and the desaminated product $C_{12}H_{14}N_3$, *m/z* 200.1188; 4,6-dimethylpyrimidin-2-amine ($C_6H_{10}N_3$), *m/z* 124.0875; the hydroxylated product of SMZ (*m/z* 295.0865) and desaminated moiety (*m/z* 264.0807) of SMZ; a compound resulting from the reduction of the hydroxylamine in the N^4 position of the hydroxylated product ($C_{12}H_{13}N_4O_3S$), *m/z* 293.0708; (4,6-dimethylpyrimidin-2-yl) sulfamic acid ($C_6H_{10}N_3O_3S$), *m/z* of 204.0443. Finally, AcSMZ photodegradation yielded 3 TPs: one resulting from the loss of SO_2 and the methyl group from the acetylated moiety ($C_{13}H_{14}N_4O$), *m/z* 243; a second one as the result of a desulfonation reaction (*m/z* 257); a third TP attributed to a structural rearrangement of the desulfonated photoproduct ($C_{15}H_{19}N_4O$), *m/z* 271.

Benzodiazepines (BZPs) are prescribed in high amounts worldwide and constitute potentially new emerging contaminants. However, the environmental persistence and fate of these pharmaceuticals, as well as their degradation products is, yet, scarcely understood. Hence, a study by Kosjek et al. (42) addressed this gap by monitoring environmental concentrations of several benzodiazepine residues and studying their removal during biological and photochemical water treatment, including the identification of stable TPs. As a result, the elucidation of eight novel diazepam (DZP) TPs and four novel oxazepam (OXA) TPs was performed. DZP biodegradation results in the formation of OXA, nordazepam (NRZ) and temazepam (TMZ), which are also human metabolites of DZP and marketed as individual pharmaceuticals. NRZ is formed by N-demethylation on N-1 of DZP, TMZ by hydroxylation of the C-3 atom and OXA comprises both transformation reactions. The newly discovered DZP TPs included 5 isomers with elemental composition $C_{16}H_{14}N_2O_2Cl$ and nominal mass of 300 Da, resulting from one single hydroxylation of DZP, and 2 isomers with elemental composition $C_{16}H_{14}N_2O_3Cl$ and nominal mass of 316 Da, resulting from double hydroxylation of DZP, all obtained by photocatalysis; the last TP identified, resulted from biotransformation of DZP and presented the elemental composition $C_{16}H_{16}N_2O_2Cl$ and a nominal mass of 302 Da.

Regarding OXA, 1 degradation product with elemental composition $C_{15}H_{12}N_2OCl$ and nominal mass 270 Da was originated by biotransformation reactions, while other 3 hydroxylated isomers, presenting the elemental composition $C_{15}H_{12}N2O_3Cl$ and a nominal mass of 302 Da, were the result of photocatalysis at pH 2.

In another study by Calisto et al. (43), the relevance of photodegradation processes on the environmental persistence of four benzodiazepines - OXA, DZP, lorazepam (LZP) and alprazolam (ALP), was investigated. These benzodiazepines were irradiated under simulated solar conditions, together with three different fractions of humic substances. A total of nineteen TPs were identified by electrospray mass spectrometry, 7 for OXA, 4 for DZP, 6 in the case of LZP and finally 2 for ALP.

Sousa et al. (44) also studied LZP phototransformation pathways under artificial UV and natural solar irradiation, through photolytic and TiO_2-assisted photocatalytic processes. Herein, fourteen compounds (excluding structural isomers) were identified as presumable LZP TPs. The proposed photodegradation mechanism included the initial opening of the diazepinone seven-membered ring, followed by a rearrangement into a highly stabilized six-membered aromatic ring and subsequent cleavage and/or hydroxylation reactions. LZP photocatalytic degradation was further assessed on a contaminated municipal effluent, where the photoproducts generated showed to be more persistent than LZP itself.

Estrogens high potency at the nanogram per litre level makes it important to assess their fate in the urban environment and evaluate the effectiveness of different processes of the urban sewer infrastructure in removing or lowering their estrogenic potential. Other chemicals such as octylphenol, nonylphenol, nonylphenol ethoxylates, bisphenol A and phthalates also express estrogenicity, even though to a lesser extent (15, 45). Limpiyakorn et al. (46) recently published a review work summarizing data collected from a total of 130 full-scale WWTP distributed over 14 countries and focusing on the fate of estrogens and estrogen potentials in the sewerage system, starting from human excretion until municipal sewage treatment facilities. Humans generally excrete 90-95% of estrogens via urine, preferentially in the conjugated forms. Conjugated estrogens usually found in female urine include estrone 3-(*β*-D-glucuronide) (E1–3G), estrone 3-sulfate (E1–3S), 17*β*-estradiol 3-glucoronide (E2–3G), 17*β*-estradiol 17-glucoronide (E2–17G), 17*β*-estradiol 2-sulfate (E2–3S), estriol 3-(*β*-D-glucuronide) (E3–3G), estriol 16α (*β*-D-glucuronide) (E3–16G) and estriol 3-sulfate (E3–3S). Glucuronide estrogens are found to be the most predominant form (47, 48). Afterwards, in the septic tanks, the dominant transformation mechanism

appears to be conjugation, with sulphate estrogens concentrations to the total conjugated estrogens, increasing from 22% in the influent to 55% in the effluent. In sewers, conjugated estrogens E1–3S, E1–3G, E2–3S, E2–17G, E2–3G, E3–3S, E3–16G, and E3–3G were found to be reduced by about 64%, 84%, 100%, 0%, 100%, 84%, 100% and 0%, respectively (47). Finally, D'Ascenzo et al. (47) were able to quantify E1–3G, E1–3S and E3–3S in municipal WWTPs samples, of which 0.7 ng L^{-1}, 9.0 ng L^{-1} and 2.2 ng L^{-1}, respectively, remained in the effluent. In another study conducted by Isobe et al. (49), E1–3S and E2–3S were found in the concentrations of 0.3-2.2 ng L^{-1} and 1.0 ng L^{-1}, respectively, in the effluent of WWTPs in Japan. On the other hand, in a batch study performed by Ternes et al. (50), the conjugated estrogen E2–17G was found to be totally cleaved under aerobic conditions by activated sludge in a German WWTP.

Moving to pesticides TPs group, Gomez-Ramos et al. (3) have identified unequivocally, with the help of a pure standard, the 2-isopropyl-6-methyl-4-pyrimidinol (IMP - *m/z* 153) as a TP of diazinon, an organophosphorous pesticide widely used in urban and agricultural applications. This TP was quantified in 2 samples from 2 different WWTPs at the concentrations of 288 and 630 ng L^{-1}. It had already been reported in surface waters and referenced as one of the major degradation products of diazinon, showing a higher genotoxic potential than diazinon itself (24, 51).

As previously mentioned, nonylphenol polyethoxylates (NPEOs) give rise to a great variety of dicarboxylic degradation products (CAPECs), particularly those containing five to eight carbon atoms (CA_{5-8}PECs). Their demonstrated presence and possible persistence in several environmental compartments is raising concern worldwide (52). However, reports on CAPECs environmental occurrence and behaviour, including their possible eco- and human toxicological effects, are still very scarce. In this context, Hoai et al. (52) investigated four model compounds of dicarboxylic metabolites (dm-CA_{5-8}P1EC) and other dicarboxylic metabolites (CA_{5-8}P1ECs) of nonylphenol polyethoxylates in wastewater, river and pure water. The dicarboxylic metabolites referred as dm-CA_{5-8}P1EC present an α,α-dimethyl configuration (expressed as "dm"), five to eight carbon atoms and a carboxyl group in the alkyl chain, besides an ethoxy acetic acid group.

Though dm-CA_{5-8}P1ECs metabolites were not detected in the collected water samples, Hoai et al. (52) could identify 21 isomers of CA_{5-8}P1ECs by CI-MS in surface and wastewater effluents in Japan: 2 isomers of the CA_5P1EC metabolite, 5 isomers of the CA_6P1EC metabolite, 6 isomers of the CA_7P1EC metabolite and 8 isomers of the CA_8P1EC

metabolite. The metabolites of CA_6P1EC and CA_8P1EC were identified as the dominant compounds, at μg L^{-1} level, from 2-15 times higher than CA_5P1EC and CA_7P1EC metabolites, leading to the conclusion that nonyl chains degradation occurs mainly through the elimination of two carbon units. Hoai et al. (52) also highlighted that dicarboxylic degradation products should play an important role when studying the behaviour of NPEOs in WWTPs, due to the high concentrations of $CA_{5-8}P1ECs$ metabolites in the studied river and WWTP effluents. Moreover, the obtained results for surface and wastewaters in Japan were compared to previous ones reported in Italy and Taiwan, where $CA_{5-8}P1ECs$ concentrations were about one and two orders of magnitude higher, respectively.

6.4. Origin and Presence of Transformation Products in Drinking Water

In their review, Mompelat et al. (4) highlighted the human health risk associated with the occurrence of pharmaceuticals and degradation products in drinking water, even at low concentrations. According to these authors, drinking water is the least studied when it deals with the occurrence, fate and behaviour of emerging pollutants' by-products, compared to environmental matrices.

Lee et al. (53) have studied the fate of steroid estrogens upon chlorination. Ortho-substituted derivatives were identified as the main by-products which displayed considerably weaker estrogenic potency. In the presence of increased levels of bromine in the raw water and low concentration of dissolved organic matter and ammonia, secondary products of 17α-ethinylestradiol (EE2) were identified, containing one or two bromine atoms in both positions (2- and 4-) of the phenolic ring. These products can be further transformed either by chlorine or bromine into a cleaved phenolic moiety (53). During the chlorination process (at pH 7) and under typical concentrations of 17α-ethinylestradiol at ng L^{-1} levels and bromine between 10-100s μg L^{-1}, the predominant products are the brominated ones (4-Br- and 2,4-diBr-17α-ethinylestradiol) until total depletion of bromine. The formation of chlorinated by-products of 17α-ethinylestradiol is faster at pH from 7-9 with higher accumulation of 2,4-dichloroderivatives. The authors advocate that the same behaviour can be extrapolated for other steroid estrogens, such as 17β-estradiol (E2), estrone (E1) and estriol, and other phenol-containing endocrine disrupting

compounds such as bisphenol A and nonylphenol, in which the presence of bromine accelerates the transformation and gives rise to less potent by-products (53).

Under ozonation conditions in water treatment works the primary oxidation end-products of metformin were identified as the hydroperoxide of metformin, methylbiguanide and 2-amino-4-imino-5-methyl-1,3,5-triazine (36). Under chlorination both substances metformin and guanylurea should undergo similar reactions. Dimethylamine was identified as the prime chlorination product but others such as: N-chlorourea, NCl_3, $NHCl_2$ and NH_2Cl can be formed by hydrolysis of the amine group and further chlorination (36). The presence of guanylurea in finished drinking water seems, however, to be unlikely if a underground passage is part of the raw water treatment train (36).

Illicit drugs have been recognized in recent years as a new group of water contaminants and a sufficient pool of data exists already on their ubiquity around the world (54). Huerta-Fontela et al. (54) have demonstrated in previous works that most illicit drugs and respective metabolites can be removed during the steps of drinking water production. Nevertheless, they can give rise to the formation of disinfection by-products. A common chlorinated by-product (3-chlorobenzo)-1,3-dioxole, was identified for both 4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxyethamphetamine (MDEA) while for 3,4-methylenedioxymethamphetamine (MDMA), 3-chlorocatechol was found (54). The chemical (3-chlorobenzo)-1,3-dioxole was found after the first chlorination step, in concentrations from 1.2 to 3 ng L^{-1}, and was eliminated after ozone and graphitic activated carbon filtration. On the other hand, 3-chlorocatechol, generated mainly after the post-chlorination step, showed to be recalcitrant and it was found in final treated waters at concentrations ranging from 0.5 to 5.8 ng L^{-1} (54).

6.5. Ubiquity and Regio-Specificity of Transformation Products

After all the information regarding the occurrence and fate of pharmaceuticals and their TPs is gathered, one of the most difficult issues is to prioritize them and extend these finds to other environmental scenario. Indeed, a great variability may be found in terms of geographical consumption, seasonal and environmental climatic conditions, contexts of water treatment, flora and fauna exposed individuals and water safety regulations.

According to the results given in the review by Mompelat et al. (4) and our own literature review most of the studies on degradation products of emerging contaminants have been produced in Germany followed by France and UK, which is not surprising as these countries lead the environmental research and host the most renowned researchers in the field. Spain and Switzerland also devote great effort to environmental research which was reflected in the present literature survey.

A variety of studies on PDBEs and respective degradation products have taken place in Asia, namely in China (55). The fast growth of the electronics manufacturing capacities for the global market and the accumulation of a large fraction of electronic waste (E-waste) from developed countries, which are submitted to uncontrolled recycling, endangered the environment with PBDEs and their by-products (55). The results presented in the previous section regarding the detection of tetrabromobisphenol A diallyl ether in an industrial area attest the relevance of brominated flame retardants contamination in China (13).

As far as ubiquity is concerned, one of the main gaps hampering wider/global perception is the lack of data in "younger" European and accession countries. The studies conducted by Loos et al. (21, 56) at the European Union Joint Research Centre (JRC) are exceptions and they proved the ubiquity of NPE1C and NP in surface- and groundwaters which were even detected at remote areas.

It is obvious from the present review that results on emerging pollutants and degradation products is mainly available from Mediterranean and Central European countries as well as from Asiatic (China and Japan) and American countries (Brazil, Canada and USA). Eastern countries contribute very little to this awareness; however, it is believed that today's global economy and globalized habits may give rise to similar (qualitative if not quantitative) contamination problems. In other words, with due exceptions, the ubiquity or regio-specificity may be more a matter of availability of studies rather than environmental reality. Furthermore, Europe is crossed by big water streams which conduct organic micropollutants from industrialized/populated areas to far distances that suffer the indirect impact of these substances.

6.6. Transformation Products of Emerging Contaminants: Fate and Behaviour

Transformation reactions mediated by biotic and abiotic factors often lead to smaller, more polar, and thus less hydrophobic molecules, which are in turn less toxic and less bioaccumulative. However, in some instances (e.g., when polar or charged parts of a molecule are cleaved off, or heteroatoms are included in the molecule), hydrophobicity is increased leading to increased toxicity. The toxic mechanism (e.g., genotoxicity) may also be altered, as often verified in disinfection by-products (6). In evaluating the toxicity of a TP, two dimensions should be appreciated: the toxicokinetics meaning the behaviour of a substance in terms of uptake, distribution, metabolism and excretion; and the toxicodynamics respecting the type and potency of interaction with the target site which prompts for a biological effect. This two components may differentiate the behaviour of parent compounds and TPs (6).

Using an effect-driven approach, Dodd et al. (57) conducted several toxicity studies with antibiotics during the oxidation by ozone, a process frequently employed in water treatment works. Similarly to roxithromycin, other antibiotics (macrolides, β-lactams, fluoroquinolones, tetracyclines, aminoglycosides, etc.) oxidized by ozone and hydroxyl radicals either did not give rise to TPs in significant amounts and/or their toxicity was much smaller than that of the parent compounds. Exception is noted for penicillin G and cephalexin, which form sulfoxides as first generation TPs retaining antibacterial activity, though further transformation leads to loss of antibacterial activity (57). In such cases where toxicity is eliminated concomitantly with the parent compounds degradation, no further identification and characterization of the TPs should be needed.

Escher and Fenner (6) compiled several reports on the toxicity of emerging pollutants and degradation products, mainly pharmaceuticals, under accelerated oxidation conditions (simulated photodegradation, photocatalysis and ozonation), where *Vibrio fischeri* and *Daphnia magna* were the preferred test species. Under photocatalysis with TiO_2 sulfacetamide, sulfathiazole, sulfamethoxazole, and sulfadiazine don't give rise to toxicologically relevant TPs. Under hypochlorite oxidation the mutagenicity of frazolidone has disappeared while the mutagenicity of nitrofurazone has been reduced. The isomerisation and polymerization of isoamylmethoxycinnamate and ethylhexylmethoxycinnamate under irradiation led to decrease in toxicity. The initial degradation 2-ethylhexyl-4-(dimethylamino) benzoate maintained the same toxicity, but

further degradation decreased toxicity. Dipyrone and three degradation products (4-methylaminoantipyrine, 4-formylaminoantipyrine, 4-acetylaminoantipyrine) were submitted to simulated solar irradiation giving rise to increased toxicity of the reaction mixture after photolysis. Similar behaviour was observed for sulfamethoxazole whose TPs displayed higher toxicity for *Daphnia magna*. The ozonation of a solution containing clofibric acid gave rise to an initial increase in toxicity. The often studied and ubiquitous anti-inflammatory drug in the environment diclofenac photodegrades to 2-[2-(chlorophenyl)amino]benzaldehyde which is more toxic than diclofenac itself due to higher bioconcentration potential (6).

Triazines in the environment generally give rise to dealkylated and hydroxy degradates which, with some exceptions, tend to be less frequently detected than the parent herbicides and occur at lower concentrations. Conversely, two well-known classes of chloroacetamide degradates, the ethane sulfonic acid (ESA) and oxanilic acid (OA) derivatives, are normally encountered at concentrations much higher than the parent herbicides. The array of possible degradation products is also much larger (58). Some studies indicate that the principal hydrolysis products that are formed have long persistence in the environment. Besides, some neutral chloroacetamide derivatives have revealed toxic attributes. Just to mention, 2-hydroxy-2',6'-diethylacetanilide and 2-chloro-2',6'-diethylacetanilide, have demonstrated mutagenic activity and the later can bind DNA. Both 2-ethyl-6-methylaniline and 2,6-diethylaniline are more teratogenic than their parent compounds (metolachlor and alachlor, respectively) while aniline metabolites, such as 2,6-dialkylbenzoquinoneimine and 2-ethyl-6-methylbenzonquinoneimine displayed genotoxic activity (58).

Nonylphenol polyethoxylates are ubiquitous pollutants whose pathway of degradation in the environment depends on the actual conditions. If there is enough oxygen present, as is the case in surface water and upper soil layers, the carboxylation of NPnEO to NP1EC and NP2EC is favoured and nonylphenol is expected to mineralize quickly. Otherwise, nonylphenol formed out of NP1EC or NP1EO may be more persistent (14, 59). Carboxylated ethoxy chains AP2ECs and AP1ECs have been observed in aerobic biodegradation. Anaerobically treated sewage sludge favours the formation of alkylphenols (59). Nonylphenol ethoxylates are known to cause estrogenic responses in aquatic organisms at concentrations similar to those at which chronic effects occur. It is believed however that, short chain analogues have increased toxicity, the highest belonging to nonylphenol (14). In a risk assessment study including degradation products

performed by Fenner et al. (14) under a typical Swiss scenario, although none of the degradation products exhibited a significant risk alone, the mixture risk quotient was 2.2 (>1). The biggest contributions to the overall risk stem from the three most toxic compounds, namely nonylphenol, NP2EO and NP1EO (14). The acids NP2EC and NP1EC exhibit lower single risks. For the special case of nonylphenol polyethoxylates, whose TPs are more toxic than the parent compound itself, the TPs account for 89% of the overall risk, according to Fenner et al. (14) estimations.

Prevedouros et al. (60) reviewed the behaviour and fate of perfluorinated compounds, concluding that these chemicals are primarily emitted to water which is their major reservoir in the environment and transport media and that they accumulate in surface waters, being carried from rivers to sea, due to their very high persistence.

6.7. Conclusions

In this section we will discuss the gaps in knowledge, needs of further research and future trends. A major conclusion highlights from the present review of the literature, building on the remarks expressed by Escher and Fenner (6): the presence of TPs in the aquatic environment is not negligible and clearly contributes to the environmental and human health risk of organic micropollutants. Based on the results of a monitoring study of surface and drinking waters which revealed similar concentrations of transformations and parent drugs with equivalent pharmacological activity, Jongh et al. (10) strengthened the relevance of monitoring TPs and including them in risk assessment.

Furthermore, there is a need to study the evolution of TPs spatially and temporarily in water streams. Also much deeper research is needed targeting the distribution of TPs in the environmental compartments, namely sediments and biota which may be regarded as sinks for toxic compounds or an alternative entrance route in the trophic chain, respectively. Monitoring of TPs in environmental matrices so far have mostly been restricted to major known by-products due to the limited availability of authentic standards both for unequivocal identification and quantification. Several authors just give qualitative or semi-quantitative information on TPs occurrence, while others have circumvented this limitation purifying degraded solutions to be used as standards.

One of the options to address the extensive work to be done on the risk assessment of TPs might be the use of a battery of bioassays including one targeting the specific mode of action of the parent compound, complemented by a nonspecific bioassay sensitive to TPs which has developed high nonspecific toxicity or a new mode of toxic. Nevertheless, chromatographic/mass spectrometric techniques remain essential tools in the elucidation of TPs due to their superior sensitivity and accuracy. In specific cases, nuclear magnetic resonance (NMR) technique is necessary for ultimate identification/confirmation of degradation products, however this technique is not yet widely available, requires purified samples and its sensitivity is inadequate for environmental levels. Hyphenation of NMR to LC instruments is rarely found in environmental laboratories.

To tackle the lack of spectra libraries and of efficient common strategies for the identification of new compounds NORMAN has established a web-based accurate mass spectra database for environmental contaminants, named MassBank database, which is open for contributions and freely accessible (61).

In the future, prioritization of the degradation products which merit inclusion in the monitoring lists of pollutants is a paramount need, as it is already being attempted for the parent emerging pollutants (62). All efforts of environmental science including analytical tasks (identification of TPs and occurrence levels), fate and behaviour studies and risk assessment should converge to supply the data to turn this goal feasible. A strong emphasis on the thorough study of the environmental behaviour of new synthetic substances, as required by REACH (Registration, Evaluation, Authorization and Restriction of Chemical substances) policy, will alleviate the work load that is left for characterizing threats perceived just once they are already established.

With the aim of anticipating the risk posed by synthetic chemicals, opposite to delayed perception of dangerous xenobiotics already widely distributed, the Environmental Specimen Banks (ESB) are being regarded as tools for retrospective evaluation of toxic substances, eventually degradation products (63). Studies of the Swedish ESB on archived guillemot eggs from the Baltic Sea allowed describing the concentration trends of brominated flame retardants and perfluorinated compounds. This ESB are also useful to gauge the success of banning uses of chemicals (63).

As noted in the workshop “Mixtures and metabolites of chemicals of emerging concern” organized by NORMAN in Amsterdam, 2009, pesticides and pharmaceuticals degradation products still remain the most studied chemicals. Research should be enlarged to other

groups. Fatta-Kassinos and Kalavrouziotis (64) highlighted the presence of degradation products of xenobiotics as a current concern related to wastewater reuse.

As an overall conclusion, the study of degradation products requires more clever and productive strategies, in order to overcome the challenge of characterizing a massive number of possible substances with uncertain human health and environmental relevance.

6.8. References

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Chapter 7

Final Conclusions and Remarks: Work Novelty, Knowledge Gaps and Future Research

Over the last few decades, the so called "emerging pollutants" have been raising increasing awareness, due to their widespread environmental occurrence and many times unpredictable (eco)toxicological effects. These pollutants are not necessarily new chemicals, but only the development of new highly sensitive analytical tools has enabled their detection, in often significantly low concentration levels. Among all emerging substances in water, special attention has been given to pharmaceutical products, owe to their demonstrated presence and persistence in environmental waters, as well as for the potential toxicological effects they may induce on non-target organisms.

In order to build knowledge on the environmental occurrence and fate of pharmaceutical compounds, our work began with the development of a multiresidue analytical method for the concurrent determination of 23 pharmaceuticals of diverse chemical nature, among the most consumed in Portugal, in wastewater samples. The main overall conclusions related to the better performance achieved with Oasis MAX cartridges, when compared to all other tested extraction adsorbents (e.g., normal-phase, ion exchange and mixed composition), allowing recovery rates generally higher than 60%. Moreover, these cartridges allowed the simultaneous extraction and cleanup of the sample, thus accelerating the experimental procedure before sample injection into the LC-tandem MS system, while reducing the variability of the results.

In a subsequent work, the developed, optimized and validated method was then applied to evaluate the impact caused by the discharge of a municipal WWTP on Febros river, a small tributary of Douro river, located in the northern region of Portugal, regarding pharmaceutical content only. Febros WWTP implication on the pharmaceutical input to Febros river was evidenced by the higher pharmaceutical load in samples collected

immediately downstream the WWTP discharge point, when compared to upstream sampling sites. Moreover, the performance of the method was assessed through the participation in two European level inter-laboratory exercises, promoted by EU-JRC and PHARMAS FP7. Most of our results fell in a satisfactory z-score range, which further confirmed the reliability of the developed procedure for monitoring pharmaceuticals in water samples.

The limitations of conventional wastewater treatments for the remediation of emerging pollutants have potentiated the development and application of advanced tertiary/quaternary treatment processes. In this field, special attention has been given to Advanced Oxidation Processes, in which hydroxyl radicals (•OH) are the responsible agents for the oxidation and mineralization of almost any organic molecule, due to their strong unselective oxidative power, yielding CO_2 and inorganic ions as final products. Therefore, subsequent work approaching the use advanced photodegradation processes for the removal of a rather recalcitrant anxiolytic drug – lorazepam, allowed to withdraw several conclusions. Firstly, when comparatively evaluating the performance of three distinct experimental phototreatment systems, lorazepam highest degradation yield was obtained by TiO_2-driven photocatalysis, using the solar pilot plant with CPCs (k = 1.49±0.03 L kJ^{-1}, for 200 mg L^{-1} TiO_2). To the best of our knowledge, never was lorazepam studied under accelerated photodegradation treatment processes. In order to provide knew information concerning lorazepam degradation mechanism, studies proceeded using high-resolution and high accuracy instrumentation, such as UHPLC/QqToF-MS, to allow the structural elucidation of lorazepam by-products, according to the concept of "diagnostic fragment ions", besides the acquisition of accurate masses. This work main conclusions included the identification of six major lorazepam degradation products, with nominal $[M+H]^+$ masses of 337, 303, 319, 275, 291 and 293 Da. Moreover, the proposed photodegradation mechanism consisted on the initial opening of the diazepinone seven-membered ring, followed by a rearrangement into a highly stabilized six-membered aromatic ring and subsequent cleavage and/or hydroxylation reactions.

Since the photocatalytic treatment proved to be quite efficient for lorazepam removal, it was further applied to the treatment of a real municipal WWTP effluent, containing several other emerging contaminants. Initial effluent physicochemical characterization revealed the presence of 22 pharmaceutical compounds in moderate concentrations (maximum of 680 ng L^{-1}, except for diclofenac ~24 μg L^{-1} and hydrochlorothiazide ~3 μg L^{-1}). A pseudo-first order kinetic model was able to successfully predict all pharmaceuticals'

degradation kinetics, while the overall treatment was considered efficient, with a complete removal of the majority of these micropollutants, except for ciprofloxacin (35%), ketoprofen (61%) and bisoprolol (77%).

However, one main issue regarding water treatment processes leading to incomplete mineralization of contaminants, is that the resulting intermediate oxidation compounds possibly formed may present even more significant toxicity, individually or in more or less complex mixtures, than parent compounds themselves. Therefore, it is crucial to always support phototreatment processes with solid toxicity results. In our case, an acute toxicity test with the species *V. fischeri* was performed, in order to confirm that the effluent toxicity did not increase significantly over the photodegradation process.

There is still a lot of missing information regarding several aspects of this environmental problematic. From occurrence data to fate in the aquatic compartment, from contamination prevention strategies to the development of end-of-pipe phototreament technology and engineering, several lacunas and doubts have to be first clarified, in order reach an agreement on the most efficient strategies to antagonize this increasing pollution trend. New legislation including these emerging contaminants must be created, but this will only be possible once provided accurate and high quality data, which in turn has to be based upon well developed and validated analytical methodologies, always associated to high performance instrumentation.

Studies on identification of degradation products are vital for proper assessment of the environmental impact. However, a question remains on how these studies can substantially contribute to the understanding of their overall fate under field conditions where “cocktail” effects in aqueous environments are much more complex with numerous organic and inorganic chemicals, including nutrients and suspended solids interacting simultaneously.

Finally, several exercises have now been running, with the purpose to prioritise the most critical questions raised concerning this pollution issue, to aid in the development of future research programs on the topic. From specific workshops regarding the environmental contamination by pharmaceutical compounds and personal care products, the “top 3” of the most relevant questions were as follows:

1) What approaches should be used to prioritise pharmaceutical compounds and personal care products for research on environmental and human health exposure and effects?

2) What are the environmental exposure pathways for organisms (humans included) to pharmaceuticals in the environment, and are any of these missed in current risk assessment approaches?
3) How can the uptake of ionisable pharmaceuticals into aquatic and terrestrial organisms and through food chains can be predicted?

In conclusion, methodologies must be harmonised, strategies must be concerted and efforts must be gathered, in order to achieve the common goal of (aquatic) environmental pollution containment and future regression.

Annex I – NORMAN list of emerging substances (most frequently discussed) (latest update approved March 2011) (8)

List built upon the data collected by NORMAN Associates, after several European surveys.

Category / class	CAS	Individual substances
Algal toxins	101043-37-2	Microcystin-LR
	111755-37-4	Microcystin-RR
	101064-48-6	Microcystin-YR
Anticorrosives	95-14-7	1,2,3-Benzotriazole
	Other	4-/5-Tolyltriazole (TTri)
	29878-31-7	4-Methyl-1H-benzotriazole
	4184-79-6	5,6-Dimethyl-1H-benzotriazole
	136-85-6	5-Methyl-1H-benzotriazole
	302-01-2	Hydrazine
	29385-43-1	Methyl-1H-benzotriazole / Tolyltriazole
	29385-42-1	Methylbenzotriazole
Antifoaming agents	126-86-3	Surfinol-104
Antifouling compounds	1002-53-5	Dibutyl tin ion
	1135-99-5	Diphenyltin ion
	28159-98-0	Irgarol
	78763-54-9	Monobutyl tin ion
	1461-25-2	Tetrabutyl tin ion
	668-34-8	Triphenyltin ion
Antioxidants	128-39-2	2,6-Di-tert-butylphenol
	98-54-4	4-tert-Butylphenol
	25013-16-5	BHA
	1948-33-0	BHQ
	128-37-0	BHT
Biocides	120-32-1	Chlorophene
	1085-98-9	Dichlofluanide
	01-01-4640	Methyl triclosan
	3380-34-5	Triclosan
Bio-terrorism / sabotage agents	76-06-2	Chloropicrin
Complexing agents	67-43-6	DTPA
	60-00-4	EDTA
	139-13-9	NTA
	10543-57-4	TAED

Detergents	20427-84-3	4-Nonylphenol di-ethoxylate (NPE2O)
	104-35-8	4-Nonylphenol mono-ethoxylate (NPE1O)
	3115-49-9	4-Nonylphenoxy acetic acid (NPE1C)
	Other	4-Nonylphenoxyethoxy acetic acid (NPE2C) 4-Octylphenol di-ethoxylate (OPE2O)
	2315-67-5	4-Octylphenol mono-ethoxylate (OPE1O)
	15234-85-2	4-Octylphenoxy acetic acid (OPE1C)
	Other	4-Octylphenoxyethoxy acetic acid (OPE2C)
	69669-44-9	C10-C14-LAS
	25155-30-0	C12-LAS
	120-18-3	Naphthalene sulphonic acid
Disinfection by-products (drinking water)	1768-31-6	1,1,1,3,3-Pentachloropropanone
	16995-35-0	1,1,1,3-Tetrachloropropanone
	632-21-3	1,1,3,3-Tetrachloropropanone
	921-03-9	1,1,3-Trichloropropanone
	867-54-9	1,1-Dibromopropanone
	534-07-6	1,3-Dichloroketone
	683-72-7	2,2-Dichloroacetamide
	74039-30-8	2,3,5-Tribromopyrrole
	79-07-2	2-Chloroacetamide
	95-57-8	2-Chlorophenol
	106-48-9	4-Chlorophenol
	62872-35-9	Acetamide, 2-chloro-2-iodo-
	590-17-0	Bromoacetonitrile
	98136-99-3	Bromochloroacetaldehyde
	62872-34-8	Bromochloroacetamide
	5589-96-8	Bromochloroacetic acid
	83463-62-1	Bromochloroacetonitrile
	34970-00-8	Bromochloroiodomethane
	74-97-5	Bromochloromethane
	135531-25-8	Bromochloronitromethane
	71133-14-7	Bromodichloroacetic acid
	918-02-5	Bromodichloronitromethane
	557-95-9	Bromodiiodomethane
	62872-36-0	Bromoiodoacetamide
	563-70-2	Bromonitromethane
	107-20-0	Chloroacetaldehyde
	107-14-2	Chloroacetonitrile

Disinfection by-products (drinking water)	638-79-9	Chlorodiiodomethane
	1794-84-9	Chloronitromethane
	4471-47-0	Cyanoformaldehyde
	84852-53-9	Decabromodiphenyl ethane
	598-70-9	Dibromoacetamide
	3252-43-5	Dibromoacetonitrile
	5278-95-5	Dibromochloroacetic acid
	1184-89-0	Dibromochloronitromethane
	594-18-3	Dibromodichloromethane
	593-94-2	Dibromoiodomethane
	74-95-3	Dibromomethane
	598-91-4	Dibromonitromethane
	79-02-7	Dichloroacetaldehyde
	3018-12-0	Dichloroacetonitrile
	594-04-7	Dichloroiodomethane
	7119-89-3	Dichloronitromethane
	5875-23-0	Diiodoacetamide
	25637-99-4	Hexabromocyclododecane (HBCD)
	116-16-5	Hexachloropropanone
	144-48-9	Iodoacetamide
	64-69-7	Iodoacetic acid
	624-75-9	Iodoacetonitrile
	75-47-8	Iodoform
	87-56-9	Mucochloric acid
	77439-76-0	Mutagen X (MX)
	79-15-2	N-Bromoacetamide
	924-16-3	NDBA
	62-75-9	NDMA
	59-89-2	NMOR
	55-18-5	N-nitrosodiethylamine (NDEA)
	86-30-6	N-Nitrosodiphenylamine (NDPA)
	10595-95-6	N-nitrosomethylethylamine (NMEA)
	100-75-4	N-Nitrosopiperidine (NPIP)
	930-55-2	N-Nitrosopyrrolidine (NPYR)
	115-17-3	Tribromoacetaldehyde
	594-47-8	Tribromoacetamide
	75-96-7	Tribromoacetic acid
	464-10-8	Tribromonitromethane
	594-65-0	Trichloroacetamide
	545-06-2	Trichloroacetonitrile

Drugs of abuse	50-36-2	Cocaine
	125-28-0	Dihydrocodeine
	561-27-3	Heroin
	57-27-2	Morphine
	76-42-6	Oxycodone
Flame retardants	3194-55-6	1,2,5,6,9,10-Hexabromocyclododecane (HBCD) (5 isomers - alpha to epsilon)
	1163-19-5	2,2',3,3',4,4',5,5',6,6'-Decabromodiphenyl ether (BDE-209)
	207122-16-5	2,2',3,4,4',5',6-Heptabromodiphenyl ether (BDE-183)
	68631-49-2	2,2',4,4',5,5'-Hexabromodiphenyl ether (BDE-153)
	207122-15-4	2,2',4,4',5,6'-Hexabromodiphenyl ether (BDE-154)
	5436-43-1	2,2',4,4'-Tetrabromodiphenyl ether (BDE-47)
	Other	Long chain polychlorinated alkanes (l-PCAs, C>17) Medium chain polychlorinated alkanes (m-PCAs, C14-17)
	32536-52-0	Technical octabromodiphenyl ether
	Other	Technical polychlorinated alkanes products
	79-94-7	Tetrabromo bisphenol A (TBBPA)
	21850-44-2	Tetrabromo bisphenol A bis (2,3 dibromopropylether)
	13674-87-8	Tri-(dichlorisopropyl)phosphate
	78-40-0	Triethylphosphate
	126-73-8	Tri-n-butylphosphate
	115-86-6	Triphenylphosphate
	115-96-8	Tris(2-chloroethyl)phosphate
	78-42-2	Tris(2-ethylhexyl)phosphoric acid
	78-43-3	Tris(dichloropropyl)phosphate
Food additives	56038-13-2	Sucralose
	102-76-1	Triacetin
	77-93-0	Triethylcitrate
Fragrances	4455-13-4	2-Ethylthioacetic acid ethylester
	67-71-0	2-Methylthioacetic acid ethylester
	646-01-5	3-Methylthiopropionic acid
	32388-55-9	Acetylcedrene

Fragrances	13171-00-1	ADBI (Celestolide)
	15323-35-0	AHDI (Phantolide)
	1506-02-1	AHTN (Tonalide)
	68140-48-7	ATII (Traseolide)
	140-11-4	Benzylacetate
	118-58-1	Benzylsalicylate
	76-22-2	Camphor
	106-02-5	Cyclopentadecanolide
	6028-61-1	Dipropyltrisulfide
	5989-27-5	d-Limonene
	105-95-3	Ethylene brassylate
	1222-05-5	Galaxolide
	127-51-5	g-Methylionone
	34902-57-3	Habanolide
	101-86-0	Hexylcinnamaldehyde
	124-76-5	Isoborneol
	125-12-2	Isobornylacetate
	119-65-3	Isoquinoline
	24851-98-7	Methyldihydrojasmonate
	119-36-8	Methylsalicylate
	83-66-9	Musk ambrette
	81-14-1	Musk ketone
	81-15-2	Musk xylene
	54464-57-2	OTNE
	80-54-6	p-t-Bucinal (Lilial)
	98-55-5	Terpineol
Gasoline additives	1634-04-4	Methyl-tert-butyl ether (MTBE)
Industrial chemicals	79-00-5	1,1,2-Trichloroethane
	75321-20-9	1,3-Dinitropyrene
	42397-64-8	1,6-Dinitropyrene
	42397-65-9	1,8-Dinitropyrene
	17164-77-1	2-(2-Naphthalenyl)benzothiophene
	149-30-4	2-Mercapto-benzothiazole
	108-42-9	3-Chloroaniline
	106-47-8	4-Chloroaniline
	17117-34-9	4-Nitrobenzanthrone
	75-07-0	Acetaldehyde
	62-53-3	Aniline
	82-05-3	Benzanthrone
	98-10-2	Benzenesulfonamide

Industrial chemicals	56-55-3	Benzo(a)anthracene
	941-57-1	Benzothiazol-2-sulfonic acid
	95-16-9	Benzothiazole
	92-52-4	Biphenyl
	101-83-7	Dicyclohexylamin (DCHA)
	122-39-4	Diphenylamine
	78-51-3	Ethanol, 2-butoxy-, phosphate (3:1)
	50-00-0	Formaldehyde
	68002-20-0	Hexa(methoxymethyl)melamine
	2082-79-3	Irganox 1076
	100-61-8	N-methyl-Aniline
	90-30-2	N-phenyl-naphthylamine
	1336-36-3	Polychlorinated biphenyls (PCBs) - Total
	100-42-5	Styrene
	51805-45-9	TCEP
	56-23-5	Tetrachloromethane
	108-88-3	Toluene
	791-28-6	Triphenyl phosphine oxide
	13674-84-5	Tris(2chloroisopropyl)phosphate (TCPP)
	1330-20-7	Xylene
Nanoparticles	7429-90-5	Aluminium fiber (nanoparticles) Aluminium metal (nanoparticles)
	1344-28-1	Aluminium oxide (powder) (nanoparticles)
	99685-96-8	Buckyballs (Fullerene C-60)
	115383-22-7	Buckyballs (Fullerene C-70)
	1317-65-3	Calcium carbonate (nanoparticles)
	1344-95-2	Calcium silicate (nanoparticles)
	1333-86-4	Carbon black (nanoparticles)
	308068-56-6 / 308068-63-0	Carbon nanotubes - coated Carbon nanotubes - multi-wall Carbon nanotubes - single-wall
	9004-34-6	Cellulose (nanoparticles)
	7440-50-8	Copper (nanoparticles)
	1302-74-5	Emery (nanoparticles)
	60676-86-0	Fused silica (nanoparticles)
	10101-41-4	Gypsum (nanoparticles)
	1317-65-3	Limestone (nanoparticles)
	546-93-0	Magnesite (nanoparticles)
	1309-48-4	Magnesium oxide (nanoparticles)

Nanoparticles	1317-65-3	Marble (nanoparticles)
	12001-26-2	Mica (nanoparticles)
	17069-72-6	Silica (crystalline) (nanoparticles)
	7631-86-9	Silica (SiO2 amorphous) (nanoparticles)
	7440-21-3	Silicon (nanoparticles)
	409-21-2	Silicon carbide (nanoparticles)
	13463-67-7	Titanium dioxide (nanoparticles)
	557-05-1	Zinc distearate (nanoparticles)
Other	1634-36-2	(1-Hydroxy-iso-propyl)acetophenone
	619-33-0	1,1-Dichloro-2,2-diethoxyethane
	151-05-3	1,1-Dimethyl-2-phenethylacetate
	96-19-5	1,2,3-Trichloropropene (TRCP)
	4773-83-5	1,2,3-Trimethyl-1H-indene
	118-12-7	1,3,3-Trimethyl-2-oxoindol
	1014-60-4	1,3-Bis(1,1-dimethylethyl)-benzene
	793-23-7	1,4-Bis(phenylmethyl) benzene
	106-46-7	1,4-Dichlorobenzene
	112-30-1	1-Decanol
	8047-67-4	1H-Indole
	111-87-5	1-Octanol
	3910-35-8	1-Phenyl-1,3,3-trimethylindane
	615-22-5	2-(Methylthio)benzothiazol
	85688-81-9	2,3-Diethyl-2,3-dimethylsuccinonitrile
	61-70-1	2,3-Dihydro-1-methyl-1H-indol
	607-99-8	2,4,6-Tribromoanisole
	118-79-6	2,4,6-Tribromophenol
	21702-84-1	2,4-Dibromoanisole
	615-58-7	2,4-Dibromophenol
	120-83-2	2,4-Dichlorophenol
	3149-12-0	2,6-Diethoxytetrahydropyran
	10396-80-2	2,6-Di-tert-butyl-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one
	719-22-2	2,6-Di-tert-butylquinone
	71758-44-6	2-[(2-Chlorophenyl)amino]benzaldehyde
	704-00-7	2-Acetylacetophenone
	95-56-7	2-Bromophenol
	100-86-7	2-Methyl-1-phenylpropan-2-ol
	2444-37-3	2-Methylthioacetic acid
	88-75-5	2-Nitrophenol

Other	1929-29-9	3-(Bromo-4-methoxyphenyl)propionic acid
	14035-33-7	3,5-Di-tert-butyl-4-hydroxyacetophenone
	3964-56-5	4-Bromo-2-chlorophenol
	104-92-7	4-Bromoanisole
	106-41-2	4-Bromophenol
	445-03-4	4-Chloro-2-(trifluoromethyl)aniline
	06-04-5359	4-iso-Propenylacetophenone
	645-13-6	4-iso-Propylacetophenone
	832-64-4	4-Methyl-phenanthrene
	98-52-2	4-tert-Butylcyclohexanol (2 isomers)
	98-53-3	4-tert-Butylcyclohexanone (2 isomers)
	2719-62-2	6-Phenyldodecane
	82304-66-3	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
	4273-98-7	Aminodiphenylsulfone
	18339-16-7	Androstenone
	84-65-1	Anthrachinone
	54460-96-7	Bis(chloropropyl)ethers
	91-22-5	Chinoline
	91-19-0	Chinoxaline
	14866-68-3	Chlorate
	88-04-0	Chlorodimethylphenol (Chloroxylenol)
	7205-98-3	Chloromethylphenylsulfone
	25167-93-5	Chloronitrobenzene (2 isomers)
	486-56-6	Cotinine
	57-12-5	Cyanides
	506-77-4	Cyanogen chloride
	3173-53-3	Cyclohexylisocyanate
	91-17-8	Decahydronaphtalene (Dekalin)
	608-27-5	Dichloroaniline
	133-53-9	Dichlorodimethylphenol (2,4-Dichloro-meta-xylenol)
	4253-89-8	Di-iso-propyldisulfide
	2078-54-8	Di-iso-propylphenol
	2591-86-8	Formylpiperidine
	74-90-8	Hydrogen cyanide
	28179-44-4	Ioxithalamique acid
	108-62-3	Metaldehyde

Other	529-19-1	Methylbenzonitrile
	614-68-6	Methylphenylisocyanate
	3112-85-4	Methylphenylsulfone
	761-65-9	N,N-Dibutylformamide
	686-07-7	N,N-Diethyldithiocarbamic acid methyl ester
	85-98-3	N,N'-Diethyl-N,N'-diphenylurea
	4128-37-4	N,N'-Di-iso-propylurea
	1696-20-4	N-Acetylmorpholine
	103-69-5	N-Ethylaniline
	5022-29-7	N-Ethylphthalimide
	26914-52-3	N-Ethyltoluenesulfonamide
	4394-85-8	N-Formylmorpholine
	98-95-3	Nitrobenzene
	1678-25-7	N-Phenylbenzenesulfonamide
	1087-02-1	p-Dicyclohexylbenzene
	127-18-4	Perchloroethylene
	85-01-8	Phenanthrene
	103-71-9	Phenylisocyanate
	529-34-0	Tetralinone
	7695-91-2	Tocopherolacetate
	634-67-3	Trichloroaniline
	13463-41-7	Zincpyrithione
Perfluoroalkylated substances and their transformation products	865-86-1	10:2 FTOH
	39239-77-5	12:2 FTOH
	1691-99-2	2-(N-ethylperfluorooctanesulfonamido)-ethyl alcohol (N-Et-FOSE)
	24448-09-7	2-(N-methylperfluorooctanesulfonamido)-ethyl alcohol (N-Me-FOSE)
	2043-47-2	4:2 FTOH
	425670-75-3	6:2 Fluorotelomer sulfonate (6:2 FTS)
	647-42-7	6:2 FTOH
	678-39-7	8:2 FTOH
	4151-50-2	N-ethylperfluorooctanesulfonamide (N-EtFOSA)
	31506-32-8	N-methylperfluorooctanesulfonamide (N-MeFOSA)

Perfluoroalkylated substances and their transformation products	25268-77-3	N-methylperfluorooctanesulfonamidoethyl acrylate (N-MeFOSEA)
	29420-49-3	Perfluorobutanesulfonate anion (PFBS)
	335-77-3	Perfluorodecane sulfonate (PFDS)
	335-76-2	Perfluorodecanoic acid (PFDA)
	307-55-1	Perfluorododecanoic acid (PFDoA)
	375-85-9	Perfluoroheptanoic acid (PFHpA)
	432-50-7	Perfluorohexane sulfonate (PFHS)
	307-24-4	Perfluorohexanoic acid (PFHxA)
	375-95-1	Perfluorononanoic acid (PFNA)
	2058-94-8	Perfluoro-n-undecanoic acid (PFUnA)
	754-91-6	Perfluorooctane sulfonamide (PFOSA)
	1763-23-1	Perfluorooctane sulfonate (PFOS)
	45298-90-6	Perfluorooctane sulfonate (PFOS) - anion
	307-35-7	Perfluorooctanesulfonyl fluoride (POSF)
	335-67-1	Perfluorooctanoic acid (PFOA)
	376-06-7	Perfluorotetradecanoic acid (PFTDA)
Personal care products	36861-47-9	4-Methylbenzylidene camphor
	1125-21-9	4-Oxoisophorone
	658051-75-3	Bayrepel
	119-61-9	Benzophenone
	87075-14-7	Butyl methoxydibenzoylmethane
	8024-53-1	Cineole
	23726-91-2	Damascone
	541-02-6	Decamethylcyclopentasiloxane (D5)
	141-62-8	Decamethyltetrasiloxane (MD2M)
	37172-53-5	Dihydromethyljasmonate
	540-97-6	Dodecamethylcyclohexasiloxane (D6)
	141-63-9	Dodecamethylpentasiloxane (MD3M)
	2400-22-4	Drometrizole
	155633-54-8	Drometrizole trisiloxane (INCI)
	5466-77-3	Ethylhexyl methoxycinnamate
	120-47-8	Ethyl-paraben
	588-68-1	Eusolex
	107-46-0	Hexamethyldisiloxane (HM or HMDS)
	118-56-9	Homosalate
	03-02-4247	Isobutyl-paraben

Personal care products	6485-40-1	Methyl-iso-propylcyclohexenone, Carvone
	99-76-3	Methyl-paraben
	134-62-3	N,N-Diethyltoluamide (DEET)
	556-67-2	Octamethylcyclotetrasiloxane (D4)
	107-51-7	Octamethyltrisiloxane (MDM)
	80135-31-5	Octocrylene
	84-15-1	o-Terphenyl
	131-57-7	Oxybenzone
	94-13-3	Propyl-paraben
	92-94-4	p-Terphenyl
	101-48-4	Viridine
Pesticides	542-75-6	1,3-Dichloropropene
	58-90-2	2,3,4,6-Tetrachlorophenol
	94-75-7	2,4 D
	95-95-4	2,4,5-Trichlorophenol
	87-40-1	2,4,6-Trichloroanisole
	88-06-2	2,4,6-Trichlorophenol
	553-82-2	2,4-Dichloroanisole
	1984-65-2	2,6-Dichloroanisole
	934-32-7	2-Aminobenzimidazole
	578-57-4	2-Bromoanisole
	84-54-8	2-Methylanthraquinone
	83-05-6	4,4?-DDA
	2642-80-0	4,4?-DDMS
	530-48-3	4,4?-DDNU
	95975-55-6	4,4?-DDOH
	74070-46-5	Aclonifen
	116-06-3	Aldicarb
	1646-88-4	Aldicarb sulfone
	834-12-8	Ametryn
	1066-51-9	Amino Methyl Phosphoric Acid (AMPA)
	61-82-5	Amitrole
	2642-71-9	Azinphos-ethyl
	25057-89-0	Bentazone
	42576-02-3	Bifenox
	314-40-9	Bromacil
	4824-78-6	Bromofos-ethyl
	1689-99-2	Bromoxynil octanoate
	63-25-2	Carbaryl

	86-74-8	Carbazole
	10605-21-7	Carbendazim
	5234-68-4	Carboxin
	1698-60-8	Chloridazon
	1897-45-6	Chlorothalonil
	15545-48-9	Chlorotoluron
	1982-47-4	Chloroxuron
	101-21-3	Chlorpropham
	5598-13-0	Chlorpyriphos methyl
	1861-32-1	Chlorthal-dimethyl
	1702-17-6	Clopyralid
	21725-46-2	Cyanazine
	36576-43-9	Cyanazine acid
	52315-07-8	Cypermethrin
	90-98-2	DBP
	62-73-7	d-Dichlorvos
	52918-63-5	Deltamethrin
	6190-65-4	Desethylatrazine
	30125-63-4	Desethylterbutylazine
	1007-28-9	Desisopropylatrazine
	13684-56-5	Desmedipham
	1014-69-3	Desmetryn
	333-41-5	Diazinon
	1918-00-9	Dicamba
Pesticides	1194-65-6	Diclobenil
	115-32-2	Dicofol
	83164-33-4	Diflufenican
	108-18-9	Diisopropylamine
	87674-68-8	Dimethenamid
	60-51-5	Dimethoat
	1420-07-1	Dinoterb
	563-12-2	Echio
	1031-07-8	Endosulfan-sulfate
	26225-79-6	Ethofumesate
	13194-48-4	Ethoprophos
	60168-88-9	Fenarimol
	55-38-9	Fenthion
	142459-58-3	Flufenacet
	69377-81-7	Fluroxypyr
	85509-19-9	Flusilazole

Pesticides	76674-21-0	Flutriafol
	65907-30-4	Furathiocarb
	1071-83-6	Glyphosate
	76-44-8	Heptachlor
	1024-57-3	Heptachlor epoxide
	23560-59-0	Heptenophos
	51235-04-2	Hexazinone
	138261-41-3	Imidacloprid
	18181-70-9	Iodofenphos
	330-55-2	Linuron
	121-75-5	Malathion
	94-74-6	MCPA
	94-81-5	MCPB
	93-65-2	MCPP (Mecoprop)
	16484-77-8	Mecoprop-P
	57837-19-1	Metalaxyl
	41394-05-2	Metamitron
	67129-08-2	Metazachlor
	2032-65-7	Methiocarb
	01-10-2635	Methiocarb sulfoxide
	16752-77-5	Methomyl
	72-43-5	Methoxychlor
	51218-45-2	Metolachlor
	19937-59-8	Metoxuron
	7786-34-7	Mevinphos
	96180-79-9	Microcystin-LA / Cyanoginosin-LA
	2212-67-1	Molinate
	825629-31-0	N-Ethyl-2-tolylsulfonamide
	53-19-0	o,p'-Dichlorodiphenyldichloroethane (o,p'-DDD)
	1113-02-6	Omethoate
	34622-58-7	Orbencarb
	19666-30-9	Oxadiazon
	77732-09-3	Oxadixyl
	76738-62-0	Paclobutrazol
	56-38-2	Parathion ethyl
	298-00-0	Parathion methyl
	40487-42-1	Pendimethalin
	52645-53-1	Permethrin
	13684-63-4	Phenmedipham

Pesticides	14816-18-3	Phoxim
	29232-93-7	Pirimiphos methyl
	67747-09-5	Prochloraz
	1610-18-0	Prometon
	7287-19-6	Prometryn
	1918-16-7	Propachlor
	709-98-8	Propanil
	139-40-2	Propazine
	60207-90-1	Propiconazole
	23950-58-5	Propyzamide
	90717-03-6	Quinmerac
	124495-18-7	Quinoxyfen
	26259-45-0	Secbumeton
	35507-37-0	Sulfonyl urea
	886-50-0	Terbutryn
	5915-41-3	Terbutylazine
	148-79-8	Thiabendazole
	59669-26-0	Thiodicarb
	57018-04-9	Tolclofos methyl
	731-27-1	Tolylfluanid
	43121-43-3	Triadimefon
	2303-17-5	Tri-allate
	52-68-6	Trichlorfon
	101-20-2	Triclocarban
Pharmaceuticals	123050-98-6	(R)-O-Desmethyl Naproxen
	57-91-0	17-alpha-Estradiol
	57-63-6	17-alpha-Ethinylestradiol
	50-28-2	17-beta-Estradiol
	37517-30-9	Acebutolol
	77-66-7	Acecarbromal
	89796-99-6	Aceclofenac
	53164-05-9	Acemetacin
	103-90-2	Acetaminophen (paracetamol)
	59-66-5	Acetazolamide
	50-78-2	Acetylsalicylic acid (aspirin)
	59277-89-3	Acyclovir
	18559-94-9	Albuterol
	51022-70-9	Albuterol sulfate
	22131-79-9	Alclofenac
	52-43-7	Allobarbital

Pharmaceuticals	28981-97-7	Alprazolam
	50-48-6	Amitryptiline
	57-43-2	Amobarbital
	26787-78-0	Amoxicillin
	69-53-4	Ampicillin
	635-12-1	Anthracen-1,4-dione
	37321-09-8	Apramycin
	77-02-1	Aprobarbital
	29122-68-7	Atenolol
	83905-01-5	Azithromycin
	1134-47-0	Baclofen
	102280-35-3	Baquiloprim
	378-44-9	Betamethasone
	83-46-5	Beta-sitosterol
	63659-18-7	Betaxolol
	41859-67-0	Bezafibrate
	66722-44-9	Bisoprolol
	1812-30-2	Bromazepam
	77-26-9	Butalbital
	58-08-2	Caffeine
	57775-29-8	Carazolol
	298-46-4	Carbamazepine
	10206-21-0	Cefacetrile
	15686-71-2	Cefalexin
	5575-21-3	Cefalonium
	21593-23-7	Cefapirin
	25953-19-9	Cefazolin
	62893-19-0	Cefoperazone
	302-17-0	Chloral hydrate
	56-75-7	Chloramphenicol
	57-15-8	Chlorobutanol
	57-62-5	Chlortetracycline
	57-88-5	Cholesterol
	85721-33-1	Ciprofloxacin
	59729-32-7	Citalopram
	81103-11-9	Clarithromycin
	37148-27-9	Clenbuterol
	882-09-7	Clofibric acid
	23593-75-1	Clotrimazole
	7081-44-9	Cloxacillin

Pharmaceuticals	76-57-3	Codeine
	8064-90-2	Cotrimoxazole
	483-63-6	Crotamiton
	50-18-0	Cyclophosphamide
	75526-90-8	Cyclophosphamide (anhydrous form)
	112398-08-0	Danofloxacin
	7261-97-4	Dantrolene
	80-08-0	Dapsone
	20830-81-3	Daunorubicin
	50-02-2	Dexamethasone
	117-96-4	Diatrizoate
	439-14-5	Diazepam
	15307-86-5	Diclofenac
	3116-76-5	Dicloxacillin
	56-53-1	Diethylstilbestrol
	98106-17-3	Difloxacin
	88637-37-0	Diphenhydramine
	57808-66-9	Domperidone
	1668-19-5	Doxepine
	25316-40-9	Doxorubicin
	94088-85-4	Doxycycline (anhydrous)
	564-25-0	Doxycycline (monohydrate)
	74011-58-8	Enoxacin
	93106-60-6	Enrofloxacin
	56420-45-2	Epirubicin
	114-07-8	Erythromycin
	128196-01-0	Escitalopram
	119141-88-7	Esomeprazole
	50-27-1	Estriol
	53-16-7	Estrone
	481-97-0	Estrone sulphate
	77-67-8	Ethosuximide
	56775-91-8	Etofibrate
	76824-35-6	Famotidine
	458-24-2	Fenfluramine
	49562-28-9	Fenofibrate
	26129-32-8	Fenofibric acid
	31879-05-7	Fenoprofen
	53746-45-5	Fenoprofen calcium salt dihydrate
	03-12-1944	Fenoterol

Pharmaceuticals	5250-39-5	Flucloxacillin
	42835-25-6	Flumequine
	51-21-8	Fluorouracil
	54910-89-3	Fluoxetine
	54739-18-3	Fluvoxamine
	54-31-9	Furosemide
	25812-30-0	Gemfibrozil
	1403-66-3	Gentamicin
	10238-21-8	Glyburide (glibenclamid; glybenzcyclamide)
	56-29-1	Hexobarbital
	58-93-5	Hydrochlorothiazide
	125-29-1	Hydrocodone
	15687-27-1	Ibuprofen
	53949-53-4	Ibuprofen 1-hydroxy
	51146-55-5	Ibuprofen 2-hydroxy
	3778-73-2	Ifosfamide
	50-49-7	Imapramine
	256-96-2	Iminostilbene
	53-86-1	Indomethacin
	66108-95-0	Iohexol
	78649-41-9	Iomeprol
	60166-93-0	Iopamidol
	73334-07-3	Iopromide
	70288-86-7	Ivermectin
	16846-24-5	Josamycin
	08-07-8063	Kanamycin sulfate
	22071-15-4	Ketoprofen
	84057-84-1	Lamotrigine
	103577-45-3	Lansoprazole
	102767-28-2	Levetiracetam
	137-58-6	Lidocaine
	859-18-7	Lincomycin
	554-13-2	Lithium carbonate
	79794-75-5	Loratadine
	846-49-1	Lorazepam
	75330-75-5	Lovastatin
	115550-35-1	Marbofloxacin
	07-06-3625	Mebeverine
	644-62-2	Meclofenamic acid

Pharmaceuticals	06-12-2898	Medazepam
	61-68-7	Mefenamic acid
	57-53-4	Meprobamate
	72-33-3	Mestranol
	657-24-9	Metformin
	61-32-5	Methicillin
	115-38-8	Methylphenobarbital
	37350-58-6	Metoprolol
	73573-88-3	Mevastatin
	13614-98-7	Minocycline
	42200-33-9	Nadolol
	985-16-0	Nafcillin
	434-22-0	Nandrolone
	22204-53-1	Naproxen
	1404-04-2	Neomycin B
	7298-73-9	N-Methylphenacetine
	1088-11-5	Nordiazepam
	70458-96-7	Norfloxacin
	1476-53-5	Novobiocin
	82419-36-1	Ofloxacin
	3922-90-5	Oleandomycin
	73590-58-6	Omeprazole
	66-79-5	Oxacillin
	14698-29-4	Oxalinic acid
	604-75-1	Oxazepam
	6452-71-7	Oxprenolol
	79-57-2	Oxytetracycline
	61869-08-7	Paroxetine
	61-33-6	Penicillin G
	87-08-1	Penicillin V
	76-74-4	Pentobarbital
	06-05-6493	Pentoxifylline
	60-80-0	Phenazone
	50-06-6	Phenobarbital
	50-33-9	Phenylbutazone
	57-41-0	Phenytoine
	13523-86-9	Pindolol
	1893-33-0	Pipamperon
	81093-37-0	Pravastatin
	50-24-8	Prednisolone

Pharmaceuticals	125-33-7	Primidone
	525-66-6	Propranolol
	479-92-5	Propyphenazone
	66357-35-5	Ranitidine
	80214-83-1	Roxithromycin
	35763-26-9	Salbutamol
	98105-99-8	Sarafloxacin
	76-73-3	Secobarbital
	309-43-3	Secobarbital sodium
	79617-96-2	Sertraline
	79902-63-9	Simvastatin
	3930-20-9	Sotalol
	1695-77-8	Spectinomycin
	8025-81-8	Spiramycin
	57-92-1	Streptomycin
	68-35-9	Sulfadiazine
	122-11-2	Sulfadimethoxine
	2447-57-6	Sulfadoxine
	127-79-7	Sulfamerazine
	57-68-1	Sulfamethazine
	723-46-6	Sulfamethoxazole
	144-83-2	Sulfapyridine
	25451-15-4	Taloxa
	846-50-4	Temazepam
	23031-25-6	Terbutaline
	60-54-8	Tetracycline
	55297-95-5	Tiamulin
	108050-54-0	Tilmicosin
	91524-16-2	Timolol
	13710-19-5	Tolfenamic acid
	27203-92-5	Tramadol
	738-70-5	Trimethoprime
	1401-69-0	Tylosin
	101312-92-9	Valnemulin
	99-66-1	Valproic acid
	52-53-9	Verapamil
	82626-48-0	Zolpidem
Plasticizers	131-56-6	2,4-Dihydroxybenzophenone
	7425-14-1	2-Ethylhexanoic acid 2-ethylhexyl ester
	85-68-7	Benzylbutylphthalate (BBP)

Plasticizers	80-05-7	Bisphenol A
	84-66-2	Diethylphthalate (DEP)
	68515-49-1, 26761-40-0	Di-isodecyl-phthalate (DIDP)
	68515-48-0, 28553-12-0	Di-isononyl-phthalate (DINP)
	131-11-3	Dimetylphthalate (DMP)
	84-74-2	Di-n-butylphthalate (DBP)
	117-84-0	Di-n-octylphthalate (DOP)
	38640-62-9	DIPN
	947-19-3	Methanone, Irgacure 184
	3622-84-2	NBBS
	872-50-4	NMP
	77-90-7	Tributylacetylcitrate
	126-71-6	Tri-iso-butylphosphate (TIBP)
	6846-50-0	TXIB
Trace metals and their compounds	7440-38-2	Arsenic
	7440-47-3	Chromium
	7440-50-8	Copper
	03-05-7440	Palladium
	12595-26-5	Silver
	74-00-2	Tetraethyllead
	75-74-1	Tetramethyllead
	7440-66-6	Zinc
Wood preservatives	51-28-5	2,4-Dinitrophenol (DNP)
	106-44-5	para-Cresol

Annex II – Analysis of acidic, basic and neutral pharmaceuticals in river waters: clean-up by 1º,2º amino anion exchange and enrichment using an hydrophilic adsorbent

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Analysis of acidic, basic and neutral pharmaceuticals in river waters: clean-up by 1°, 2° amino anion exchange and enrichment using an hydrophilic adsorbent

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A fast and sensitive LC-MS/MS method suitable to monitor a set of 24 pharmaceuticals belonging to 9 therapeutical families was developed using an analytical column of reduced dimensions and an appropriate SPE procedure for acidic, basic and neutral analytes. Furthermore, a preliminary assessment of the occurrence and levels of these substances in surface waters of the Leça and Douro rivers, located in the north of Portugal, was conducted. Among 17 different SPE adsorbents tested, some of them often neglected, the JTBaker H_2Ophillic provided the best recoveries (average 86%). When compared to the amino and quaternary ammonium adsorbents, the 1°, 2° amino proved the most suitable clean-up media for surface water samples prior to enrichment by SPE. The overall method provided limits of detection generally below 5 ng L^{-1} and a precision below or around 10% RSD in three non-consecutive days at 500 ng L^{-1} and around 12% at 50 ng L^{-1} concentration levels. The confirmatory capabilities of the method developed are especially welcome either through the MS^3 spectra or the isotopic pattern.

The first known results regarding the occurrence of pharmaceuticals in Leça river confirmed its expected high contamination load and the successful selection of the target pharmaceuticals. Concentrations up to 770 ng L^{-1} of bezafibrate, 925 ng L^{-1} of paracetamol, 389 ng L^{-1} of hydrochlorothiazide and 283 ng L^{-1} of furosemide were measured. The most ubiquitous in both seasons, February and June 2009, were bisoprolol, furosemide, bezafibrate and gemfibrozil. In Douro river the abundance and contamination level of pharmaceuticals was much lower which gives a clear indication that the hydraulic features of the river provide enough attenuation of the several contamination sources whereas Leça is highly impacted by insufficiently treated anthropogenic effluents.

Keywords: pharmaceuticals; surface water; LC-MS/MS; hydrophilic SPE; anion exchange clean-up; Leça and Douro rivers

1. Introduction

A number of studies beginning in the 1990s have reported the presence of pharmaceuticals in surface water, groundwater and drinking water in concentrations ranging from a

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few ng L^{-1} to tens of μg L^{-1} [1–4]. Generally, these pollutants are amenable to biotic and/or abiotic degradation; however, the world-wide dissemination and frequent release sources, which often exceed their degradation rate, turn them into pseudo-persistent pollutants [5,6]. The most important entry sources in the environment are household residues, waste water treatment plants (WWTPs), hospitals, industrial units, land application of biosolids and intensive animal-breeding farms [6–11].

The presence of pharmaceuticals in the environment raises particular awareness since they are bioactive substances produced to modulate the functioning of vital organs and systems in living organisms [7]. Therefore the growth, physiology and reproduction of exposed individuals may be impaired. Among the best characterized detrimental effects of this sort are the disruption of the endocrine system in wildfowl originated by estrogens and a wide range of other chemicals (pesticides, phthalates, alkyphenols, fragrances, etc.) and the selection of multi-resistant strains of pathogenic microorganisms that results from uncontrolled use of biocides (antibiotics and antivirals) and deficient treatment of anthropogenic effluents [12–15]. Nonetheless, pollution by cocktails of parent substances, metabolites and degradation products is even more worrying than pharmaceuticals considered individually because mixture toxicity effects are virtually unforeseeable [5].

Often, pharmaceuticals are found in surface waters with higher frequency and concentration levels than other recognized priority pollutants (see data in [16]). Besides, in the preparatory stage of Directive 2008/105/EC several pharmaceuticals were subject to review for the identification as priority substances (carbamazepine, clotrimazole, diclofenac, amidotrizoate and lopamidol) and currently an Impact Assessment of the Review of the Priority Substances list including diclofenac, ibuprofen, 17-α-ethinylestradiol and 17-β-estradiol is ongoing [17].

Against this background, research is urgently needed to build up knowledge on the occurrence of pharmaceuticals as well as for monitoring of the removal, partition and ultimate fate of these pollutants in the environment allowing to uncover their potential ecotoxicological and human health impact [1,5,18]. Reliable, high throughput and multiresidue analytical methods based on powerful equipments (namely liquid chromatography-tandem mass spectrometry (LC-MS/MS) and efficient enrichment materials for pharmaceuticals in water (such as hydrophilic polymeric adsorbents) are indispensable tools to this aim [1–3,12,13]. Furthermore, pharmaceuticals, in particular, are characterised by ranging from acidic (carboxylic acids, e.g. bezafibrate, diclofenac) to basic (secondary amines e.g. bisoprolol, paroxetine), therefore inclusive methodologies yet selective against interferences must be developed to analyse the most environmentally relevant pharmaceuticals.

The challenge of managing conflictive properties embraces not only the sample pre-treatment protocol (extraction and clean-up), considered the bottleneck step, but also separation, ionization and detection [16]. Solid-phase extraction (SPE) is particularly well adapted to multi-residue analysis, because of the great variety of sorbents available, their larger capacity as compared to microextraction techniques, moderate consumption of organic solvents and easy automation [18]. Hypercrosslinking, functionalization and copolymerisation of styrene-divinylbenzene materials have been exploited to improve the extraction of polar analytes along with a few novel polymers [19,20]. A definitive trial including the largest array of adsorbents ever tested (seventeen, some of them often neglected) for the extraction of acidic, neutral and basic pharmaceuticals from water was

undertaken in this work. The following adsorbents were tested, among others: Abselut Nexus, Chromabond Easy, Bakerbond H_2Ophilic, StrataX, Evolute ABN and Resprep RDX.

The clean-up procedure constitutes a critical step for the accurate determination of pharmaceuticals and has been addressed by some authors to tackle matrix effects [6,8]. A previous work where a systematic evaluation of clean-up strategies was carried out demonstrated the complexity of this task [21]. The options may range from using normal phase, ion exchange or mixed-mode retention but the final decision ought to take into account the diverse nature of the analytes and the load of the matrix, in order to eliminate the unwanted substances while preserving the target analytes. Often the most efficient cleaners also eliminate the pharmaceuticals [21]. Therefore, a clean-up protocol targeted for acidic, basic and neutral pharmaceuticals in surface water of medium matrix load is proposed considering the best sorbent material and its application stage.

In summary, this work was mentored by two objectives: (i) developing a fast and sensitive LC-MS/MS method suitable to monitor a set of important pharmaceutical substances using an analytical column of reduced dimensions and an appropriate SPE procedure (extraction and clean-up) for acidic, basic and neutral analytes, and (ii) preliminary assessment of the occurrence and levels of these substances in surface waters of the Leça and Douro rivers, located in the north of Portugal. A list of 24 most consumed pharmaceuticals in Portugal belonging to 9 different therapeutic categories was selected as representative compounds.

2. Experimental

2.1 *Reagents and chemicals*

The pharmaceuticals determined in this work and respective therapeutic categories were the following: zolpidem, bromazepam, lorazepam, alprazolam, diazepam (anxiolytics); paracetamol, naproxen, ketoprofen, ibuprofen, diclofenac, nimesulide, (analgesic/antipyretics); amoxicillin, ciprofloxacin, azithromycin (antibiotics); bezafibrate, gemfibrozil, simvastatin (lipid regulators); hydrochlorothiazide, furosemide (diuretics); fluoxetine, paroxetine (anti-depressives), omeprazol (anti-ulcer agent); indapamide (anti-diabetic) and bisoprolol (cardiotonic). The analytical standards were purchased from Fluka, Sigma and Riedel-de-Haën (Sigma-Aldrich, Spain), except the benzodiazepines which were supplied by LGC Standards (Barcelona, Spain). Two isotopically labelled compounds were used as external standards: paracetamol-D4 (LGC Standards) and fluoxetine-D5 (Sigma-Aldrich).

Individual stock standard solutions were prepared in methanol at a concentration around 500 mg L^{-1} of the free acid or base and stored at $-18^{\circ}C$ in the dark and renewed yearly. A mixture of all pharmaceuticals with a concentration of 5 mg L^{-1} was prepared as needed in methanol, at least every four months. A working solution at 500 μg L^{-1} concentration was prepared daily in methanol : water (25 : 75, v/v) and analysed to assess the separation efficiency and response.

LC mobile phases were prepared with ultrapure Milli Q water (Millipore, Molsheim, France), methanol LC-MS analyzed reagent (JTBaker, Deventer, The Netherlands) and formic acid 50% (Fluka, Buchs, Switzerland).

Table 1. Set of adsorbents tested for the preconcentration of pharmaceuticals and clean-up of environmental samples.

Adsorbents for preconcentration	Mass (for 6 mL)	Supplier
Abselut Nexus	200 mg	Varian
Bond Elut ENV	1000 mg	Varian
Bond Elut PPL	500 mg	Varian
Bakerbond H_2OPhilic	200 mg	JTBaker
Bakerbond H_2OPhobic	200 mg	JTBaker
Bakerbond SDB-1	200 mg	JTBaker
Chromabond C18	500 mg	Macherey-Nagel
Chromabond Easy	500 mg	Macherey-Nagel
Chromabond HRX	500 mg	Macherey-Nagel
Chromabond HRP	500 mg	Macherey-Nagel
Evolute ABN	200 mg	Biotage/IST
Isolute ENV+	200 mg	Biotage/IST
Lichrolut EN	500 mg	Merck
Oasis HLB	200 mg	Waters
Oasis MCX	150 mg	Waters
Resprep RDX	500 mg	Restek
Strata X	500 mg	Phenomenex
Adsorbents for clean-up	**Mass (for 3 mL)**	**Supplier**
Bakerbond Amine	500 mg	JTBaker
Bakerbond 1°, 2° Amino	500 mg	JTBaker
Bakerbond quaternary ammonium	500 mg	JTBaker
Diol	500 mg	JTBaker

2.2 *Solid phase extraction procedure*

SPE was performed using an automated device ASPEC XL from Gilson (Middleton, USA) fitted to accommodate 6 mL cartridges. The set of adsorbents tested for extraction (see Table 1) include C18, modified polystyrene-divinylbenzene and proprietary polymeric materials specially designed for the analysis of polar pharmaceutical substances. The characteristics of some of the adsorbents can be found in Weigel's paper [19]. Comparison trials were performed under basic conditions for conditioning (8 mL of methanol), percolation (10 mL min^{-1}) and elution (8 mL of methanol). For clean-up of environmental samples weak anion exchange (WAX, amino and primary/secondary (1°, 2°) amino), strong anion exchange (SAX, quaternary ammonium) and normal phase (diol) on silica substrate were evaluated (see Table 1).

Under the optimised conditions, conditioning of the extraction cartridges was performed with 3 mL of methanol followed by 3 mL of acidified Milli Q water at pH 2 with HCl. 200 mL of standards and samples acidified at pH 2 were percolated through the JTBaker H_2Ophilic cartridge at a flow rate of 10 mL min^{-1}. The adsorbent was allowed to dry under vacuum in an Analytichem International SPE manifold (Varian). The analytes were then eluted with 6 mL of methanol, the deuterated external standards were added to obtain a final concentration of 500 μg L^{-1}, the extract was evaporated to dryness under a gentle stream of nitrogen and redissolved in 200 μL of methanol : water (25 : 75, v/v) for LC-MS/MS analysis. Samples requiring clean-up were acidified at pH 2 and percolated

directly through Bakerbound 1°, 2° Amino adsorbent before enrichment in the SPE material. The clean-up procedure was done manually under vacuum with 3 mL cartridges packed with 200 mg of adsorbent.

2.3 *Protocol for LC-MS/MS analysis*

Analysis of pharmaceuticals was performed using a LC-MS/MS system composed of two Varian 210 HPLC pumps, a Varian 500 MS ion trap mass spectrometer and a ProStar 410 autosampler, all from Varian (Walnut Creek, CA, USA). The system was mounted with an analytical column of short dimensions, Pursuit UPS C18 (2.1 mm i.d. × 50 mm, 2.4 μm) from Varian and a guard column of the same characteristics (2.1 mm i.d. × 10 mm, 3 μm). The equipment was fitted with a 10 μL sample loop.

Chromatographic separation was achieved using an elution gradient of LC-MS grade methanol and 10 mM formic acid in Milli Q water as mobile phases. The program started with 25% methanol, rising to 75% methanol in 8 min without delay, then rising to 100% methanol at 10 min and holding until 13 min run time. The program returned to the initial conditions in 1 min and the column was allowed to stabilize for 8 min. The flow rate was 0.3 mL min^{-1} and the column temperature was 35°C.

The ESI source parameters (ionization polarity, drying gas temperature, needle voltage and capillary voltage) and the detector storage and fragmentation conditions (RF loading voltage and collision induced dissociation (CID) voltage) were optimized on a per analyte basis by infusion of authentic standards. The purpose was accumulating the maximum amount of precursor ion in the detector followed by CID fragmentation until a few percent of the precursor ion was remaining. Positive electrospray ionization was selected whenever possible since it gave better sensitivity in this instrument and ionization is favoured under acidic mobile phase. Table 2 assembles the instrumental conditions employed.

Confirmation of positive results in real samples was made by comparison of the MS/MS spectra against authentic standards and also by setting two to three qualifiers and 20% tolerance criteria. Isotopic ratios in halogenated compounds were also used to improve confirmation. Nevertheless for several compounds just one product ion is generated, e.g. paracetamol, bisoprolol, furosemide, nimesulide, ketoprofen, bezafibrate, diclofenac, ibuprofen and gemfibrozil. Therefore, pharmaceuticals occurring in real samples were also confirmed by MS^3 spectra. The instrumental conditions for the analysis of azithromycin were such to accumulate the double charged ion and fragment it to a single charged product which is possible for a few molecules improving the analytical confidence and the sensitivity. Instrumental control and data processing was performed by a personal computer running the Varian MS Workstation version 6.9.1.

2.4 *Method validation*

After the optimization of the method in the extraction and analysis perspectives, full validation was performed encompassing sensitivity, linear range, precision and accuracy features. The limits of detection (LODs) were estimated as the concentration giving a signal-to-noise ratio of 3 (S/N 3) and the LOQs a S/N 10. Precision was studied as repeatability and intermediate precision (on three non consecutive days) with a spiking level of 500 ng L^{-1} in Milli Q water and two surface waters. Accuracy was determined calculating the method's recoveries and through a detailed study of matrix effect along the

Table 2. Instrumental conditions employed in LC-MS/MS analysis of pharmaceuticals.

Pharmaceuticals	Retention time (min.)	Acquisition segment (min.)	Drying Gas Temperature	Needle voltage (V)	Ionization mode	Precursor ion (m/z)	Product ions (m/z)	Capillary Voltage (V)	RF Loading (%)	CID amplitude (V)
Amoxicillin	0.398	0.0–2.3	300°C	5000–4630	ESI+	366	(349), 208, 160	47	93	0.86
Hydrochlorothiazide	0.871				ESI−	296	(269), 205, 271	84	128	1.10
Paracetamol	0.949				ESI+	152	(110)	41	60	0.81
Paracetamol D4	0.97				ESI+	156	(114)	60	54	0.65
Ciprofloxacin	1.319				ESI+	332	(314), 288, 315	65	88	1.30
Zolpidem	2.64	2.3–5.7	350°C	3450	ESI+	308	(263), 235, 236	102	87	1.35
Azithromycin	3.724				ESI+	375	(591.5)	42	90	1.25
Bisoprolol	3.776				ESI+	326	(116)	80	87	0.86
Omeprazole	5.26				ESI+	346	(198), 180, 289	48	86	0.93
Furosemide	5.869	5.7–6.7	350°C	4000–4600	ESI−	329	(285)	53	117	1.10
Indapamide	5.972				ESI−	364	(189), 233, 190	53	161	1.10
Paroxetine	6.117				ESI+	330	(192), 151	78	85	0.90
Bromazepam	6.361	5.7–7.5			ESI+	316	(288), 263, 209	87	87	1.10
Fluoxetine D5	6.52	6.7–7.5	350°C	5300	ESI+	315	(153)	54	85	0.65
Fluoxetine	6.936				ESI+	310	(148)	52	86	3.00
Alprazolam	7.887	7.5–8.85	250°C	5000–5000	ESI+	309	(281), 274, 241	92	87	1.28
Lorazepam	8.023				ESI+	321	(303), 305, 275	65	88	0.90
Nimesulide	8.26				ESI−	307	(229)	53	103	0.90
Ketoprofen	8.589	7.5–10.0			ESI+	255	(209)	75	76	0.75
Diazepam	8.971	8.85–10.0	250°C	3700–5000	ESI+	285	(257), 154, 222	87	81	1.10
Naproxen	8.932				ESI+	231	(185), 174	42	77	0.65
Bezafibrate	9.306				ESI−	360	(274)	63	100	0.60
Diclofenac	10.537	10.0–11.0	300°C	−5000	ESI−	294	(250)	36	111	0.75
Ibuprofen	10.712				ESI−	205	205*	33	57	0.00
Gemfibrozil	11.491	11.0–13.0	250°C	3530–5540	ESI−	249	(121), 127	48	67	0.80
Simvastatin	12.188	11.7–13.0	350°C	3530–5540	ESI+	441	(325), 315, 310	126	66	1.00

m/z window is 3 m/z except: amoxicilin, bromazepam, alprazolam, naproxen is 4 m/z; fluoxetine, lorazepam is 5 m/z; ibuprofen, gemfibrozil is 6 m/z
High mass eject factor is 100% except: paracetamol, paracetamol D4, fluoxetine, fluoxeine D5, ibuprofen is 50%; lorazepam, gemfibrozil is 80%
Fluoxetine: CID frequency −4; The product ion between parenthesis was used for quantitation. *M − H was used for quantitation

analytical procedure. Unless otherwise stated, (absolute) recoveries means the ratio expressed in percentage of the signal obtained in a sample (subjected to SPE and eventually clean-up) relative to the signal expected without any interference of any sort (direct injection of a standard). Matrix effect observed when analyzing real samples is probably the major source of inaccuracy of the results, therefore the extension of this phenomenon was assessed in two ways: effect on recoveries and ionization suppression effect. To estimate this effect four types of solutions were analyzed: standard solution (StdSol), SPE extract of spiked Milli Q water (SPESol), SPE extract of spiked real samples (SampSol) and spiked extract (after SPE) of real sample (ExtrSol). Spiking with pharmaceuticals was always performed at 500 ng L^{-1} concentration level.

Absolute recoveries in Milli Q water and real samples were determined as:

$$\mathrm{Re}\,c = \frac{\mathrm{SPESol\,(orSamplSol\,)} \times 100}{\mathrm{StdSol}} \tag{1}$$

Ionization effect in the electrospray source (ESI) was determined as:

$$\mathrm{IonizEffect} = \frac{(\mathrm{ExtrSol} - \mathrm{StdSol}\,) \times 100}{\mathrm{StdSol}} \tag{2}$$

Matrix effect on recoveries was determined as:

$$\mathrm{EfectRe}\,c = \left(\frac{\mathrm{SampSol}}{\mathrm{ExtrSol}} - \frac{\mathrm{SPESol}}{\mathrm{StdSol}}\right) \times 100 \tag{3}$$

Additionally the loss of analyte in the clean-up step was determined comparing the response of Milli Q water solutions submitted to clean-up + SPE and just SPE and; samples spiked before and after clean-up. These trials also allowed investigating if acidification ought to be performed before or after clean-up. The physico-chemical characteristics of four river water matrices tested (1 Ave river, 2 Leça river and 1 Douro river) are given in Table 3. Leça Reg. is a clean matrix, Ave and Douro are medium loaded and Leça Pte Pedra is a highly polluted river water sample.

2.5 *Sampling scheme in Leça and Douro rivers*

The assessment of the occurrence of pharmaceuticals in surface waters was carried out in two very distinct rivers in the north of Portugal. Leça river is a small water stream of 45 km

Table 3. Physico-chemical characteristics of the river waters matrices tested in the clean-up and matrix effect studies.

	Ave river	Leça river Reg.	Leça river Pte Pedra	Douro river
TOC (mg L^{-1})	1.96	1.41	22.23	2.30
NH4+ (mg L^{-1})	0.512	0.107	43.35	0.032
NO2− (mg L^{-1})	0.042	0.03	0.094	0.235
NO3− (mg L^{-1})	9.50	8.8	17.00	6.4
Conductivity (μS cm^{-1})	146	94.4	371.5	310
pH	6.83	6.16	7.44	7.79
Turbidity (NTU)	1.71	0.81	16.35	4.64
Dissolved Oxygen (mg L^{-1})	7.34	6.84	2.21	7.89

long and average flow of $3.4\,m^3\,s^{-1}$ that receives in its path different inputs of agricultural and industrial origin as well as domestic effluents from a large urban population in the neighbourhood of Oporto, contributing to its high contamination status. Douro river is an international river with headwaters in Spain and mouth in Oporto with a stream of average $450\,m^3\,s^{-1}$ (ranging from 200 to $710\,m^3\,s^{-1}$) running a 927 km course. It is source of drinking water for the population living in the metropolitan area of Oporto.

The monitoring scheme in Leça river included ten sampling stations, one in the headwaters, as control, and so forth until the mouth before and after every contamination source (villages, tributaries, WWTPs). The same rationale was applied in Douro river with a total of 12 sampling stations beginning from 45 km towards the mouth. Samples were collected in 1 L amber glass bottles and transported to the laboratory in a refrigerated environment. Samples were processed immediately or at latest three days after sampling and were kept always at 4°C. The samplings took place in the 23 February and 15 June 2009 in Leça river and 4 March and 16 June 2009 in the case of Douro river.

3. Results and discussion

3.1 *LC-MS/MS optimization*

Instrumental optimization began by setting up the appropriate electrospray ionization source parameters, MS/MS conditions and the chromatographic gradient to analyse a mixture of acidic, basic and neutral pharmaceuticals at the maximum sensitivity and resolution. Just the most critical aspects will be discussed, briefly, which derive from the challenge of analysing the above analytes in the same run. After an initial optimization by direct infusion of the standards, two observations highlighted: some analytes gave an extremely broad chromatographic peak under methanol/water mobile phase while others required better fine tuning for improving the sensitivity. The former group include, noticeably, ciprofloxacin, azithromycin, bisoprolol, paroxetine and fluoxetine. Aqueous mobile phases buffered at the pH of 2.9, 3.2, 3.7 and 4.6 were tested using formic acid and ammonium formate as additives in a concentration of 5 and 10 mM. A mobile phase composed of formic acid 10 mM in water (pH 2.9) revealed essential for obtaining good peak shape and resolution of the abovementioned pharmaceuticals. The tremendous improvement in peak shape is demonstrated in Figure S1. In general, peak widths at half height ($W_{0.5}$) of the studied pharmaceuticals ranged then from 4.5–11.2 s. Acidification of the mobile phase also contributed to improve the ionization efficiency of several analytes, therefore ESI+ was the preferred ionization mode, namely for amoxicillin, paracetamol, ketoprofen and naproxen, which are normally analysed under ESI−. A representative MS/MS chromatogram of a $500\,\mu g\,L^{-1}$ standard solution obtained under the above conditions is given in Figure 1. A fast and efficient separation was achieved with a total chromatographic time of 13 min and a 22 min cycle time, owing to the use of a 50 mm and 2.4 μm particle size column. MS/MS segments with the least number of analytes (≤ 5) were scheduled to keep the required number of data points per peak. To address the improvement of sensitivity for some compounds, further optimization was attempted changing the precursor m/z isolation window and the high mass ejection factor. These conditions were given as footnote to Table 2. It should be noted that a larger isolation window also allowed collecting the isotopic pattern of some halogenated benzodiazepines in the precursor ion and MS/MS spectra, providing better confirmation quality.

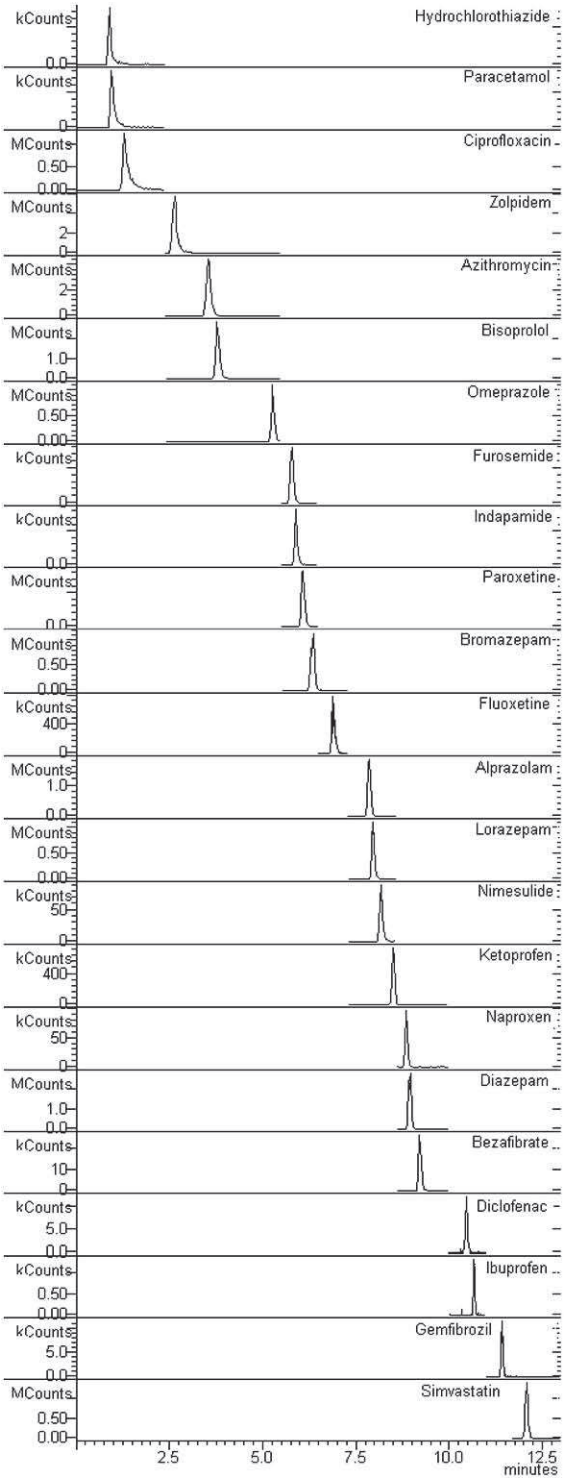

Figure 1. Representative LC-MS/MS chromatogram of a standard solution of the 23 relevant pharmaceuticals at 500 $\mu g\,L^{-1}$ concentration.

3.2 *Comprehensive adsorbent comparison*

Surface modification of styrene-divinylbenzene and synthesis of divinylbenzene-N-vinylpyrrolidone and methacrylate-divinylbenzene co-polymers have been pursued to better retain polar environmental pollutants, such as pharmaceuticals, in SPE cartridges [20]. We have performed a comprehensive evaluation of 17 SPE materials available nowadays, some of them neglected in previous studies [19], for the enrichment of a wide array of pharmaceuticals. Irrespective of their order, the five adsorbents that performed worst in a first round were: Chromabond C18 and HRP, Lichrolut EN, Bond Elut ENV and Isolute ENV+. The remaining 12 were then systematically compared in four replicates with an average relative standard deviation (RSD) per cartridge of 5.7 to 20% and the ranking of average recoveries/median recoveries at 500 ng L^{-1} concentration level was as follows: JTBaker H_2Ophilic (89/92%), JTBaker H_2Ophobic (86/96%), Abselut Nexus (86/90%), Resprep RDX (80/87%), Oasis HLB (80/85%), JTBaker SDB1 (73/77%), Bond Elut PPL (69/78%), Strata X (67/67%), Oasis MCX (66/81%), Chromabond HRX (62/61%), Chromabond Easy (58/62%) and Evolute ABN (56/64%). Of these, the best five are represented in Figure 2 for the whole group of test compounds. For the majority of pharmaceuticals the five adsorbents are comparable, namely for antidepressives and anxiolytics, nevertheless five cases must be highlighted: paracetamol and simvastatin are extracted much more efficiently by JTBaker H_2Ophilic and H_2Ophobic while for ciprofloxacin the H_2Ophilic is just superpassed by Abselut Nexus with an abnormally high recovery rate. On the contrary, hydrochlorothiazide and ibuprofen are better extracted by Oasis HLB and Resprep RDX. The efficiency obtained for simvastatin, paracetamol and ciprofloxacin has driven the selection of JTBaker H_2Ophilic as the most appropriate for the set of pharmaceuticals. The full results for the 12 best adsorbents are given in Table S1 (Supplementary Information).

According to the literature survey performed by Kostopoulou and Nikolaou until 2008 [6], the most effective SPE adsorbents for the enrichment of pharmaceuticals in aqueous samples were Isolute ENV+, Oasis HLB, Oasis MAX, Oasis MCX, Strata-X, LiChrolut C18 and LiChrolut EN. The most popular pre-concentration technique is undoubtedly SPE with a few applications of solid-phase microextraction (SPME) using fibres coated with polyacrylate and carbowax/divinylbenzene [6]. Nevertheless, the limits of detection never rival those of SPE [6]. On the other hand, van de Steene concluded that among Oasis HLB, C8, Phenyl, Strata-X and Strata SCX, the former was the best cartridge for the extraction of nine basic pharmaceuticals, mostly antimycotics, but it also led to intense matrix effect (non selective extraction) [8]. The study of Weigel *et al.* [19] also included some specialized polystyrene-divinylbenzene based adsorbents and co-polymers with noteworthy similarities to our results. The extraction efficiencies of Abselut Nexus, Chromabond Easy and JTBaker SDB1 for the commonly tested pharmaceuticals, although different in the figures, follow the same trend. The recoveries we obtained were generally higher probably because the samples were acidified. That can be important for acidic substances such as bezafibrate, diclofenac and ibuprofen but also for fluoxetine and related analytes as it will be demonstrated later. The above references show that most of the tested cartridges in the present work, namely the most efficient ones JTBaker H_2Ophilic, H_2Ophobic and Resprep RDX, are usually disregarded.

Hierarchical cluster analysis (HCA) of the recoveries achieved by the twelve adsorbents revealed that JTBaker H_2Ophilic, H_2Ophobic and Abselut Nexus have a similar extraction profile, so as Oasis HLB and Resprep RDX. JTBaker SDB1 and Bond Elut PPL cluster

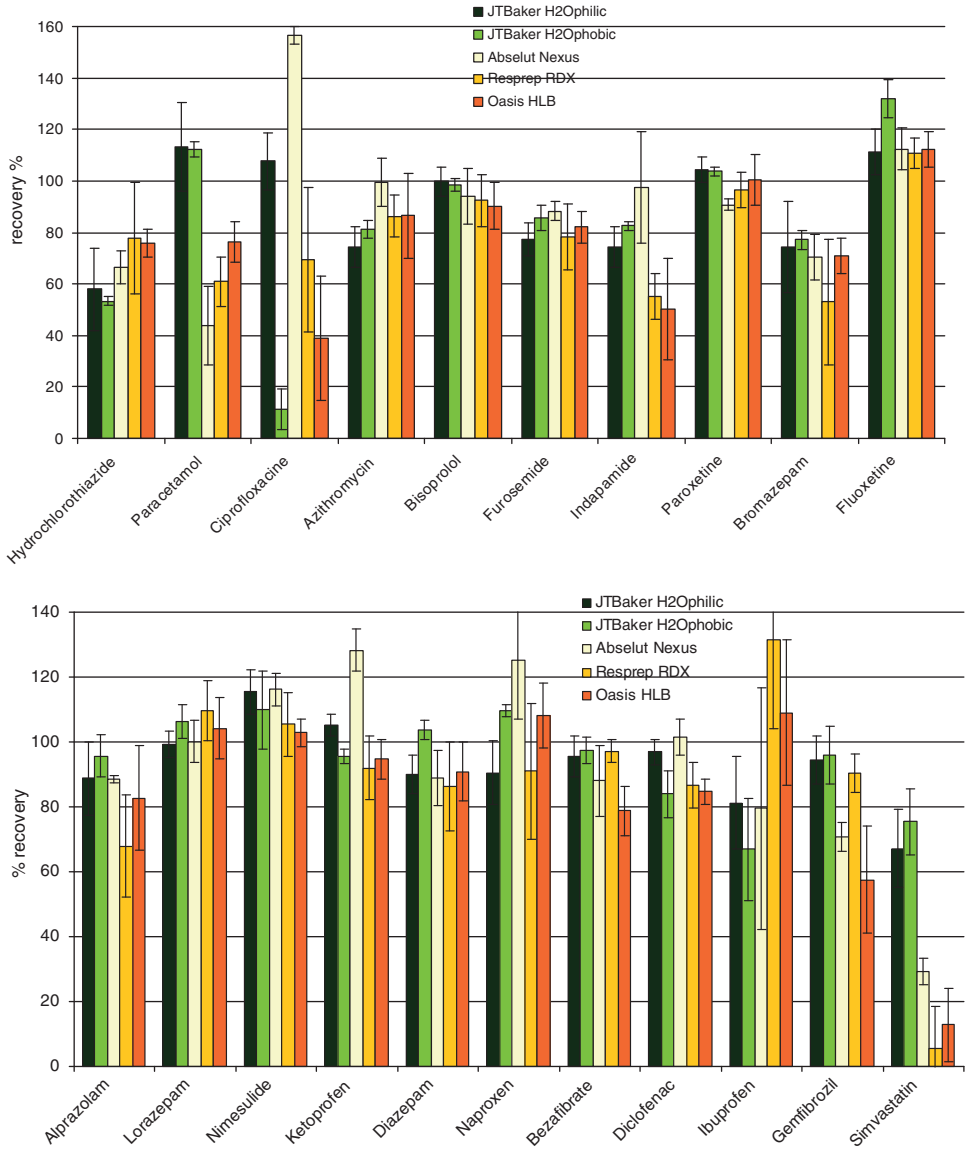

Figure 2. Recovery rates of the five most efficient SPE adsorbents for the enrichment of pharmaceuticals in Milli Q water at 500 ng L^{-1} concentration ($n = 5$). Error bars represent the standard deviation. Omeprazole was not represented since it is not extracted at pH 2 and amoxicillin gives erratic results due to its unstable character.

together and at a later stage a link is formed with the previous two. The two Chromabond phases HRX and Easy also link together and at a much farther stage they join the remaining group other than the JTBaker H_2Ophilic's. The respective dendrogram is given in Figure S2. This information can be useful when deciding from different brands since it shows how the adsorbents compare among them for a representative list of pharmaceuticals. Not to undervalue, of course, the other way round of how the substances behave

regarding the different adsorbents. The respective dendrogram is given in Figure S3. Chemical family affinity is apparent for anti-inflammatories (Group 1) and antidepressives (Group 2), however benzodiazepines scatter between two major groups where anti-inflammatories and lipid regulators are present (Groups 1 and 3). Bromazepam clusters in a more heterogeneous group (Group 4) composed of hydrochlorothiazide, azithromycin and indapamide. Two aspects should be noted: chemical variability is also present among benzodiazepines expressed in their different chromatographic retention times (from 6.37 to 8.97 min) and; the similar or dissimilar behaviour of bromazepam with the other group members is not clearly seen in Figure 2 because only five adsorbents are displayed whereas HCA encompassed all of them. For Group 1 all five adsorbents present in Figure 2 are equally good with average recoveries above 95%. For Group 3 JTBaker H_2Ophilic, H_2Ophobic and Nexus are preferred giving average recoveries between 88 and 92%. For Group 4, Nexus is the most suitable with average recovery of 83%. Group 2 approaches the case of Group 1 except that Oasis MCX is totally unsuitable (Table S1). Of superior evidence, HCA demonstrated that simvastatin, paracetamol and ciprofloxacin showed the most dissimilar pattern thus outweighed the selection of JTBaker H_2Ophilic as the most suitable adsorbent.

3.3 *Effect of sample pH, flow rate and breakthrough volume of the adsorbents*

Sample pH is a very important parameter when enriching substances containing diverse chemical moieties, such as the pharmaceuticals, in SPE adsorbents by reversed-phase interaction. This assumption was proved by testing the following sample pHs: 2, 3, 4, 7.3 and natural pH 5.4. The results obtained are given in Figure 3. The antibiotics azithromycin and ciprofloxacin require acidification to the lowest pH (pH 2) to improve recovery rates while paroxetine and fluoxetine are efficiently extracted at a moderately acid pH (pH 4). Bisoprolol benefits from acidification in a much smaller scale. On the contrary, omeprazole becomes ionized at low pH and extraction is reduced to complete loss. The extraction of the remaining pharmaceuticals is not significantly affected by sample pH and they well tolerate a pH of 2, therefore this value was considered the most appropriate to undertake the analysis, although omeprazole cannot be analysed.

The flow rate of sample percolation was studied between 5 and 20 mL min^{-1}. Generally, the extraction efficiency was independent of the flow rate except for selected compounds. A continuous loss in retention capacity was observed for azithromycin, ciprofloxacin, paroxetine and fluoxetine, with a more intense drop changing from 5 to 10 mL min^{-1} for antibiotics (18 and 22%, respectively) than antidepressives (7 and 9%, respectively). Bisoprolol and bromazepam lost retention strength just at 15 and 20 mL min^{-1} flow rate. Interestingly, the retention efficiency of simvastatin increased three fold at 20 compared to 5 mL min^{-1}. It has not been proved whether simvastatin degrades during 40 min of sample extraction or if adsorption to tubing is reduced at 20 mL min^{-1}. A 10 mL min^{-1} flow rate was finally chosen corresponding to 25 min sample processing time compared to 45 min at 5 mL min^{-1}.

Subsequently, the breakthrough volume was determined under the conditions above. The results in Figure 4 show a good proportionality between the extracted volume and the analytical response at a constant concentration of pharmaceuticals for the majority of analytes. Therefore, the breakthrough volume is above 1000 mL and the retention capacity is independent of the volume of sample. Nevertheless, paracetamol starts leaking from the

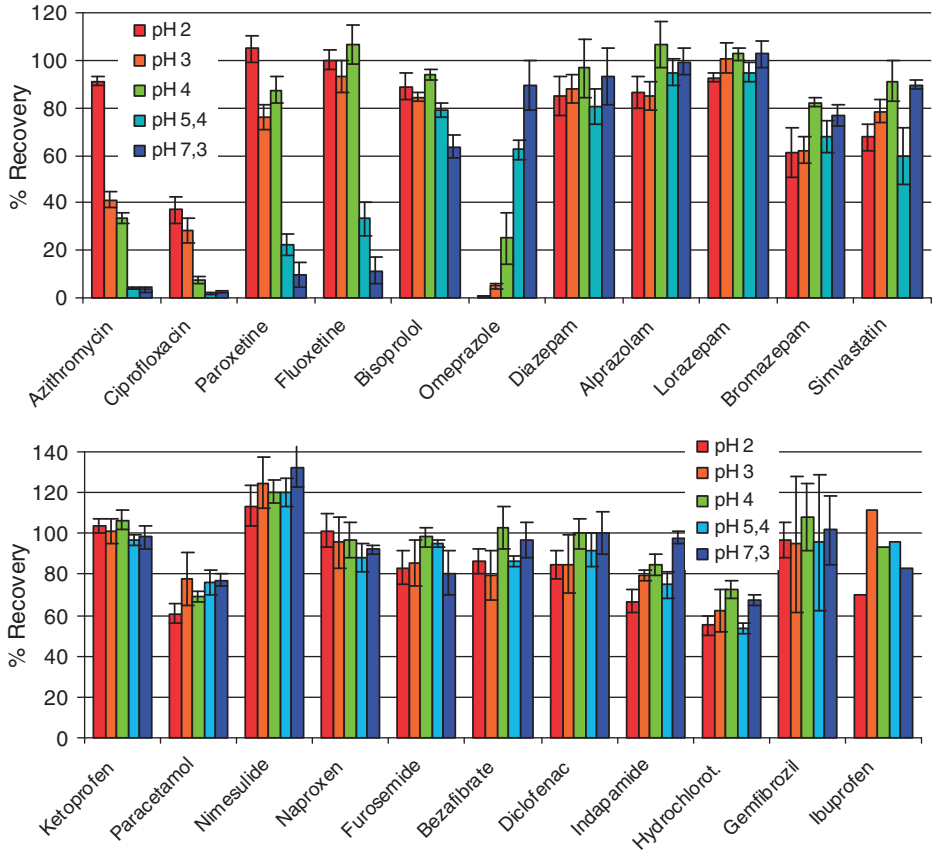

Figure 3. Effect of sample pH on the retention of some critical and representative pharmaceuticals in the JTB H_2Ophilic adsorbent ($n = 4$). Error bars represent the standard deviation of the mean. Tests were performed at a concentration level of 500 ng L^{-1} in Milli Q water.

cartridge at sample volumes around 200 mL, which is related to its hydrophilicity. Omeprazole, on the other hand, is not possible to enrich on the adsorbent under acidic conditions and the recovery is low at 100 mL volume and is further lost. In view of the above results and the necessary sensitivity of pharmaceutical analysis in surface waters, 200 mL of sample are SPE processed and eluted in 2 steps of 3 mL of methanol (1 min-. waiting time in between). The dry residue is redissolved in 200 µL of a mixture of methanol/water 35 : 65 and injected for LC-MS/MS analysis.

3.4 *Clean-up procedure to overcome matrix effects*

Evaluating matrix effects is very important when developing an LC–MS/MS method. The results of van de Steene *et al.* [8] prove that matrix effects are very difficult to tackle and combined multiple approaches are necessary to reduce their impact in precision and accuracy. Normalization of erroneous analyte response, either signal suppression or enhancement, caused by co-eluting compounds originated from the matrix can be

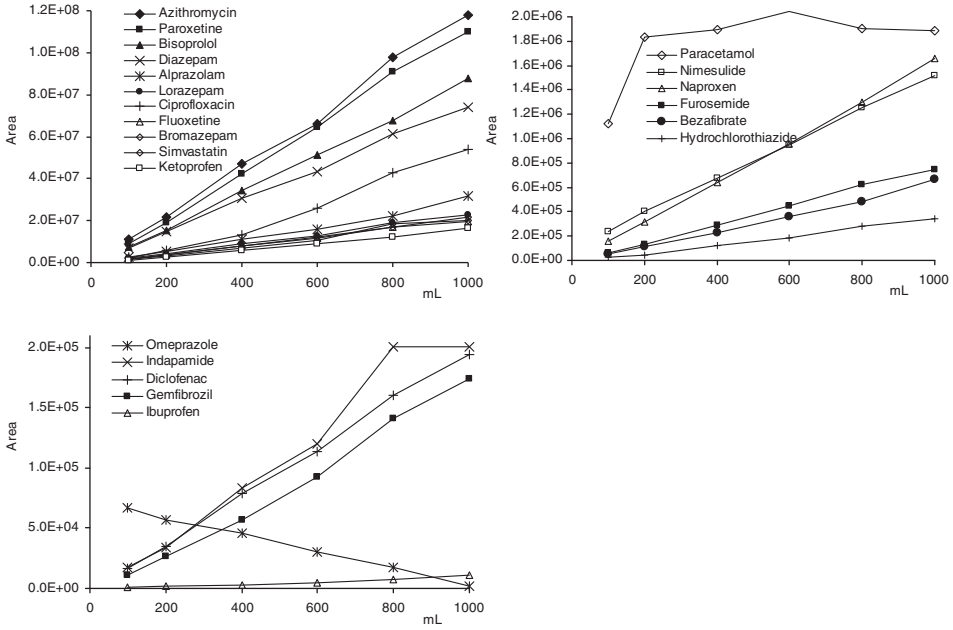

Figure 4. Study of breakthrough volume of the JTBaker H_2Ophillic adsorbent for several pharmaceuticals in a concentration of 500 ng L^{-1} in Milli Q water.

endeavoured by several methods: use of isotopically labelled internal standards, removal of interfering substances by clean-up, selective extraction of different types or standard addition for calibration and calculation of recoveries. Selective extraction, and clean-up in particular, extend the life-time of the chromatographic column [22]. Sample to sample variability is another issue to keep in mind: compensation by recoveries calculated for each sample or use of isotopically labelled internal standards are the best options to achieve accurate results.

The pharmaceuticals most affected by matrix effect (mainly ionization suppression rather than interference in enrichment efficiency) with average (relative) recovery reduction above 30% were: hydrochlorothiazide, azithromycin, furosemide, indapamide, fluoxetine, alprazolam, naproxen and diclofenac, considering the four matrices in Table 4. Signal enhancement was observed for lorazepam (18%). More detailed information on the type of matrix effect (either ionization suppression or effect on recoveries) obtained according to the calculations explained in section 2.4 can be found in Table S2). Indeed, ionization suppression could reach 46% signal reduction in the dirtiest sample. Additionally, azithromycin and bisoprolol suffered peak broadening and increase of retention time whereas paroxetine and fluoxetine were just affected by retention time deviation. Retention time shifting is critical in MS/MS methods scheduled in segments. Fluoxetine D5 was used to monitor retention time variation of antidepressives while paracetamol D4 was used for compensation of matrix effect on the native paracetamol. For the remaining pharmaceuticals correction by recoveries on related matrix was used spiking the sample with pharmaceuticals at 500 ng L^{-1} concentration. Additionally, clean-up was employed in the dirtiest samples.

Table 4. Recoveries of pharmaceuticals in Milli Q water and surface water of the Ave, Leça and Douro rivers at 500 ng L^{-1}. Douro water was submitted to clean-up with different anion exchange adsorbents to evaluate their suitability.

		Recoveries (%) without clean-up				Recoveries (%) with clean-up			
	Milli Q ($n=4$)	Ave ($n=3$)	Leça Reg. ($n=3$)	Leça Pte Pedra ($n=3$)	Douro ($n=6$)	Leça Pte Pedra + 1°, 2° amino ($n=3$)	Douro + 1°, 2° amino ($n=6$)	Douro + NH2 ($n=6$)	Douro + SAX ($n=6$)
Hydrochlorothiazide	50	16	19	21	12	33	28	16	11
Paracetamol	68	50	62	29	38	36	44	37	35
Ciprofloxacin	87	73	81	61	70	81	107	100	95
Zolpidem	98	96	97	88	104	92	94	96	103
Azithromycin	68	25	58	0.1	56	51	48	48	47
Bisoprolol	101	97	95	87	94	90	90	94	89
Furosemide	76	54	68	33	20	39	41	33	25
Indapamide	70	33	45	7	12	35	33	19	15
Paroxetine	99	77	77	64	77	86	89	83	36
Bromazepam	75	64	61	61	65	74	58	61	57
Fluoxetine	109	55	57	52	93	67	104	89	47
Alprazolam	89	61	63	55	56	66	62	64	56
Lorazepam	97	118	113	117	113	93	108	112	122
Nimesulide	110	130	133	98	47	89	85	76	66
Ketoprofen	101	88	90	89	81	91	77	73	68
Diazepam	92	90	86	81	85	84	82	80	75
Naproxen	102	71	73	57	53	66	57	41	35
Bezafibrate	96	75	85	58	49	72	59	51	52
Diclofenac	86	71	52	36	60	49	65	58	43
Ibuprofen	81	82	105	62	82	77	102	109	87
Gemfibrozil	86	95	91	75	80	74	81	70	84
Simvastatin	46	28	19	2	50	39	46	36	31
Average Recoveries	**86**	**69**	**73**	**57**	**63**	**67**	**71**	**66**	**58**

Conversely to Vieno *et al*. [22], in our studies selective wash of the cartridge did not reduce significantly matrix effects, even with 20% methanol in water (data not shown). Clean-up of the extract with normal phase adsorbents was attempted however florisil retains irreversibly the majority of analytes, as mentioned by Weigel *et al*. [19] and Fontanals *et al*. [20], while diol is quite inefficient. Much effort was then put in sample clean-up with anion exchange gathering from the previous knowledge on this strategy [21]. The results of the assays performed on river water samples can be found in Table 4. Leça Pte Pedra is the most loaded matrix since it is located downstream two WWTPs. As a first remark, adjustment of the sample to pH 2 has to be performed prior any clean-up otherwise azithromycin (16%), paroxetine (9%) and fluoxetine (17%) do not percolate quantitatively (% loss) the ion exchange adsorbents. The quaternary amine (SAX) clean-up prior to JTBaker H_2Ophili c enrichment was the least efficient: visually and analytically the clean-up was the weakest and a specific study also demonstrated that undesired retention of some analytes takes place. The 1°, 2° amino (WAX) clean-up showed advantage over the NH_2, which was demonstrated by better recoveries and less breakthrough of dirty material. Although the gain in analytical response is not outstanding (10% higher recoveries in Leça river and 8% in Douro river, on average), the clean-up has a very positive impact on chromatographic stability, thus reducing the possibility of false negatives, and obviously protecting the column from deterioration due to bulk material. Besides, an individual recovery improvement of 51, 37, 28 and 22% was observed for azithromycin, sinvastatin, indapamide and paroxetine, respectively, in Leça river and 38, 37, 21 and 21% for nimesulide, ciprofloxacin, furosemide and indapamide, respectively, in Douro river. The described strategy with 1°, 2° amino might imply a loss in the adsorbent of furosemide, indapamide, bezafibrate between 4-9% calculated in Milli Q water while for the remaining analytes it is meaningless. In Table S2 results are given for Douro river sample. The mass of adsorbent to be used should be as little as required (200 mg) to prevent the above effect and no better clean-up was achieved with 500 mg. As mentioned by van de Steene *et al*. [8] sample clean-up for pharmaceutical analysis is very tricky and a more exhaustive procedure may vanish also the analytes. Therefore we preferred a softer procedure with anion exchange. The several approaches tested by the above authors gave divergent results for different analytes, thus an overall good methodology was not clearly found [8]. These authors propose a clean-up based on NH_2 adsorbent but the option was to perform SPE first and then WAX on the extract in chloroform/methanol (80 : 20) solvent, conversely to our strategy. Subsequent elution with an organic solvent may carry interferences alongside with the analytes.

If acidification of the sample to pH 2 was not a requisite, the anion exchange retention of organic acids composing the dissolved organic matter would be improved and their subsequent enrichment on the H_2Ophili c cartridge would be minimized. However, proper recovery of some selected pharmaceuticals forced *ex ante* acidification, by several reasons already discussed.

3.5 *Evaluation of the method performance*

The extraction efficiency of the method expressed as recoveries in Milli Q and surface water, with or without clean-up with 1°, 2° amino adsorbent was already given in Table 4. Matrix effects were also already discussed. Additionally, the following figures of merit are given in Table 5: precision (as repeatability and intermediate precision in three non

Table 5. Figures of merit of the proposed method including a sample clean-up using 1°, 2° amino followed by enrichment of the pharmaceuticals in H_2Ophilic adsorbent. Results were obtained in Milli Q water, complemented with the repeatability in spiked surface waters of Leça Parada and Douro L (500 ng L^{-1}) and Leça Reguenga (50 ng L^{-1}). Linearity was also tested in Leça Reguenga water (10–500 ng L^{-1}).

Pharmaceuticals	LOD, S/N3 (ng L^{-1})	LOQ, S/N10 (ng L^{-1})	Repeatability (% RSD), $n=5$	Intermediate Precision (% RSD), $n=18$	Determination coefficient (r^2), $n=8$	Repeatability in Leça Parada (% RSD), $n=4$	Repeatability in Douro L (% RSD), $n=5$	Repeatability in Leça Reguenga (% RSD), $n=6$	Determination coefficient (r^2) Leça Reguenga
Hydrochlorothiazide	9	30	7.8	26.2	0.996	2.5	9.4	17.8	0.997
Paracetamol	6	20	17.3	11.8	0.997	9.2	6.9	7.2	0.994
Ciprofloxacin	10	33	11.1	26.6	0.989	10.5	9.4	10.6	0.998
Zolpidem	2	5	4.0	6.1	0.998	7.7	4.0	3.5	0.999
Azithromycin	4	13	8.0	16.8	0.990	6.0	9.2	12.8	0.991
Bisoprolol	1	3	5.6	7.6	0.998	1.7	8.2	9.5	0.999
Furosemide	5	17	6.4	10.7	0.997	20.5	11.6	15.8	0.987
Indapamide	4	13	8.0	17.5	0.985	6.9	17.3	25.2	0.992
Paroxetine	3	10	5.1	10.8	0.997	7.5	12.6	6.3	0.997
Bromazepam	3	10	7.9	16.7	0.996	8.5	11.5	9.9	0.998
Fluoxetine	6	20	8.9	9.4	0.995	11.7	10.8	8.9	0.999
Alprazolam	3	10	11.3	17.6	0.988	2.8	4.5	8.0	0.999
Lorazepam	4	13	4.0	6.5	0.998	5.6	4.0	11.9	0.999
Nimesulide	3	10	6.9	9.4	0.988	12.3	10.8	12.7	0.996
Ketoprofen	3	10	3.3	4.5	0.999	4.0	3.3	8.0	0.994
Diazepam	4	13	6.0	7.1	0.995	8.7	4.1	9.0	0.996
Naproxen	14	43	9.8	14.0	0.995	11.2	10.0	18.3	0.990
Bezafibrate	3	10	6.3	8.6	0.997	15.7	11.1	18.7	0.987
Diclofenac	5	17	3.9	7.3	0.994	15.1	28.1	13.8	0.988
Ibuprofen	50	165	24.2	27.2	0.980	7.6	21.1	17.2	0.990
Gemfibrozil	10	33	7.4	8.2	0.997	13.2	4.8	12.9	0.994
Simvastatin	3	10	12.0	20.5	0.991	20.2	19.7	12.6	0.990

consecutive days), limits of detection (LODs) and linear range. Recoveries in Milli Q water were generally above 80%, exceptions noted for hydrochlorothiazide and paracetamol due to their water solubility and, furosemide and indapamide which apparently are more sensitive analytes. For some unknown reason, the recovery of simvastatin never exceeded 50%. Tests on adsorption to tubing, incomplete elution and insufficient redissolution of the dry extract didn't provide any clue. Degradation by interconversion of the cyclic and open ester form is an hypothetic explanation.

Limits of detection verified in real samples were generally below 5 ng L^{-1}. The LOD of ciprofloxacin was raised to highlight from a small background peak. The sensitivity in ESI+ is typically better than in ESI−. The fragmentation of ibuprofen is very erratic, therefore no CID energy was applied to be able to collect the $[M-H]^-$ ion and the high LOD is a consequence of this limitation. The precision of the method is very good and typical of a SPE procedure. Repeatablity in Milli Q water varied between 4 and 17.3% RSD, except ibuprofen (24%), with an average value of 8.4% RSD while intermediate precision varied between 4.5 and 27.2% RSD with an average value of 13.2% RSD. No relevant difference was observed in spiked surface waters at both spiking levels (500 and 50 ng L^{-1}, except for analytes detected in ESI− (furosemide, indapamide, nimesulide, bezafibrate, diclofenac, gemfibrozil) and simvastatine, which display worse precision. At 50 ng L^{-1} precision is worse about 3% relative to 500 ng L^{-1} level.

The linear range evaluated from the determination coefficients of the linear regression model (average of eight calibrations) is generally very good in the LOQ-500 ng L^{-1} concentration range, particularly for the benzodiazepines, bisoprolol and the phenylpropanoic acid anti-inflammatories. Two compounds deserve special attention: ciprofloxacin gives a concave calibration plot while nimesulide gives a convex one therefore linear range is reduced to LOQ-100 ng L^{-1}. The linearity was also tested in a surface water sample (Leça Reguenga) and the determination coefficients are similar to those found in Milli Q water, so the linearity was not prejudiced by matrix effect. These figures of merit are perfectly adapted for monitoring acidic, basic and neutral pharmaceuticals in surface waters that can range from tens to hundreds ng L^{-1}. Conversely to our strategy, Vieno *et al.* have discriminated the analysis of acidic [23] and base/neutral pharmaceuticals [22] into two separate methods.

3.6 *Assessment of pharmaceuticals in surface water*

To demonstrate the applicability of the developed method and assess the presence of those pharmaceuticals in surface waters, Leça and Douro rivers were sampled in February/March and June, 2009. The results obtained are presented in Table 6. An illustrative chromatogram of sample Pte Moreira collected in June is shown in Figure 5.

The samples of Leça river upstream Pte Ermesinde didn't contain any quantifiable amount of pharmaceuticals. Paracetamol was present in downstream stations in considerable amounts in February while azithromycin and hydrochlorothiazide appeared in June. Bisoprolol and furosemide appeared in both seasons, generally at higher concentrations in June than in February, the former pharmaceutical in a 10-times lower concentration. Vieno *et al.* reported removal rates of β-blockers in WWTPs from 11%, for metoprolol, up to 76%, for atenolol [22], therefore these pharmaceuticals reach the receiving waters, as occurred with bisoprolol. On the other hand, ciprofloxacin only appeared in one sample due to its breakdown in the WWTPs, as documented by the same

Table 6. Results of the occurrence of pharmaceuticals in the last five sampling stations in Leça river in February and June 2009 (in $ng\,L^{-1}$).

	5 stations upstream Pte Ermesinde		Pte Ermesinde		Pte Parada		Pte Pedra		Pte Moreira		Pte Matosinhos	
	February	June	February	June	February	June	February	June	February	June	February	June
Hydrochlorothiazide	–	–	–	–	–	57.8	–	389	–	352	86.7	196
Paracetamol	–	–	–	37.6	173	–	368	–	925	–	491	22.5
Ciprofloxacin	–	–	–	–	–	–	–	–	–	–	–	59.3
Azithromycin	–	–	–	–	–	136	–	163	–	147	–	94.7
Bisoprolol	–	–	–	–	15.4	9.80	11.9	24.0	11.9	26.0	9.49	19.1
Furosemide	–	–	–	–	119	69.2	114	192	110	283	–	227
Lorazepam	–	–	–	–	–	–	–	49.1	–	41.3	21.1	25.8
Ketoprofen	–	–	–	–	–	7.90	–	–	–	11.9	–	–
Naproxen	–	–	–	–	–	86.0	–	136	–	91.3	–	–
Bezafibrate	–	–	–	–	770	19.1	706	10.9	334	21.2	256	10.2
Diclofenac	–	–	–	–	56.8	22.8	–	–	–	–	–	–
Gemfibrozil	–	–	–	–	–	43.1	35.0	59.8	43.3	91.2	–	74.2
Simvastatin	–	–	–	–	42.9	–	–	–	–	–	–	-

Notes: a dash (–) means that the analyte was not quantitated (< LOQ).

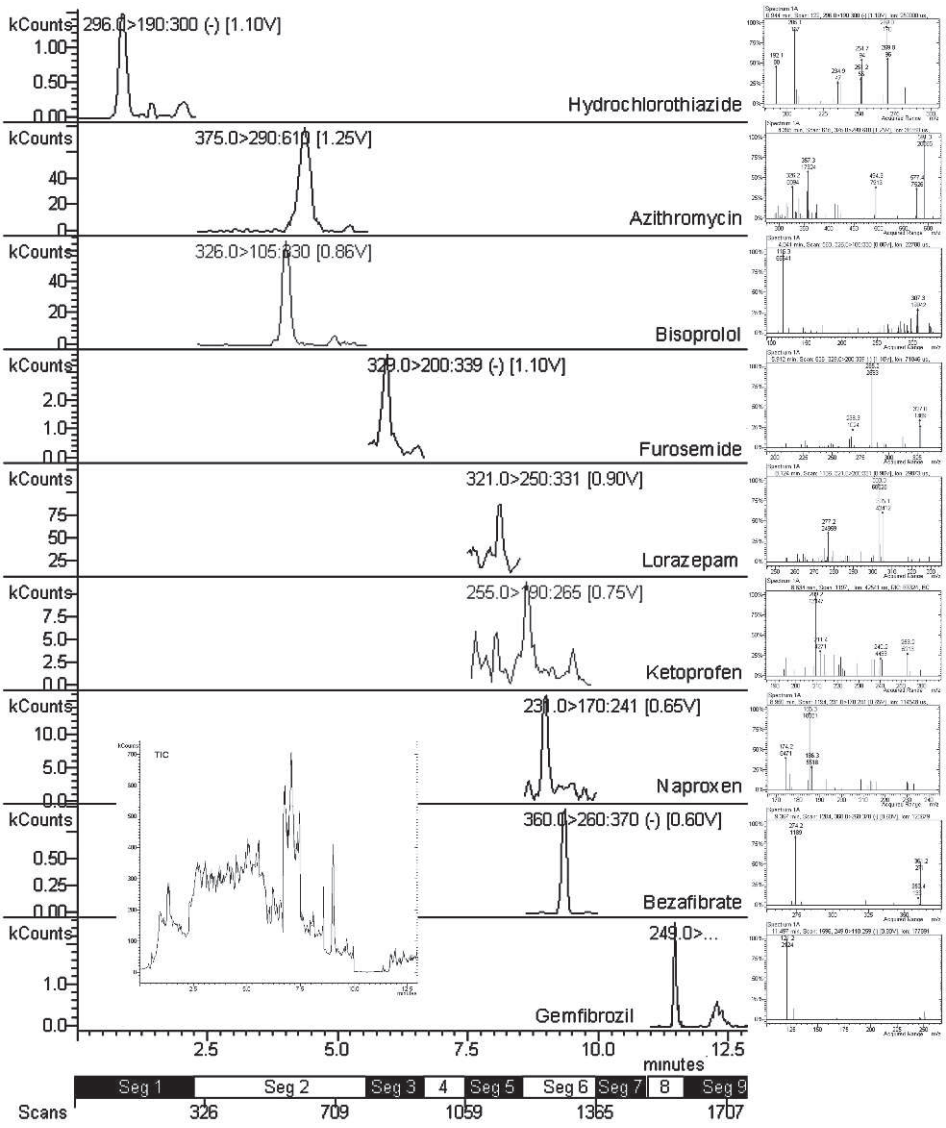

Figure 5. Extracted ion chromatogram of the sample Pte Moreira collected in June 2009 showing the quantified pharmaceuticals. The insert represents the total ion chromatogram (TIC).

authors [22]. Lipid regulators are a class with high prevalence in Leça river. A considerable concentration of bezafibrate (from 256–770 ng L^{-1}) was quantified in February decreasing 15–70 times in June while gemfibrozil was always present at around 35–90 ng L^{-1}. Ketoprofen, diclofenac and simvastatin appeared sporadically. Rodil *et al.* have reported levels of four pharmaceuticals in effluents discharged to surface waters at levels above 500 ng L^{-1}, associated with low removal rates in the WWTPs: diclofenac (23%), bezafibrate (54%), naproxen (63%) and ibuprofen (85%) [18]. Despite the different removal rates of ibuprofen and ketoprofen (10%) the levels in one effluent were not

so distinct. In Douro river none of the pharmaceuticals was measured in February while in June one sample collected in the outskirts of Oporto contained hydrochlorothiazide (31 ng L^{-1}) and another collected in the estuary contained paracetamol (63 ng L^{-1}) and ketoprofen (11 ng L^{-1}).

As noted by Madureira *et al.* [7] the concentrations of pharmaceuticals are typically higher in dry seasons rather than in winter, especially in the small stream of Leça river that carries a considerable portion of WWTP effluents in dry periods. Two punctual cases depart from this trend: paracetamol, that is normally removed by the biological treatment in WWTPs [6] and bezafibrate which was infrequent in later samplings.

To clarify whether the presence of pharmaceuticals in Leça river is just apportioned by the WWTPs, the monitoring network will be complemented by sampling the three tributaries: Leandro, Boi Morto and Arquinho streams. High levels of paracetamol conjugated with a high ratio of caffeine/carbamazepine may be an indication of untreated domestic sewage [24–26]. Although agricultural and domestic pollution sources are convened to both rivers the dimension of Douro provides a much higher dilution capacity. Leça is critically affected by pharmaceuticals since: (i) current WWTPs are not prepared to remove completely such organic substances [22], (ii) some of them are recalcitrant in the environment, (iii) they are intensively discharged in the river and (iv) the dilution ratio is scarce. The presence of the reported pharmaceuticals was unequivocally confirmed by MS/MS and MS^3 complemented with isotopic ratios.

4. Conclusions

The aim of this work consisting of the simultaneous analysis of acidic, basic and neutral pharmaceuticals was successfully attained with a judicious selection of the most appropriate SPE adsorbent, which turned out to be JTBaker H_2Ophilic. Paracetamol, ciprofloxacin and simvastatin have driven this option. Acidification was demonstrated to be essential for efficient extraction and chromatographic resolution of the set of pharmaceuticals. A tailor-made clean-up for the reduction of matrix effects in medium/high loaded surface waters was accomplished with the 1°, 2° amino adsorbent that provided a satisfactory clean-up without irreversible retention of the analytes. The employed clean-up benefited recoveries and was essential for the consistency in peak shape and retention time of antibiotics, antidepressives and bisoprolol. The developed method provided limits of detection generally below 5 ng L^{-1} and precision generally below 15% RSD. Amoxicilin is a very unstable compound that although much consumed is not likely to appear in environmental samples whereas omeprazol cannot be determined under acid conditions.

Eleven out of twenty two pharmaceuticals were quantified in Leça river. Diuretics and lipid regulators are categories of concern. Paracetamol was atypically present and lorazepam is also scarcely reported in surface water. Douro river revealed much lower levels of pharmaceuticals although several anthropogenic pollution sources are present, which can be attributed to its hydraulic features.

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Annex III – Report of the intercalibration exercise under PHARMAS Project: "Ecological and human health risk assessments of antibiotics and anti-cancer drugs found in the environment"

PHARMAS

Ecological and human health risk assessments of antibiotics and anti-cancer drugs found in the environment

Contract n° 265346

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"Report of the intercalibration exercise"

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S1	Reviewed			**R1**	Restricted to pharmas partners	**x**		Internet	
S2	Pending for review	**x**		**R2**	Restricted to European. Commission			Paper	
S3	Draft for comments								
S4	Under preparation								

Abstract

An inter-laboratory exercise was organized under the PHARMAS EU project, by the Advanced School of Public Health (EHESP), in order to evaluate the reproducibility of analytical method for the measurement of antibiotics (ATB) in waters. For the first time, this type of exercise has been organized in Europe, and using different kind of analytical methods and devices. In this exercise thirteen laboratories from five countries (Canada, France, Italy, the Netherland and Portugal) participated, and a total number of 78 samples were distributed.

During the exercise, 2 testing samples (3 bottles of each) prepared from tap water and river water respectively, spiked with ATB, were sent to the participants and analyzed along one month.

A final number of 77 (98.7 %) testing samples were analyzed. Depending on substances analysis by each participant, 305 values in duplicate were collected, and the results for each sample were expressed as the target concentration.

A statistical study was initiated using 611 results. The mean values, standard deviations (σ), variances ($\sigma 2$) and, upper and lower warning limits (UWL and LWL) between concentration-result from the participant laboratory at different intervals were obtained.

In this exercise, 24 results (3.93 % of accounted values) were outliers according with the Zscore values and the Dixon test. The outlier results were excluded.

In order to establish the stability of testing samples along the exercise, differences between variances obtained for every type of sample at different intervals were evaluated. The results showed not significant variations and can be considered that all samples were stable during the exercise.

The goals of this inter-laboratory study were to evaluate the repeatability (r) and reproducibility (R) when different laboratories conduct the analysis, the influence of different matrix samples, the variability between different methods, and to determine the rate at which participating laboratories successfully completed the tests initiated.

1. Introduction

The presence of antibiotics in environmental samples has been reported extensively. Antibiotic are among the most investigated class of pharmaceuticals in the environment. More recently, they have been detected in drinking water (Bruchet et al., 2005; Ye et al., 2007; Focazio et al., 2008; Benotti et al., 2009, Vulliet et al., 2009; Watkinson et al., 2009). However, concentrations are very low and accurate and analytical methods need to be controlled in order to insure a good uncertainty of the data obtained required for a robust assessment of exposure scenarios of human people.

In this context, an inter-laboratory comparison exercise (announcement and recommendation have been reported in annex 1) was conducted among thirteen laboratories covering different EU countries. 2 samples were analyzed along one month. The samples correspond to 3 glass bottles of tap water for the first and 3 glass bottles of river water (filtered sample from La Vilaine river in Brittany, France) for the second.

The principal aims of the interlaboratory study were:

- To evaluate the variability of results between different laboratories

- To evaluate the rate at which participating laboratories successfully completed the exercise

- To evaluate the capacity and variability in front of complex real samples

2. Participants profile

Interlaboratory exercise is an opportunity for one laboratory to assess their performances by comparison with several other laboratories and to quantify the modification necessary to improve them.

13 laboratories (annexe 2) participed to the exercise. 5 countries are represented (Canada, France, Italy, The Netherland, Portugal). 1 (one) laboratory used two analytical methods and was considered as 2 different ones. Then 14 “labs” have been considered. For reasons of anonymity, they have been numbered randomly from lab1 to lab14.

50% have already participated to EIL

1/3 of laboratories have less than 2 years of experience in the analysis of ATB in water

25% have more than 6 years of experience in the analysis of ATB in water

40% have between 3 and 5 years of experience in the analysis of ATB in water

92% perform routine analysis of ATB in their lab (83% of which several times a week and 17% at least once every 6 months).

Analytical know-how: Figure 1 show the % of laboratories being able to analysis the ATB proposed.

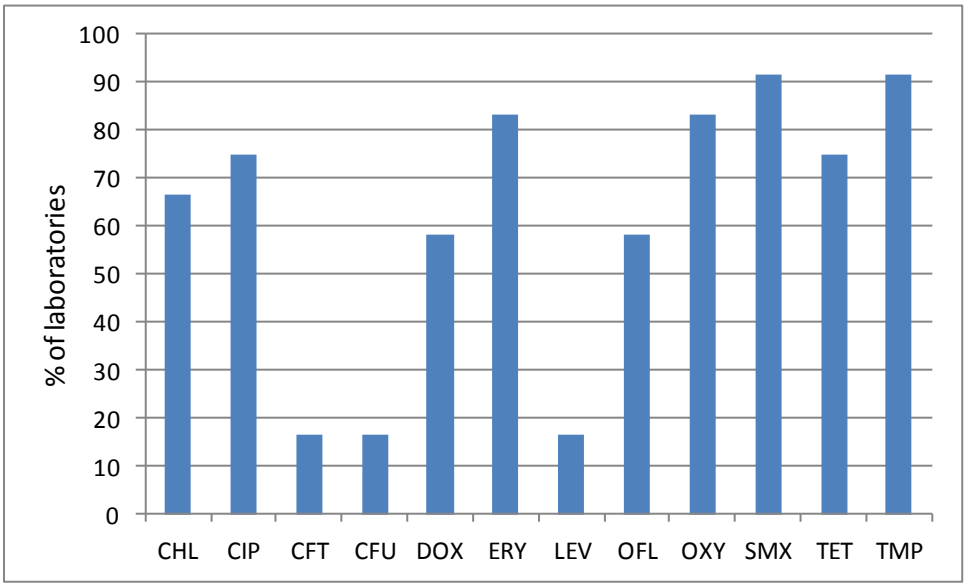


Figure 1 : Analytical Know-how of the participating laboratories

Performances of the whole consortium are reported in the Table 1. They take into account the limit of detection of each laboratory. Min and Max correspond to the lower and higher value of LOD among the laboratories. Median and mean have been calculated on the basis on the average of the 14 values of LOD declared by the laboratories. n is the number of laboratories analyzing the corresponding substance.

Table 1: Analytical performances of the participating laboratories

		LOD repartition (ng/L)			
	n	**Min**	**Max**	**Mean**	**Median**
Erythromycin (ERY)	10	1	500	80.3	10
Trimethoprim (TMP)	10	1	50	16.6	5.5

Sulfamethoxazole (SMX)	9	1	50	13	10
Ciprofloxacin (CIP)	8	1	100	32.13	20.5
Oxytetracycline (OXY)	7	1	50	26.9	25
Ofloxacin (OFL)	7	1	20	12.2	11
Tetracycline (TET)	6	1	100	39.5	37.5
Chlortetracycline (CHL)	5	1	200	48.4	11
Doxycycline (DOX)	3	5	100	51.7	50
Levofloxacin (LEV)	2	5	50	27.5	27.5
Cefuroxim (CFU)	1			50	
Ceftriaxone (CFT)	0				

When available, the substances were analyzed by non accredited method except ERY in three laboratories and SMX in two laboratories. Only one laboratory is accredited for 8 substances.

Considering the different performances and capabilities of the participating laboratories and the capacity of the host laboratory to produce the reference samples, the following antibiotics were chosen (Table 2)

Table 2 : Final substances studied during the exercise

Class of antibiotic	Molecule	Acronym	CAS registry number
Fluoroquinolones	**Ofloxacin**	OFL	83380-47-6
	Ciprofloxacin	CIP	85721-33-1
Sulfonamide	**Sulfamethoxazole**	SMX	723-46-6
Pyrimidin (associated to sulfonamide)	**Trimethoprim**	TMP	738-70-5
Macrolide	**Erythromycin**	ERY	114-07-8

3. Material preparation

3.1. Standard preparation

Acetonitrile (HPLC gradient, Baker), methanol (HPLC gradient, Baker), formic acid (Baker), NH_4OH 25% (Carlo Erba), sodium nitrite 99% (ACS Reag. PhEur, Merck) and ultrapure water were used for the preparation of standards solution.

Standards are commercialized under powder form with purity between 97 to 99,9%.
Trimethoprim standard was purchased from VWR (certified quality,Dr. Ehrenstorfer GmbH, Bgm.-Schlosser-Str. 6 A, 86199 Augsburg, Germany)
Standards of other antibiotics were purchased from Sigma Aldrich. Life durations are not certified by the provider but 3 years of preservation are insured by a certificate.

Mother solutions are solutions of individual compounds prepared in Methanol. CIP and OFL solutions are prepared as 0.5 g/L; the other ones at 1g/L. CIP solution requires addition of formic acid for a better solubility.

Daughter solutions (DS) are prepared in acetonitrile. A first DS (DS1) at 10mg/L followed by a second DS (DS2) at 0.5mg/L are prepared by dilution of the mother solutions.
DS are preserved at -20°C.

The characterization (stability and homogeneity test) of the reference sample has been conducted by UPLC/MS/MS using internal standard.
Internal standard solutions have been produced from the following marked molecule: Ofloxacin-d3, Trimethoprim-13C, Ciprofloxacin-13C-15N, Sulfamethoxazole-13C, Erythromycin-13C purchased from Sigma Aldrich (TMP, ERY and CYP are solutions in 1.2 ml flask)
Mother solutions of OFL-d3 and SMX-13C are prepared in Methanol (1g/L) followed by a dilution in acetonitrile up to solution at 10mg/L.

ERY-13C, TMP-13C, CIP-13C-15C are prepared directly in acetonitrile (12pp).

A solution standard of reference marked compound at 0.5 mg/L is finally prepared in acetonitrile.

3.2. Reference sample preparation

Two reference samples (called sample A and sample B) were prepared. Sample A and sample B correspond to treated and filtrated surface water respectively spiked with the 5 antibiotics of interest. The characteristics of the matrices are presented in Table 3.

Table 3: Characteristics of the matrices used to prepared sample A and B

Parameters	Raw water after filtration (0.7µ)	Distributed water (LERES tap)
pH at 20 °C	7.95	7.85
Conductivity at 25 °C (µS/cm)	516	697
TOC (mg/L)	7.3	1.3
DOC (mg/L)	7.0	1.3
Total chlorine (mg/L)	< 0.1	0.2
Free chlorine (mg/L)	< 0.1	< 0.1

In order to minimize sources of variation, samples A and B were collected, homogenized and prepared at EHESP-LERES, Rennes, France. River water (70L) was collected and transported to the laboratory where it was filtered through 0.7 µm glass fibre filters. Treated water (70L) was directly collected at the tap of laboratory. Afterwards, waters were homogenized, spiked and sub-sampled for homogeneity and stability testing.

Samples A and B were then transferred into 1 L polyethylene bottles (approx. 900 mL of each sample); 3 bottles of each samples for each laboratories. They were shipped on dry-ice to the participant laboratories on October, 5th, 2011. A total number of 39 bottles was sent to 13 participants (3 of each sample). The samples arrived to participant laboratories in 24 to 72 hrs in frozen state.

4. Homogeneity of samples

To assure and confirm the quality of reference sample preparation, homogeneity tested by following the guidelines of the ISO 13528. Treated (sample A) and surface water (sample B), were sub-sampled after the spiking and homogenisation, where ten subsamples per sample were taken from different layers in the container. Two parallels were analysed per each sample, in total 30 samples were analysed per each sample. The homogeneity was statistically evaluated by using the comparison of the between-sample standard deviation to total standard deviation. According to the ISO 13528 standard, the ratio of the between-samples standard deviation to the total standard deviation must be below 0.3.

The between-sample standard deviation has been calculated as follow:

- $x_{t,k}$ are the data (t represent the sample and k the parallel)
- the mean of the samples is defined as $x_{t,} = (x_{t,1} + x_{t,2})/2$

- the range of the duplicate as wt = | $x_{t,1}$ - $x_{t,2}$ |
- the general mean is given by: $\bar{x} = \sum x_t / g$
- the standard deviation of the mean of the samples: $s_x = \sqrt{\sum (x_t - \bar{x}_.)^2 / (g-1)}$
- the standard deviation of the samples: $s_w = \sqrt{\sum w_t^2 / (2g)}$

Finally, between-sample standard deviation can be calculated as follow

$$s_s = \sqrt{s_x^2 - (\frac{s_w^2}{2})}$$

Table 4 represents the calculation of the between-sample standard deviation for each target in both samples.

Table 4 : Ratios between Ss* (between-samples standard deviation) and σ (standard deviation of the round calculated from the results of the laboratories) for all the homogeneity controls

Target	Sample A			Sample B		
	s_s	σ	s_s /σ	s_s	σ	s_s /σ
ERY	7.1	43.1	0.16	11.7	252.9	0.04
OFL	2.7	21.6	0.13	9.6	83.1	0.12
CIP	ND			11.0	62.4	0.18
SMX	2.6	6.8	0.38	2.8	26.7	0.1
TMP	4.5	12.8	0.35	0.8	33.0	0.02

* details of the calculation are shown in annex 3

Thus, the results of the controls showed that the samples are not significantly different for that homogeneity target. It can be concluded that the reference samples A and B may be considered homogeneous; this means that all the manufactured samples may be considered homogeneous for a given proficiency test. It can be noted that, since homogeneity between the samples depends largely on the nature of the product.

Ciprofloxacin was not detected in sample A

5. Stability of samples

The verification of the stability was assessed by using the same protocol as for the verification of the homogeneity. Temperature preservation and storage time were assessed for the evaluation of the stability of the reference samples.

3 sub-samples of each type of water were randomly selected and preserved at room temperature (20°C ± 2) and +4°C during two, seven and ten days (total of 18 samples)

Each antibiotic were measured in each sub-samples in duplicate. The average concentration ($\bar{y}$) is compared with the average concentration of all sub-samples used for the stability and homogeneity tests ($\bar{x}$). The total standard deviation has been calculated from the results of the exercise for each antibiotic (**Table 5** and **Table** 6). Detail of calculation is given in annex 3

Table 5 : Values for verification of stability in treated water

SAMPLE A: Treated Water					
		OFL	TMP	SMX	ERY
$\bar{x}$		28.63	43.76	26.81	43.03
σ		21.6	12.8	5.7	43.1
$\bar{y}$	Room T° Day 2	33.81	40.67	24.29	44.74
	Room T° Day 7	28.43	43.04	24.45	38.04
	Room T° Day 10	30.43	42.36	23.87	41.81
	4°C Day 2	25.86	42.42	25.47	46.97
	4°C Day 7	26.13	43.23	25.19	41.92
	4°C Day 10	20.33	41.8	24.45	41.79

Table 6: Values for verification of stability in treated water

SAMPLE B: Surface Water						
		OFL	TMP	CIP	SMX	ERY
$\bar{x}$		208.96	121.06	179.35	116.21	164.85
σ		83.1	33	62.4	26.7	252.9
$\bar{y}$	Room T° Day 2	234.2	106	211.44	104.48	162.09
	Room T° Day 7	200.81	120.63	156.5	112.07	171.81
	Room T° Day 10	207.27	117.77	168.3	104.59	162.02
	4°C Day 2	234.65	117.94	215.3	112.41	169.14
	4°C Day 7	205.91	123.7	169.1	119.72	184.86
	4°C Day 10	206.37	120.48	168.24	118.14	178.04

The stability of the reference sample is correct if $|\bar{x} - \bar{y}| \leq 0.3\hat{\sigma}$

The results showed that the reference samples can be considered as stable, both at room temperature and at +4°C. The relation mentioned above has been verified in 80% of the measurement.

6. Sample distribution

The reference samples were dispatched on October 5th 2011 by express service.

7. Analytical methods

No standard recommendations have been sent to the participants neither for the sample treatment (extraction, preconcentration) nor for the analytical method.

Table 7 lists the participant laboratories as well as the main characteristics (when sent) of the analytical methods used

Table 7 : Methods used by the different participants

Participant	Pre-treatment	Extraction	Extraction solvent	Internal standard	Chromatography	column
1	before extraction 2.5 mL EDTA solution of 2.5 g/L added (pH8), and pH changed to 3.2 with 100 uL formic acid	SPE Oasis HLB	n.c	Ciprofloxacin 13C3 Sulfamethoxazole 13C6	UHPLC-MS-MS	Acquity BEH 50x2.1 mm; 1.7 um
2	none	SPE Oasis HLB	MeOH/ Ethyl acetate (50/50)	13 C compound	UPLC-MS/MS	Hypersil Gold 1.9µm-2.1*100mm
3	none	SPE reverse phase	n.c	none	LC-MS/MS	Acquity HSS-T3
4	none	SPE ENVI CHROM-P 250mg	MeOH	Carbamazepine D10	HPLC-MS/MS	XBridge C18 3.5 um, 2.1*150 mm Column
5	acidification pH2	SPE JTBaker H2Ophilic	n.c	none	HPLC-MS-MS	Varian Pursuit UPS (2.1 mm x 50 mm x 2,4 um)
6	n.c	n.c	n.c	n.c	HPLC-MS/MS	Zorbax eclipse plus C18
7	n.c	SPE Oasis HLB	acetone/méthanol/acétonitrile	yes	HPLC-MS/MS	Acquity BEH C18, 2.1*150mm, 1.7µm
8	n.c	Polymeric reversed phase	n.c	yes	HPLC-MS/MS	C18
9	n.c	SPE Oasis HLB (pH 7)	MeOH	Yes	HPLC-MS/MS	Agilent ZORBAX eclipse plus C18 (3,5 um

						2,1*100mm)
10	n.c	SPE Oasis HLB	acetonitrile	Carbamazepine D		Polaris
11	cooling + dark	SPE Polymer	n.c	yes	HPLC-MS	C18
12	cooling + dark	SPE Polymer	n.c	yes	HPLC-MS/MS	C18
13	pH 6.95+/- 0.2	SPE water HLB	n.c	13C3-analog	HPLC-MS/MS	Thermo Betasil, 2.1x100m
14	pH 7	SPE Oasis HLB	methanol	13 C compound	RLC-MS/MS	

n.c : not communicated

8. Evaluation procedure

- The statistical evaluation was executed according to ISO 13528 "Statistical methods for use in proficiency testing by interlaboratory comparisons".

For each series the mean value (X), the standard deviation (σ), coefficient of variation (CV), standard error of the mean (σ_M), median (M), the minimum (Min) and maximum (M) values of each series as well as the 95% confidence interval have been calculated or reported.

As an acceptance criteria for each result were used the z-score function according with the "Laboratory Accreditation & Audit Protocol: Food Inspection Directorate"3,4, following the AOAC & ISO & IUPAC directives.

- The Z values where calculated according to the following expression:

$$Z = \frac{X_{lab} - \mathrm{X}}{\sigma}$$

Where:

$X_{lab=}$ result for a laboratory

X = mean values between all laboratories

σ = Standard deviation in the correspondent population (accounting the results obtained for the different laboratories in this series in front of this sample)

The results whose Z value was over 3 was directly excluded and when the Z-score value was between 2 and 3 was applied the Dixon Test with a 5% of Significance Level.

- In addition, laboratory biases (D) were estimated for each results (or average of results) reported by each participants. D corresponds to the difference between the value (or average value) of laboratory (x) with the reference value (X)

$$D = x - X$$

According to ISO 13528, the biases were classified into three categories: $D \geq 3.0\sigma$ indicating an "action signal", $2.0\sigma \leq D < 3.0\sigma$ considered as "warming signal" and $-2.0\sigma \leq D < 2.0\sigma$ indicating "acceptable value". The outlier results were excluded from the calculation of D.

- Distribution of data produced by the participants for each substance was displayed in box plot (a.k.a. tuckey and whisker diagram). This standardized representation is based on five numbers: minimum, first quartile, median, third quartile, and maximum. In the simplest box plot the central rectangle spans the first quartile to the third quartile. A segment inside the rectangle shows the median and "whiskers" above and below the box show the locations of the minimum and maximum values (Figure 2: **General scheme of simple Box plot**).

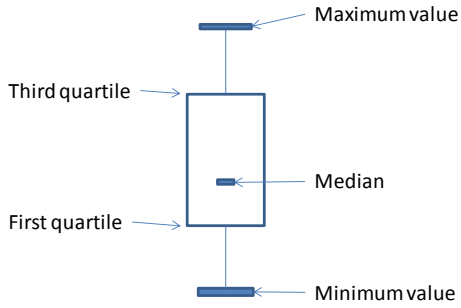


Figure 2: General scheme of simple Box plot

- Degree of variation of the data obtained between the different participants but also obtained in a same laboratory was estimated by the calculation of the coefficient of variation. It is expressed in % and represents the ratio of the standard deviation to the mean.

$$CV = 100 * \frac{\sigma}{\bar{x}}$$

9. Results

A total number of 13 participants took place of this study, by using the methods detailed in Table 7. One participant using two analytical methods, we have considered 14 series of data. Each participant received 3 bottles of sample A (spiked treated water) and 3 bottles of sample B (spiked surface water). Statistical analysis was performed by considering the spiking value as the reference value of the antibiotic of concern (Table 8).

Table 8 : Reference value for each antibiotic in the two matrices

	Sample A : treated water					Sample B : Surface Water				
	ERY	OFL	SMX	TMP		ERY	CIP	OFL	SMX	TMP
Spiking level (ng/L)	55	40	30	50		200	180	200	100	150

The five anibiotics have not been measured by all laboratories. Table 9 shows the repartitions of the antibiotics of interest by laboratories.

Table 9 : Substances and laboratories

Lab / Subst.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ERY														
CIP														
OFL														
SMX														
TMP														

A total number of 611 results were collected (annex 4). The mean values, standard deviations (σ), coefficient of variation, standard error of mean, median, lower and higher values between results from the participant laboratories at different intervals were calculated.

Notice that ciprofloxacin in sample A was determinate only in two laboratories. The number of data is too weak to be considered in the statistical analysis. Consequently, CIP in sample B (surface water) have been only investigated.

9.1. z score

z score values were used to calculate the outliers within the results. The calculation gave 24 outliers (3.9% of the total number of results, see Table 10). The outliers were not distributed evenly across all labs. Only 4 labs produced outliers and only on one substance.

Figure 3 presents the absolute values of z score after exclusion of the outliers. According to the difference of the analytical protocols used, the number of outliers obtained in this exercise can be considered low. The sample matrix with the higher number of outlier was treated water.

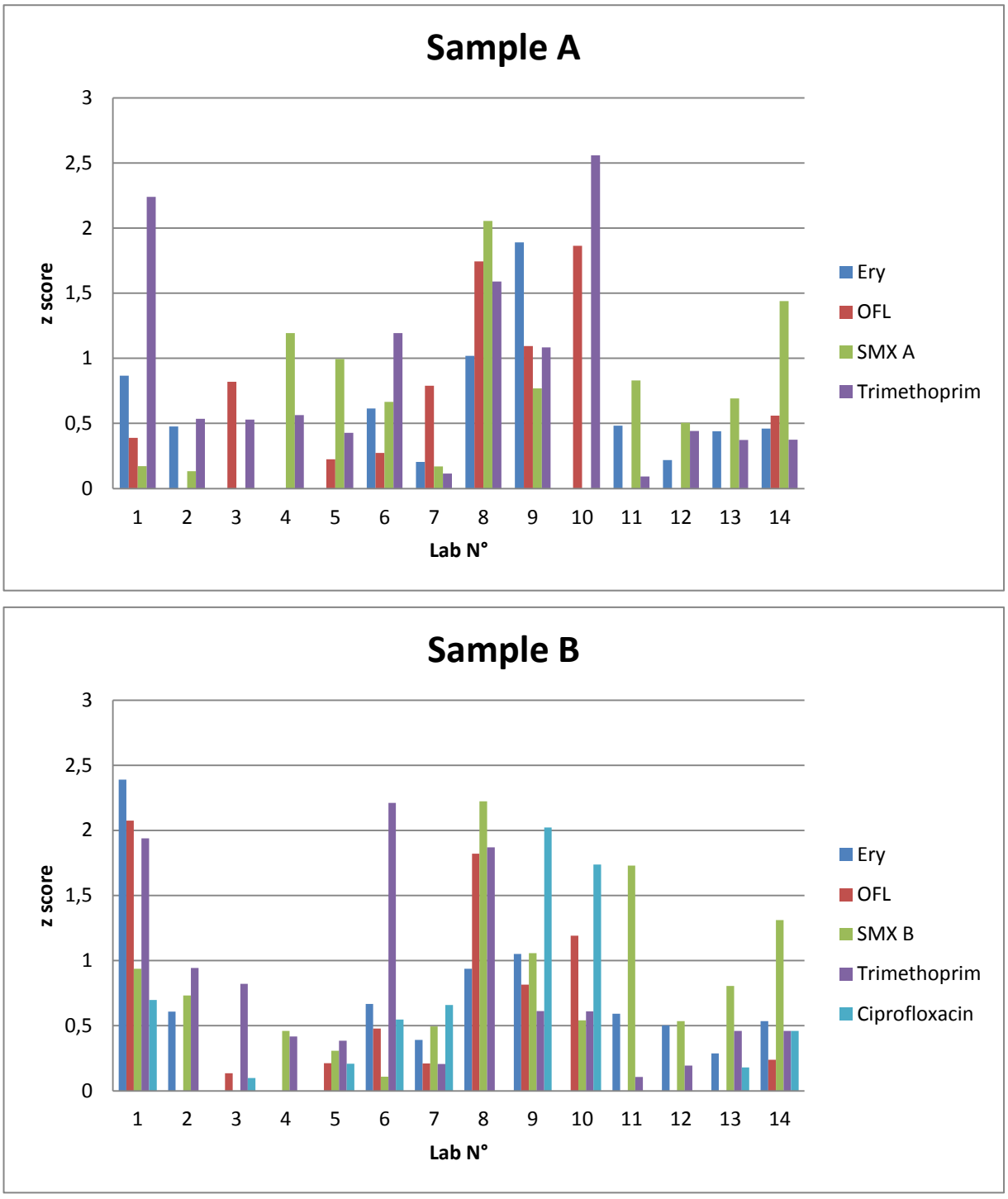


Figure 3 : z score absolute values

The outlier values were excluded for the final data treatment and the statistical parameters (mean values, standard deviation, variance, coefficient of variation) were calculated. Table 10 shows the corrected statistical values (after outlier exclusion) obtained for each compound in the different samples.

Table 10 : Statistical values corrected after outlier exclusion for each compound in the different types of water: mean (x), standard deviation (σ), coefficient of variation (CV), standard error of mean (σ_M), median (M), minimum value (Min), maximum value (Max).

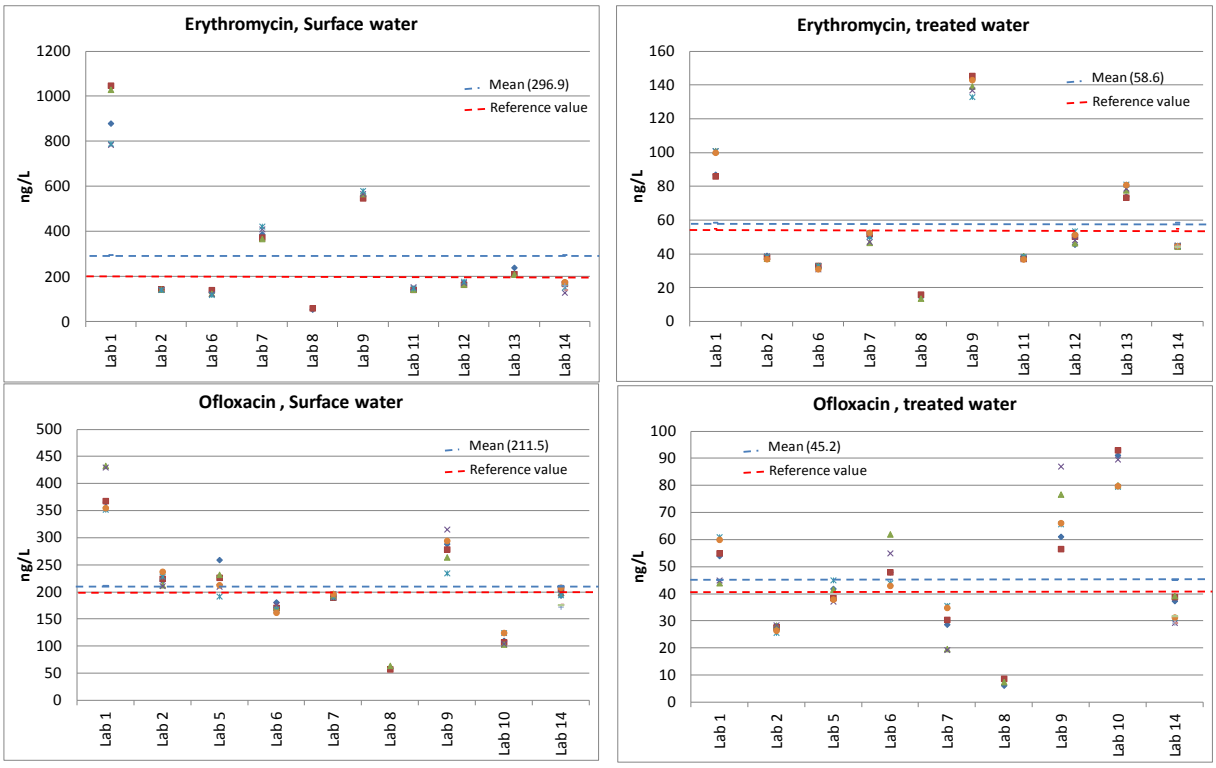
Substance *Matrix*	**Spiking level (ng/L)**	**Nbr of accepted results**	**x (ng/L)**	**σ (ng/L)**	**CV**	**σ_M (ng/L)**	**M (ng/L)**	**Min (ng/L)**	**Max (ng/L)**	**95% confidence interval**		**Nbr of outlier**
										from	**to**	
Cyprofloxacin												
Surface Water (B)	180	55	137.96	62.44	0.45	8.42	135.03	22.59	298.54	121.12	154.80	0
Erythromycin												
Treated water (A)	55	60	58.64	34.66	0.59	4.47	45.73	13.66	145.45	49.70	67.59	6
Surface water (B)	200	58	296.87	252.89	0.85	33.21	170.47	54.74	1048	230.46	363.28	6
Ofloxacin												
Treated water (A)	40	54	45.20	21.64	0.48	2.95	40.43	6.19	93.05	39.31	51.09	0
Surface Water (B)	200	54	211.51	83.12	0.39	11.31	197.78	57.49	433.0	188.89	234.14	0
Sulfamethoxazole												
Treated water (A)	30	70	20.0	5.67	0.28	0.68	19.83	5.64	30.51	18.64	21.35	6
Surface water (B)	100	76	87.06	26.73	0.31	3.07	89.28	26.86	127.07	80.96	93.16	0
Trimetoprim												
Treated water (A)	50	80	39.91	12.77	0.32	1.43	39.43	18.86	74	37.05	42.76	4
Surface water (B)	150	80	105.79	32.98	0.31	3.69	110.70	30	178	98.41	113.16	2
Total		**587**										**24**

9.2. Results by molecules and matrices

Results for the different samples expressed in ng/L, mean and reference values are plotted in Figure 4.

For all molecules, the tendency is the same in the two matrices concerning the comparison of the mean of all values and the reference value. Two behaviors can be distinguished: ERY and OFL show higher mean than the reference value; SMW, TMP and CIP show the contrary.

The deviation between the two values (calculated by the ration between the observed mean concentration and the reference one) is important for ERY in surface water (148%) and SMX in treated water (66%). It corresponds to 106%, 88% 71%, 77% in surface water for OFL, SMX, TMP, CIP respectively and to 106%, 113%, 80% in treated water for ERY, OFL, TMP. This difference can be considered as representative in the case of SMX in treated water and TMP in surface water because the value is higher than the standard deviation (Table 10).



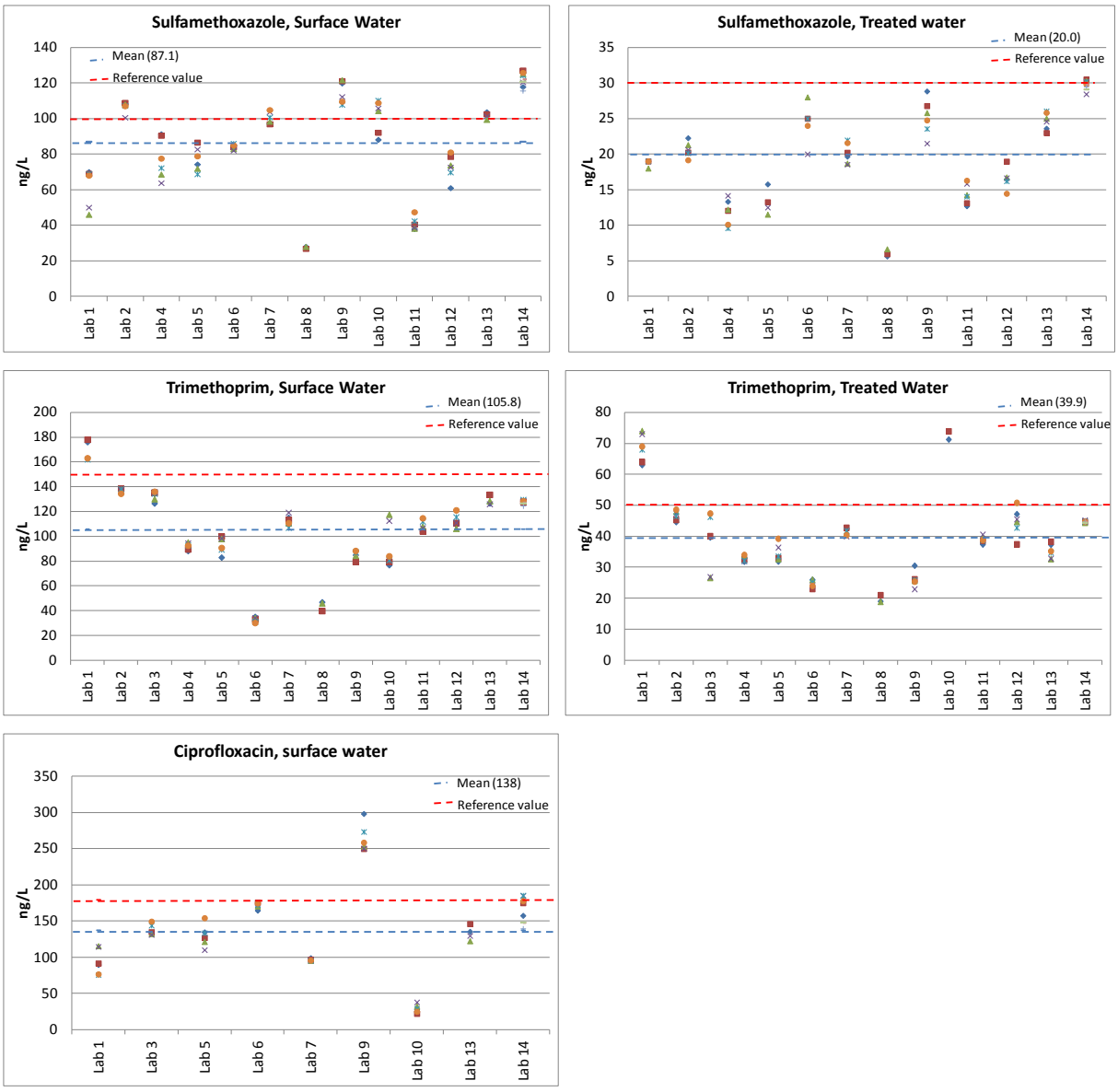


Figure 4: Results obtained for each participant for Erythromycin, Ofloxacin, Sulfamethoxazole, Trimethoprim and Ciprofloxacin expressed in ng/Lin the different samples, and mean values of results.

These data can be represented by using Tuckey diagram representing the different characteristics of the values for each molecule in the two matrices (Figure 5) illustrating the distribution of the data.

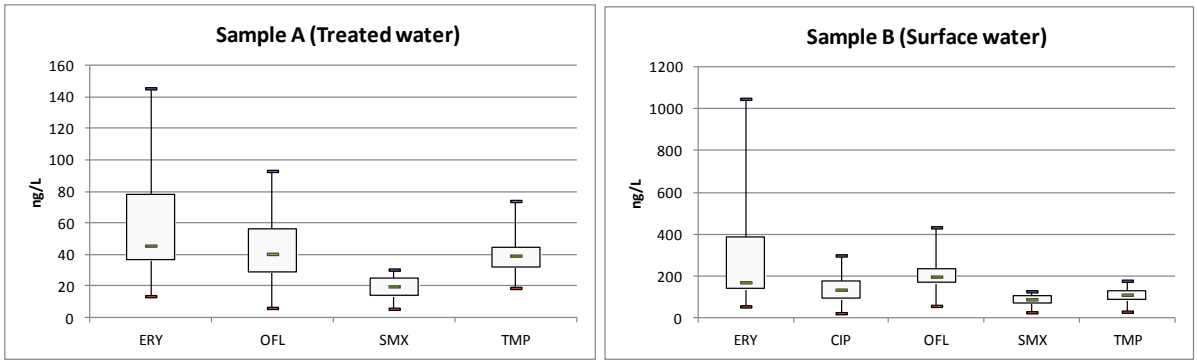


Figure 5 : Tuckey diagram for the repartition of the value for treated water and surface water

9.3. Coefficients of variation

A comparison of the CV values (Figure 6) in both matrices (surface and treated water) for all laboratories resulted in a weak difference. The highest CV was observed for erythromycin in both matrices. It is particularly high in surface water (highest concentration).

The lowest CV was observed for sulfamethoxazole and trimethoprim and is similar in the two matrices in spite of a difference in the spiked concentration.

According to the heterogeneity of the methods employed we can consider the CV relatively low for the targets except for erythromycin

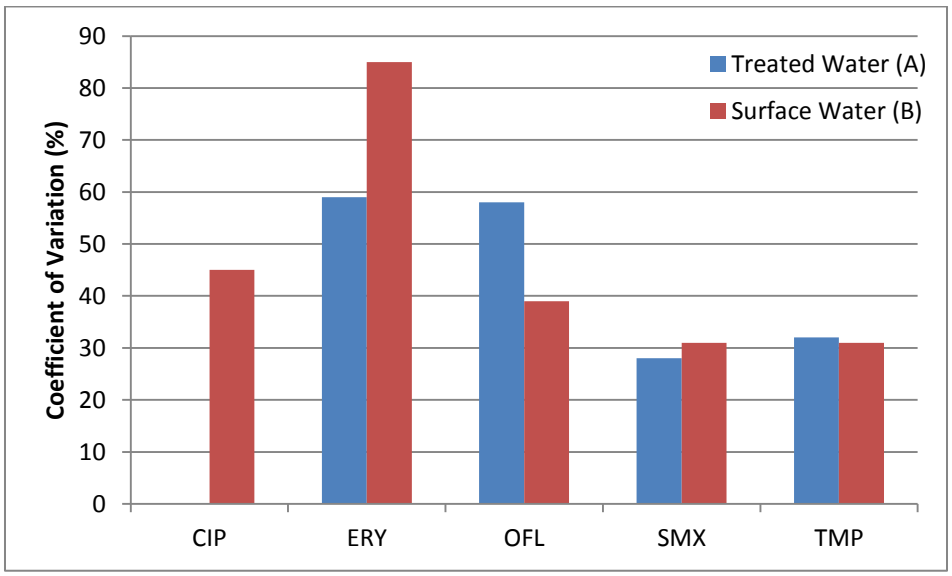


Figure 6 : Coefficient of variation for all laboratories

Several laboratories have used the same methodology in particular, same extraction cartridges (Oasis HLB) and chromatography techniques (HPLC/MS/MS). CV between such laboratories has been calculated (Figure 7). Except for ciprofloxacin in surface water, and trimethoprim in

treated water, CV is lower than considering all laboratories, showing an influence of the analytical procedure in the representativity of the results.

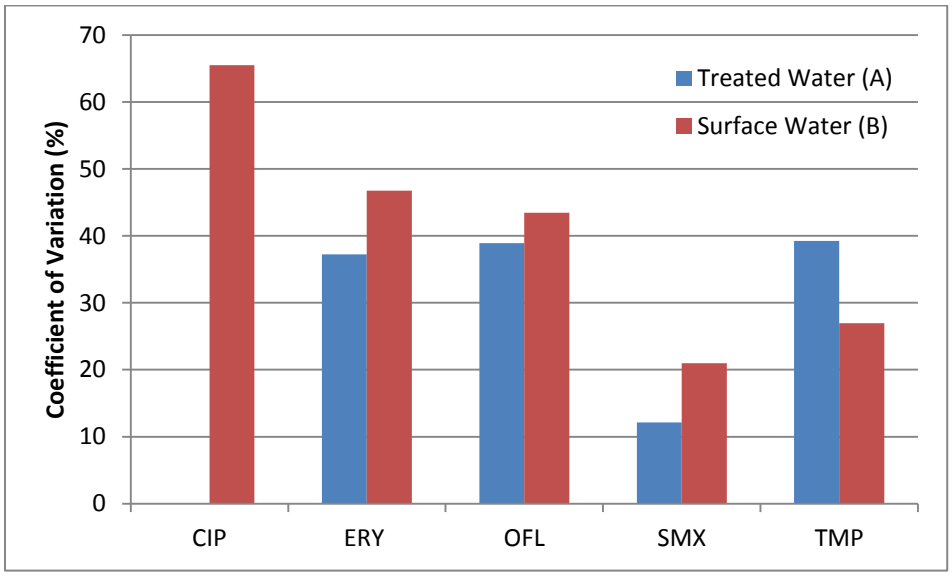


Figure 7: CV of laboratories using oasis HLB and HPLC/MS/MS as extraction cartridge and chromatography techniques respectively

9.4. Laboratory performances

Intralaboratory repeatability has been evaluated for each substance in the two matrices and for each laboratory by the calculation of the coefficient of variation (Figure 8 and Figure 9).

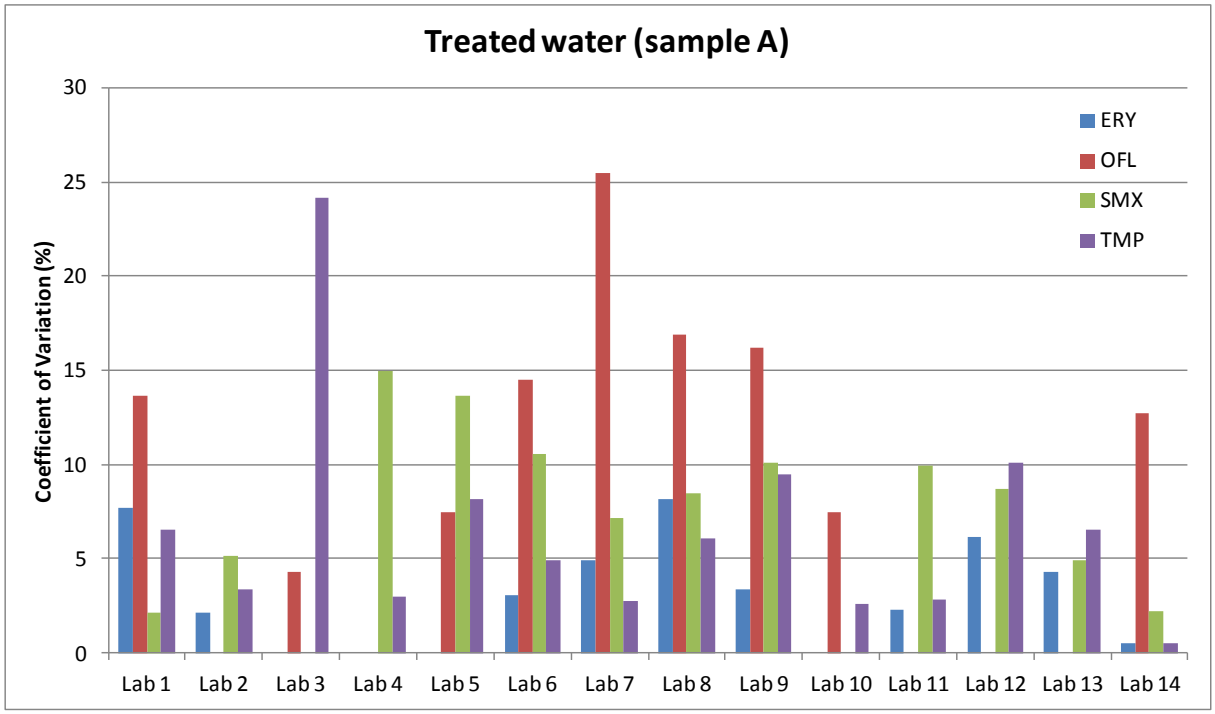


Figure 8: Intralaboratory repetability in sample A (tap water)

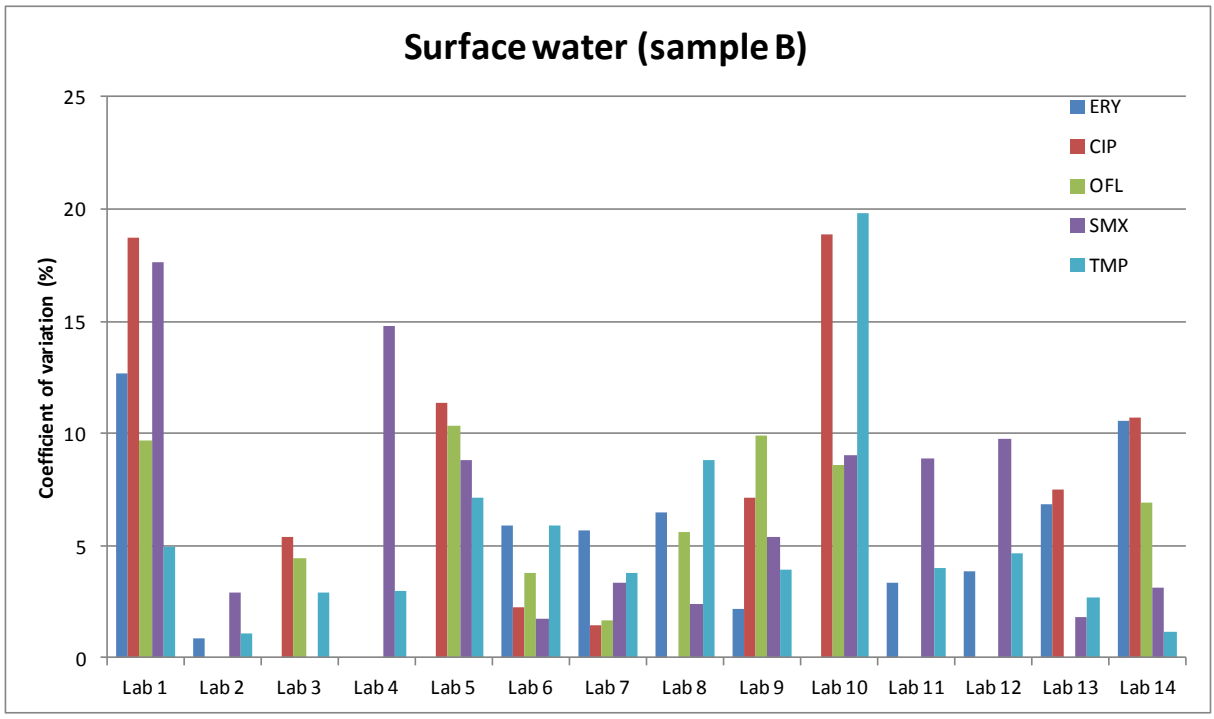


Figure 9: Intralaboratory repetability in sample B (surface water)

Good intralaboratory reproducibility has been observed. CV varies from 0.5 to 25.5 % (median 6.6%) and to 0.8 to 19.8 % (median 5.6%) for sample A and B respectively. This result illustrates the important capability of the different laboratories to produce representative data.

On the second hand, the difference between the produced values by each laboratory and the reference value was estimated by the calculation of the bias.
By considering all values, the ISO 13528 classification of the laboratory biases resulted in 85% of acceptable values, 11% of value to be survey and 5% of not acceptable values

Furthermore, the average performance of the laboratories can be divided up the following (Table 11). The detail of the calculation is reported in annex 5.

- 43% of the laboratories have the whole result considered as acceptable
- 36% of the laboratories have at minimum one value to be survey
- 14% of the laboratories have at minimum one value not acceptable
- 7 % of the laboratories have at minimum one value to be survey and one value not acceptable

Table 11 : Laboratory biases for each values reported by the participant laboratories in the two matrices (surface (SW) and treated (TW) water). 0, 1 and 2 correspond to acceptable values, warming signal and action signal respectively (grey boxes correspond to non produced data)

	ERY		CIP.	OFL		TMP		SMX	
	TW	SW	SW	TW	SW	TW	SW	TW	SW
Lab 1	0	1	0	0	1	0	0	0	0
Lab 2	0	0				0	0	0	0
Lab 3			0	0	0	0	0		
Lab 4						0	0	2	0
Lab 5		0	0	0	0	0	0	1	0
Lab 6	0	0	0	0	0	0	2	2	0
Lab 7	0	0	0	0	0	0	0	0	0
Lab 8	0	0	0	0	1	1	2	2	1
Lab 9	0	0	0	0	0	0	0	0	0
Lab 10			1	1	0	0	0		0
Lab 11	0	0				0	0	1	1
Lab 12	0	0				0	0	1	0
Lab 13	0		0			0	0	0	0
Lab 14	0	0	0	0	0	0	0	0	0

10. Conclusion

Thirteen participants from five different countries took part in the interlaboratory exercise organized in the frame of Pharmas Project. 77 testing samples were analyzed to determine concentration of selected antibiotics and 611 results (including parallels, excluding <LOD) were collected for the data evaluation. The final number of 587 values was pooled out for further data analysis, where 24 of them (3.9 %) were determined as outliers according to classical approach (z score).

The sample matrix yielding the highest number of outliers was, treated water (66%)

According to the scheme of the ILE (no recommendation of the analytical procedure), the global coefficients of variation between the participants are relatively low except for erythromycin in the two matrices and ofloxacin in treated water.

The intralaboratory coefficients of variation (repeatability) show better result for each lab for the two matrices (below than 20%).

The estimation of the laboratory biases (D) showed 5 results outside the range $-3.0\ \sigma < D < 3.0\ \sigma$ ("action signals"), while 11 were "warning signals", falling outside the range $-2.0\ \sigma < D < 2.0\ \sigma$.

Between the 14 participating laboratories 6 laboratories showed an excellent performance (5 out of 6 using internal standard), never reaching the range outside $-2.0\ \sigma < D < 2.0\ \sigma$, 2 laboratories with only one warming signal. Only three laboratories showed action signals (2 out of 3 using internal standard).

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Annex IV – Treatment of a pesticide-containing wastewater using combined biological and solar-driven AOPs at pilot scale

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Treatment of a pesticide-containing wastewater using combined biological and solar-driven AOPs at pilot scale

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HIGHLIGHTS

- 19 Pesticides were quantified in a wastewater representing 14–19% of total DOC.
- Preliminary biological oxidation (BO) resulted in 41–56% reduction of DOC.
- No significant decrease of pesticides content was observed after the BO.
- Pesticides degradation was achieved using a solar photo-Fenton (SPF) reaction.
- The amount of energy needed for pesticides degradation using a SPF was 8 $kJ_{UV}\,L^{-1}$.

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ABSTRACT

The present work focuses on treatment of a pesticide-containing wastewater resulting from phytopharmaceutical plastic containers washing, combining a preliminary biological pre-treatment step, using an immobilized biomass reactor (IBR), with further advanced oxidation processes (AOPs). Heterogeneous (TiO_2/UV and TiO_2/H_2O_2/UV, both with and without acidification) and homogeneous (UV, H_2O_2/UV, Fe^{2+}/H_2O_2/UV and Fe^{2+}/H_2O_2) systems were tested using a solar pilot plant with compound parabolic collectors (CPCs). The wastewater exhibited a moderate organic load (COD = 1662–1960 mg $O_2\,L^{-1}$; DOC = 513–696 mg $C\,L^{-1}$), high biodegradability (BOD_5 = 1350–1600 mg $O_2\,L^{-1}$) and nineteen pesticides were quantified in the range of 0.02–45 mg L^{-1}, representing 14–19% of total DOC. Due to its high biodegradability, a biological treatment was performed prior to AOPs, leading to a COD, DOC and BOD_5 reduction of 46–54%, 41–56% and 88–90% respectively, resulting in a recalcitrant wastewater with a residual pesticide content corresponding to 24–34% of DOC. The photo-Fenton reaction, performed with an initial iron concentration of 140 mg $Fe^{2+}\,L^{-1}$, leading to an average dissolved iron concentration of 14 mg L^{-1} after $FePO_4$ precipitation, proved to be the most efficient process, showing an initial reaction rate 8.4, 8.7 and 5.1 times higher than for H_2O_2/UV, TiO_2/H_2O_2/UV-without and with acidification systems, respectively. The reaction required 167 mM of H_2O_2 and 21 $kJ_{UV}\,L^{-1}$ to achieve 86% mineralization and only 8 $kJ_{UV}\,L^{-1}$ to eliminate eighteen of the nineteen pesticides initially quantified to levels below the respective quantification limit. Despite the Fenton reaction revealed a slower mineralization profile, it can be quite efficient for significant pesticide abatement compared to the other AOPs employed.

1. Introduction

One of the main current worldwide concerns is the growth of surface and groundwater pollution. In particular, water contamination by pesticides is a severe problem. The risk inherent to pesticide pollution is prominent due to their generally high solubility in water, low-sorption affinity to soils, toxicity, chemical stability, bioaccumulation and low biodegradability [1]. The major sources of pollution by pesticides are drainage waters from intensive agriculture, including water from washing pesticides containers and application equipment, and effluents from agricultural industries and pesticide manufacturing plants [2,3].

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EU legislation is being continually updated to protect and improve water quality, as is the case of Water Framework Directive (WFD), which establishes the guidelines for community action in the field of water policy [4]. A list of 33 Priority Substances, which represent a significant risk to or via the aquatic environment, including pesticides (alachlor, atrazine, chorfenvinphos, chlopyrifos, diuron, endosulfan, isoproturon, hexachlorocyclohexane, pentachorophenol, simazine and trifluraline) was later defined by the European Commission [5].

Among the different approaches used to achieve pesticide elimination, and considering their bio-recalcitrant character, advanced oxidation processes (AOPs) have been recognized as especially efficient when compared to conventional technologies [6–8]. AOPs are chemical oxidation processes characterized by the production of extremely reactive and unselective oxidants, mostly hydroxyl radicals ($HO^{\bullet}$). They are able to oxidize and mineralize almost any organic compound, even bio-recalcitrant ones. AOPs' versatility is enhanced by the fact that there are many different ways of producing $HO^{\bullet}$. Heterogeneous photocatalysis using titanium dioxide (TiO_2) and solar UV, possibly combined with hydrogen peroxide (H_2O_2), and homogeneous processes such as Fenton (Fe^{2+}/H_2O_2) and photo-Fenton ($Fe^{2+}/H_2O_2/UV$) reactions have proved to be useful tools for the treatment of pesticide-containing wastewaters. The photo-Fenton process was the most efficient one [3]. AOPs' main disadvantage is their high cost associated with energy production and reactants consumption, which can be substantially reduced if solar-driven AOPs are used. In addition, combination of AOPs with a low-cost biological pre-treatment, capable of reducing from the outset the biodegradable fraction, turns the overall process less expensive and, therefore, more economically attractive. In previous studies, AOPs were usually applied as a preliminary step to enhance the biodegradability of pesticide-containing wastewaters before the biological treatment [2,9–13]. However, these studies dealt mainly with individual pesticide solutions [14–18] and simulated mixtures of some pesticides [2,12,13,19–23], thus disregarding all other compounds potentially present in such a complex matrix as the real wastewater effluent.

A prominent example of AOPs application to the treatment of a real pesticide-contaminated wastewater is the one performed since 2004 at ALBAIDA *Recursos Naturales y Medio Ambiente* S.A, in Spain. This company selectively collects empty plastic pesticide containers from greenhouses in El Ejido, Almeria (southeastern Spain) for recycling. The containers are shredded and washed, producing rinse water contaminated with pesticides, with an initial Chemical Oxygen Demand (COD) ranging between 200 and 500 mg L^{-1}. In this effluent, it was also possible to detect and quantify twelve pesticides, with the highest individual concentration of about 1000 μg L^{-1}. The treatment strategy adopted was a combination of a solar photo-Fenton, performed in a photocatalytic reactor based on solar compound parabolic collectors (CPCs), with a further biological treatment, which consisted basically of two immobilized biomass reactors (IBRs) [24,25].

In Portugal, management of empty phytopharmaceutical plastic containers, resulting from agriculture, is accomplished by VALORFITO, designation by which is known the Integrated Management System for Agricultural Packaging and Residues (http://www.valorfito.com/). This company ensures the containers periodic collection and provides an appropriate final destination (treatment/recycling/energy recovery), according to current legislation. This collection focus only on primary containers of plant protection products, i.e., containers that are in direct contact with plant protection products, classified as hazardous waste. In 2010, there were 416 operational reception spots and around 221 tons of containers were collected. The current plastic containers treatment is managed by EGEO – *Tecnologia e Ambiente* S.A. (http://www.egeo.pt/) and performed in EGEO-SISAV-CIRVER facilities, where the containers are screened, shredded into small pieces, granulated and washed using a steam system. The washed plastics are recycled and the hazardous sludge (constituted by the paper from the labels, pesticides and other compounds related to the composition of the commercial product) is disposed in a landfill.

This study focuses on the development of an alternative solution for phytopharmaceutical plastic containers recycling, in order to avoid the production of hazardous sludge and further disposal in landfills. The strategy adopted includes: (i) a preliminary plastic containers washing with tap water, resulting in a wastewater contaminated with different pesticides; (ii) biological oxidation of the resulted pesticide-containing wastewater, using an IBR, in order to eliminate the biodegradable organic carbon fraction; (iii) degradation of the recalcitrant pesticides using different solar AOPs in heterogeneous (TiO_2/UV or TiO_2/H_2O_2/UV, both with and without acidification) or homogeneous (UV, H_2O_2/UV, Fe^{2+}/H_2O_2/UV or Fe^{2+}/H_2O_2) phases, at pilot plant scale with CPCs.

2. Experimental methodology

2.1. Chemicals

AOPs were performed employing titanium dioxide (Degussa, P25, 80% anatase and 20% rutile), hydrogen peroxide (50% (w/v), 1.10 g cm^{-3}, Quimitécnica, S.A.), iron sulfate heptahydrate (Panreac) and sulphuric acid (96%, 1.84 g cm^{-3}, Pronalab) for pH adjustment. Photo-treated wastewater was neutralized with commercial grade sodium hydroxide (30% (w/v), 1.33 g cm^{-3}, Quimitécnica, S.A.). pH control in the biological reactor was performed with sodium hydroxide and sulphuric acid.

Ultrapure and pure water for analyses was obtained using a Millipore® system (Direct-Q model) and a reverse osmosis system (Panice®), respectively.

2.2. Analytical determinations

Evaluation of hydrogen peroxide concentration during experiments was performed by the metavanadate method, based on reaction of hydrogen peroxide with ammonium metavanadate in acidic medium, which results in the formation of the red–orange color peroxovanadium cation, with maximum absorbance at 450 nm [26]. pH and temperature were measured using a pH meter (HANNA HI8424). Conductivity was determined by a conductivity meter (WTW LF538). Dissolved oxygen was measured by means of a dissolved oxygen meter (CrisonOxi 45). Turbidity was measured with a turbidimeter (Merck Turbiquant 3000 IR). COD concentration was measured by Merck® Spectroquant kits (ref: 1.14541.0001). Dissolved organic carbon (DOC) was measured in a TC-TOC-TN analyzer equipped with ASI-V autosampler (Shimadzu, model TOC-V_{CSN}), as CO_2, using a NDIR detector calibrated with standard solutions of potassium hydrogen phthalate (total carbon) and a mixture of sodium hydrogen carbonate/sodium carbonate, anhydrous (inorganic carbon). Total dissolved nitrogen was measured in the same TC-TOC-TN analyzer coupled with a TNM-1 unit (Shimadzu, model TOC-V_{CSN}), calibrated with standard solutions of KNO_3, by thermal decomposition and NO detection by chemiluminescence. Biochemical oxygen demand (BOD_5) was determined according to OECD-301F test described in Standard Methods Manual using an OXITOP® system (manometric respirometry) [27]. Total polyphenols concentration was measured by spectrophotometry at 765 nm, using Folin–Ciocalteau reagent (Merck) [28]. Polyphenols content is expressed as mg L^{-1} of caffeic acid. Total dissolved iron concentration was determined by colorimetry with 1,10-phenanthroline, according to ISO standard 6332. Nitrite, nitrate, sulfate, phosphate, fluoride, chloride and bromide were quantified by ion

chromatography (Dionex ICS-2100; column AS 11-HC 4 × 250 mm; suppressor ASRS®300 4 mm). Ammonium, lithium, sodium, potassium, magnesium and calcium were analyzed by ion cromatography (Dionex DX-120; column: CS12A 4 × 250 mm; suppressor: CSRS®300 4 mm). Isocratic elution was done with 30 mM NaOH/20 mM methane sulfonic acid, at a flow rate of 1.5/1.0 mL min^{-1}, for anions/cations analyses, respectively. Total suspended solids (TSS) and volatile suspended solids (VSS) were measured by gravimetry, according to Standard Methods Manual [27]. UV–Vis spectrum between 200 and 700 nm, absorbance at 450 nm (metavanadate method) and 510 nm (phenanthroline method) were obtained using a UNICAM Helios α spectrophotometer. All samples were pre-filtered through 0.45 μm Nylon, VWR membrane filters before analysis. Quantitative analysis of pesticides was performed by LC-MS/MS. Samples were first diluted (dilution factor of 1/20) and subsequently injected in the HPLC (Waters Alliance HT 2795; analytical column Waters Atlantis dC18, 3 μm, 2.1 × 100 mm), coupled to a mass spectrometer (Micromass Quattro micro). The mass detector was operated in MRM mode (Multiple Reaction Monitoring), after thorough selection of the specific transitions for each pesticide, according to the procedure described by Carvalho et al. [29]. The final optimized method allowed the concurrent detection of 31 pesticides and degradation products, during chromatographic runs 20 min long.

2.3. Experimental set-up

2.3.1. Biological oxidation system

The biological oxidation system is composed by a conditioner flat-bottom tank (50 L) and an IBR (45 L). The conditioner tank is equipped with a pH control unit (CRISON, electrode and PH27P controller) and a mechanical stirrer (TIMSA) for pH adjustment, using either H_2SO_4 or NaOH dosed by means of two metering pumps (DOSAPRO MILTON ROY, GTM series, model A). The IBR is a flat-bottom container packed with 62 units of propylene rings (nominal diameter 50 mm), colonized by activated sludge from a municipal wastewater treatment plant (Freixo WWTP). The bioreactor is also equipped with a dissolved oxygen control unit (CRISON, electrode and OXI49P controller) and air is supplied by a blower (compressor-HAILEA model V-20; air flow rate = 20 - L min^{-1}; ceramic air diffuser) for maintaining dissolved oxygen concentration in the system in the selected range (0.5–2 mg O_2 L^{-1}).

2.3.2. Solar CPC pilot plant

Heterogeneous (TiO_2/UV, TiO_2/H_2O_2/UV) and homogeneous (UV, H_2O_2/UV, Fe^{2+}/H_2O_2/UV, Fe^{2+}/H_2O_2) experiments were conducted under sunlight in a pilot plant constituted by CPCs, installed at the roof of the Chemical Engineering Department, Faculty of Engineering, University of Porto (FEUP), Portugal. The plant has a total irradiation area of 0.91 m^2, two recirculation tanks (10 and 20 L), two recirculation pumps (20 L min^{-1}), two flow meters, five polypropylene valves, connecting polypropylene tubing and an electric board for process control [30]. The solar collectors are made-up of four borosilicate tubes (Schott-Duran type 3.3, Germany, cut-off at 280 nm, internal diameter 46.4 mm, length 1500 mm and thickness 1.8 mm) connected by polypropylene junctions. The pilot plant can be operated in two batch ways: using the total CPCs area (0.91 m^2) or using 0.455 m^2 of CPCs area individually, giving the possibility of performing two different experiments at the same time and under

Table 1
Physical/chemical characteristics of the wastewater resulting from the phytopharmaceutical plastic containers washing and after the biological treatment.

Parameter	1st Washing	1st BT[a]	2nd Washing	2nd BT[a]	3rd Washing	3rd BT[a]	BT Mixture[b]	ELV [DL n.° 236/98]
pH	7.2	7.8	6.7	7.7	7	7.4	7.6	6.0–9.0
Conductivity (μS cm^{-1})	739	924	786	905	781	845	926	–
Dissolved oxygen (mg L^{-1})	3.0	6.7	3.8	7.2	3.2	7.1	7.2	–
Turbidity (NTU)	188	42	44	59	43	51	48	–
COD (mg O_2 L^{-1})	1662	818	1960	1094	1932	1136	892	150
DOC (mg C L^{-1})	513	217	665	298	696	335	303	–
BOD_5 (mg O_2 L^{-1})	1350	220	1550	100	1600	140	160	40
BOD_5/COD	0.81	0.27	0.79	0.09	0.83	0.12	0.18	–
Polyphenols (mg caffeic acid L^{-1})	16	22	24	6	22	18	13	–
Nitrite (mg NO_2^- L^{-1}/mg N–NO_2^- L^{-1})	18/5.4	6.5/2.0	34/10	7.7/2.3	5.3/1.6	7.6/2.3	7.9/2.4	–
Nitrate (mg NO_3^- L^{-1}/mg N–NO_3^- L^{-1})	32/7.2	15/3.3	<0.01/<0.002	<0.01/<0.002	<0.01/<0.002	<0.01/<0.002	<0.01/<0.002	50/11
Ammonium (mg NH_4^+ L^{-1}/mg N–NH_4^+ L^{-1})	39/30	91/71	41/32	111/86	33/26	88/68	106/83	–
Total dissolved nitrogen (mg N L^{-1})	118	99	144	107	132	111	106	15
Dissolved iron (mg (Fe^{2+} + Fe^{3+}) L^{-1})	2.0	<1	2.5	<1	2.3	1.1	1.3	2
Sulfate (mg SO_4^{2-} L^{-1})	68	93	93	121	107	124	134	2000
Phosphate (mg PO_4^{3-} L^{-1})	7.5	133	21	46	23	27	81	–
Fluoride (mg F^- L^{-1})	0.8	0.9	1.9	1.7	1.1	2	1.7	–
Chloride (mg Cl^- L^{-1})	58	73	77	83	92	86	97	–
Bromide (mg Br^- L^{-1})	1.2	1.4	3.2	2.7	5.4	3.1	2.9	–
Lithium (mg Li^+ L^{-1})	0.1	0.2	0.2	0.3	0.3	0.3	0.3	–
Sodium (mg Na^+ L^{-1})	82	100	80	82	84	79	87	–
Potassium (mg K^+ L^{-1})	37	85	34	61	36	55	66	–
Magnesium (mg Mg^{2+} L^{-1})	18	40	34	37	39	40	40	–
Calcium (mg Ca^{2+} L^{-1})	112	91	128	114	126	120	110	–
TSS (mg L^{-1})	82	np	62	np	66	np	126	60
VSS (mg L^{-1})	62	np	52	np	58	np	104	–

ELV – Emission limit values.
np – Analysis not performed.
[*] BT, effluent after biological treatment.
[**] BT Mixture, effluent resulting from three independent replicates biological treatment batches.

the same solar radiations conditions. The plant is mounted on a fixed platform tilted 41° (local latitude), with south orientation. The intensity of solar UV radiation is measured by a global UV radiometer (ACADUS 85-PLS), mounted on the pilot plant at the same angle, which provides data in terms of instantaneous radiation (W_{UV} m^{-2}). Eq. (1) allows to calculate the amount of accumulated UV energy ($Q_{UV,n}$, kJ L^{-1}) received on any surface in the same position with regard to the sun in a time interval Δt per unit of volume of water inside the reactor:

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

where t_n is the time corresponding to n-water sample, V_t is the total effluent volume, A_r is the illuminated collector surface area and $\overline{UV}_{G,n}$ is the average solar ultraviolet radiation measured during the period Δt_n. All experiments were conducted from August to November 2011, during sunny and cloudy days.

2.4. Experimental procedure

2.4.1. Pesticides-containing wastewater

Phytopharmaceutical plastic containers from VALORFITO, after being granulated into small pieces in EGEO-SISAV-CIRVER facilities, were washed with tap water in a proportion of 0.15 kg of plastics per liter of water. Three washing batches were performed. The main physical/chemical characteristics and the pesticide content of these three washing batches are summarized in Tables 1 and 2, respectively.

2.4.2. Biological oxidation

The biological reactor was inoculated with 80 L of wastewater collected at an aerated activated sludge reactor from Freixo WWTP. After 5 days of recirculation in batch mode at 250 L h^{-1}, biomass was completely fixed on supports, as confirmed by volatile suspended solids (VSS) analysis. The remaining wastewater at the biological reactor (conditioner tank + IBR) was discharged and a volume of 75–86 L of pesticide-containing wastewater was added to the conditioner tank and then pumped to the IBR, which operates as an up-flow reactor at a recirculation rate of 250 L h^{-1} (EHEIM pump) between conditioner tank and IBR. The pH and dissolved oxygen concentration was controlled in a range of 6.5–7.5 and 0.5–2 mg O_2 L^{-1}, respectively. Three biological pre-treatment batches were performed and mixed in order to obtain the volume of wastewater necessary for all AOPs post-treatment experiments. The main physical/chemical characteristics and pesticide content of each of the three bio-treated wastewaters and resulting mixture are summarized in Tables 1 and 2, respectively.

2.4.3. Solar AOPs

A volume of 16 L of wastewater resulting from biological treatment was added to the recirculation tank of the CPC units (illuminated volume = 5.1 L; 0.455 m^2) and homogenized by turbulent

Table 2
Pesticide content of the wastewater resulting from the phytopharmaceutical plastic containers washing and after the biological treatment.

Pesticides	1st Washing (μg L^{-1})	1st BT[a] (μg L^{-1})	2nd Washing (μg L^{-1})	2nd BT[a] (μg L^{-1})	3rd Washing (μg L^{-1})	3rd BT[a] (μg L^{-1})	BT Mixture[b] (μg L^{-1})	LOQ (μg L^{-1})	LOD (μg L^{-1})
S-Metolachlor	40652	25686	45328	33127	41754	32685	32888	50	15
2,4-D[c]	33552	33778	43645	42171	38898	39468	36785	660	200
MCPA[d]	29052	26018	37398	39944	38752	35601	33596	330	100
Imidacloprid	14837	15083	17166	15627	17119	14457	15676	205	62
Alachlor	8741	5120	10841	7391	12328	8836	7209	80	24
Terbuthylazine	8509	5968	10218	7982	9411	7065	8093	30	9
Isoproturon	7423	6289	13670	12626	15577	12950	11864	50	15
Bentazone	6500	7350	9320	10090	12300	10660	9780	45	14
Tebuconazole	5927	4311	6104	4694	5801	4744	4636	50	15
Atrazine	5330	4430	12240	9200	13960	11940	10110	165	50
Linuron	1995	1117	2305	1260	2206	1772	1580	220	67
Metobromuron	1302	1119	1610	1713	1416	1565	1458	115	35
Dimethoate	1200	1277	1906	1622	1980	1935	1459	130	39
Diuron	804	729	1741	1211	1733	1270	1185	90	27
Metribuzin	930	826	923	883	738	696	702	45	14
Metalaxyl	428	361	466	448	624	653	484	45	14
Chlorotoluron	319	357	1462	1337	2525	1824	1189	130	39
Simazine	245	255	581	542	684	584	387	70	21
Terbuthylazine–desethyl	20	19	22	29	30	23	19	20	6
Benalaxyl	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	35	11
Carbaryl	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	55	17
Carbofuran	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	25	8
Cymoxanil	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	250	76
Deisopropylatrazine	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	225	68
Desethylatrazine	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	245	74
Methidathion	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	165	50
Propanil	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	190	58
Propazine	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	35	11
Propylenethiourea	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	1400	424
Pyrimethanil	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	185	56
Triclopyr	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	3330	1009

LOQ – Limit of quantification.
LOD – Limit of detection.
[a] BT, effluent after biological treatment.
[b] BT Mixture, effluent resulting from three independent replicates biological treatment batches; [1]2,4-D - (2,4-dichlorophenoxy)acetic acid; [2]MCPA – (4-chloro-2-methylphenoxy)acetic acid.
[c] 2,4-D – (2,4-dichlorophenoxy)acetic acid.
[d] MCPA – (4-chloro-2-methylphenoxy)acetic acid.

recirculation, during 15 min, in darkness (a first control sample was taken for further characterization).

For heterogeneous photocatalytic tests (TiO_2/UV and TiO_2/H_2O_2/UV), after taking the first sample, titanium dioxide was added up to a concentration of 200 mg L^{-1} and the mixture recirculated for more than 15 min. For TiO_2/UV tests, a second sample was collected just before uncovering the CPC units, in order to evaluate the pollutants adsorption onto the catalyst surface. In the case of TiO_2/H_2O_2/UV experiments, a second sample was as well collected and hydrogen peroxide (500 mg L^{-1}) was added to the mixture TiO_2/wastewater, just before uncovering the CPCs. The two heterogeneous photocatalytic tests were repeated adjusting the pH in the range of 2.6–2.9 with sulfuric acid, in order to eliminate carbonates and bicarbonates and compare the results with the photo-Fenton tests. In all cases, samples were taken at successive time intervals to evaluate the progress of the photocatalytic oxidation.

For the photo-Fenton tests, pH was adjusted to 2.6–2.9 with sulfuric acid and another sample was taken 15 min later to confirm this value. Afterwards, iron salt (20, 40, 140 or 160 mg Fe^{2+} L^{-1}) was added, the mixture was well homogenized for 15 min and a third sample was taken for iron concentration control. Finally, the first dose of hydrogen peroxide (500 mg L^{-1}) was added, the CPCs were uncovered and samples were taken at different time intervals to evaluate the degradation process.

Regarding the Fenton experiment, reaction occurred in the dark (CPC units were not uncovered) at pH 2.8 and with 140 mg Fe^{2+} L^{-1}.

The H_2O_2/UV test was performed with no acidification and no iron addition. The UV test was carried out with no acidification and no iron and hydrogen peroxide addition.

Whenever hydrogen peroxide was used, its concentration was maintained between 200 and 500 mg L^{-1}, during the entire runs, through the addition of small amounts of hydrogen peroxide to compensate the consumption.

3. Results and discussion

3.1. Characteristics of the wastewater resulting from phytopharmaceutical plastic containers washing

The wastewater resulting from phytopharmaceutical plastic containers washing presented a light yellow color, associated to a moderate organic content (COD = 1662–1960 mg O_2 L^{-1}; DOC = 513–696 mg C L^{-1}) and high biodegradability (BOD_5 = 1350–1600 mg O_2 L^{-1}; BOD_5/COD = 0.79–0.83) (Table 1). The soluble nitrogen concentration was in the range of 118–144 mg N L^{-1} (5.3–34 mg NO_2^- L^{-1}; <0.01–32 mg NO_3^- L^{-1}; 33–41 mg NH_4^+ L^{-1}; 75–104 mg dissolved organic nitrogen L^{-1}). The effluent also showed a neutral pH (6.7–7.2) and a low concentration of polyphenols (16–24 mg caffeic acid L^{-1}). The conductivity was moderate, corresponding to low/moderate concentrations of dissolved iron, sulfate, phosphate, fluoride, chloride, bromide, lithium, sodium, potassium, magnesium and calcium. The values of COD, BOD_5, total dissolved nitrogen, dissolved iron and TSS exceeded the wastewater emission limit values (ELVs) imposed by the Portuguese legislation (Table 1) [31].

Table 2 presents pesticide content in the raw washing wastewater and after biological treatment. Taking into account the LC–MS/MS analytical method quantification limits, nineteen out of thirty-one pesticides were quantified (some characteristics of these pesticides are presented in the supporting information data). Four pesticides are present in concentrations above 10,000 μg L^{-1} and nine of them always between 1000–10,000 μg L^{-1}, representing 14–19% of the DOC. In ALBAIDA's plant, twelve pesticides have been detected, but in concentrations near or below 1000 μg L^{-1}: malathion (1065 μg L^{-1}), dimethoate (877 μg L^{-1}), pyrimethanil (850 μg L^{-1}), imidacloprid (695 μg L^{-1}), thiacloprid (563 μg L^{-1}), azoxystrobin (491 μg L^{-1}), metalaxyl (192 μg L^{-1}), carbofuran (168 μg L^{-1}), spinosyn A (140 μg L^{-1}), tebufenozide (74 μg L^{-1}), bupirimate (38 μg L^{-1}) and fenamiphos (36 μg L^{-1}) [3]. Furthermore, the ALBAIDA's phytopharmaceutical plastic containers were washed using less water, in a proportion of 0.833 kg of plastics per liter of water [24], compared with 0.15 kg of plastics per liter of water used in the present study.

3.2. Preliminary biological treatment of the wastewater

Since the effluent presented a high biodegradability, a biological treatment using an IBR was performed prior to the AOPs. Table 1 also shows the physical/chemical characteristics of the bio-treated wastewater (single and mixed samples). A significant decay in COD (46–54%), DOC (41–56%) and BOD_5 (88–90%) was observed, meaning that the biodegradable organic matter fraction was almost completely removed. The biological degradation reaction rate was 4–8 mg C L^{-1} h^{-1} (28–37 mg O_2 L^{-1} h^{-1}, in terms of BOD_5) during the first 40 h and, afterwards, DOC profile remained approximately constant and BOD_5/COD ratio decreased to 0.18, revealing the recalcitrant character of the residual organic matter. When glucose was added to the bioreactor, after DOC stabilization, it was completely consumed (according to DOC values), proving that the pesticides did not cause any significant inhibition to biomass. A strong decrease of dissolved organic nitrogen (considering the difference between the total dissolved nitrogen and inorganic nitrogen species (nitrates, nitrites and ammonia)) was observed (the denitrification process was not considered), possibly to its decomposition into ammonium, which increased around 157%. Phosphate concentration also increased, probably due to oxidation of organophosphorus compounds. Conductivity raised 18–25%, mainly due to ammonium, sulfate and phosphate ions. As shown in Table 2, no significant decrease of pesticides concentration was observed after the biological treatment, which further supports their recalcitrant character and the need of an additional AOP step. However, for some pesticides, such as, S-metolachlor, alachlor and terbuthylazineno, it was observed a 30–40% reduction of its concentration in the IBR, possibly associated with the adsorption on the biofilm. After the biological treatment, pesticide content represented 24–34% of DOC.

3.3. Advanced oxidation processes

3.3.1. UV and TiO_2/UV systems

A first approach using AOPs for the treatment of wastewater from the washing of plastic containers for phytopharmaceutical included a photochemical system, using only solar UV-light and heterogeneous photocatalysis using TiO_2 combined with solar UV-light, with and without preliminary acidification. Preliminary acidification of neutral wastewater to pH 2.8 was performed in order to evaluate the effects resulting from the elimination of carbonates and bicarbonates in the form of CO_2, since these compounds are associated with $HO^{\cdot}$ scavenging, leading to formation of less reactive species [32]. It should also be underlined that catalyst concentration (200 mg TiO_2 L^{-1}) is the optimum concentration for the photoreactors used in this work, with an internal diameter of 46.4 mm [33], which has recently been supported by accurate modeling of radiation field in a CPC solar photoreactor by a six-flux absorption scattering model (SFM), showing that TiO_2 in the concentration of 200 mg L^{-1} is able to absorb 100% of the solar UV photons [34].

All systems led to low mineralization percentages (17–23%) after 51–52 kJ_{UV} L^{-1} (Fig. 1). For TiO_2/UV-with acidification system, it is visible a DOC abatement after pH adjustment, which can be attributed to the formation of high amounts of foam and possible

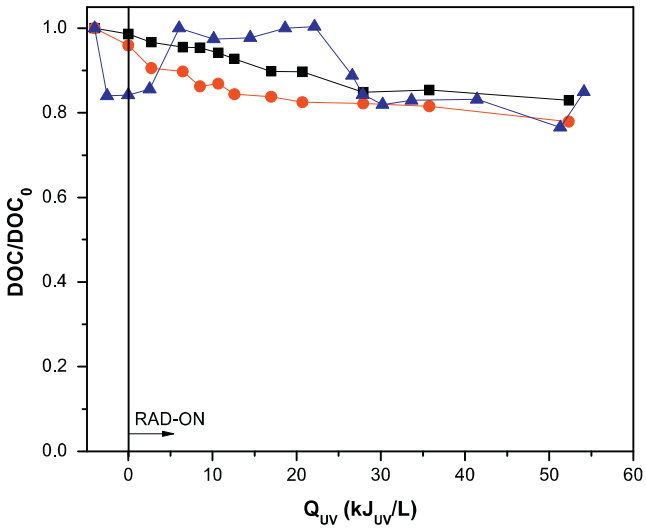

Fig. 1. Wastewater DOC/DOC$_0$ ratio evolution profile for the following systems: ■, □ – UV; ●, ○ – TiO_2/UV-without acidification; ▲, △ – TiO_2/UV-with acidification to pH 2.8, as a function of the accumulated UV energy per liter of wastewater.

precipitation of some compounds, probably responsible for the retention of 16% of the initial DOC. To further support this theory, and also through the analysis of Fig. 1, it can be inferred that DOC was redissolved during the photocatalytic reaction, and the initial DOC concentration value was achieved after 6 $kJ_{UV}\,L^{-1}$.

Fig. 2 depicts the degradation profiles of several pesticides, under three photocatalytic experimental conditions. In the cases of UV and TiO_2/UV-without acidification (7.9 < pH < 8.3) experiments, and for the majority of the analyzed pesticides, concentrations remained almost constant over the entire phototreatment process, up to an accumulated UV energy of ca. 52 $kJ\,L^{-1}$. One exception was terbuthylazine–desethyl, which concentration at the end of the photodegradation reaction was approximately twice the initial one. This can be easily understood considering that terbuthylazine–desethyl is the desethylation product of terbuthylazine, also present in the initial sample, and thus its concentration increased as terbuthylazine was being degraded [35]. For both experiments, pesticide content accounted for ca. 30% of the DOC at the beginning and at the end of the reaction, which confirms the low pesticide removal. However, for the TiO_2/UV system with preliminary acidification and after the same accumulated UV energy, it was possible to observe a degradation rate over 90% for eighteen pesticides, thirteen of which have been removed to levels below the respective quantification limits. A minimum removal of 70% was achieved for the other pesticides, excluding the case of terbuthylazine–desethyl, which registered a significant increase (11 times), due to the aforementioned reasons. In this photocatalytic system, 37% of the initial DOC was due to the pesticide content, while after 51 $kJ_{UV}\,L^{-1}$ the pesticide load contributed only to 3% of the DOC.

The obtained results denote a low photoactivity of the TiO_2 surface at alkaline/neutral pH. This can be associated, on the one hand, with interactions of carbonates and bicarbonates at the TiO_2 surface, working as scavengers of $HO^{\bullet}$, and, on the other hand, to the modification of the catalyst surface, since for pH < pzc (pzc – point of zero charge) the predominant specie is $–TiOH^{2+}$ [32], which will interact with the partially degraded molecules negatively charged. Malato et al. [21] also refer that with the generation of intermediate degradation products, through pesticide degradation, these can work as competitors on the surface of TiO_2 particles, which can further explain the low mineralization values achieved in these three experiments.

3.3.2. UV/H_2O_2 and TiO_2/H_2O_2/UV systems

A second experimental approach to pesticide-containing wastewater treatment involving AOPs included the addition of H_2O_2, as an oxidant, to the systems used in Section 3.3.1. (Fig. 3). H_2O_2 can work as an electron scavenger, avoiding the electron/hole recombination and further improving the reaction rates [36].

Comparing H_2O_2/UV and TiO_2/H_2O_2/UV-without acidification (6.6 < pH < 7.7) systems, no significant difference was observed in terms of mineralization profiles. The mineralization followed a pseudo-first-order kinetic model, with kinetic constants, k, of 0.010 ± 0.001 and 0.009 ± 0.001 L kJ_{UV}^{-1} (R^2 = 0.936 and 0.907, S_R^2 = 280 and 605 $mg^2\,C\,L^{-2}$) and initial reaction rates, r_0, of 3.0 ± 0.2 and 2.9 ± 0.2 mg C kJ_{UV}^{-1}, for H_2O_2/UV and TiO_2/H_2O_2/UV-without acidification systems, respectively. H_2O_2 consumption profile showed a linear correlation with accumulated UV energy per unit volume of wastewater, with H_2O_2 consumption rates, $k_{H_2O_2}$, of 0.65 ± 0.03 and 0.99 ± 0.02 mmol H_2O_2 kJ_{UV}^{-1} (R^2 = 0.913 and 0.987, S_R^2 = 30 and 8.7 mM^2) for H_2O_2/UV and TiO_2/H_2O_2/UV-without acidification systems, respectively. For the TiO_2/H_2O_2/UV-with acidification experiment, it was once again observed an initial DOC abatement (33%) after pH adjustment to 2.8, due to the formation of large amounts of foam, later on leading to a DOC increase as it was being redissolved (Fig. 3). The photocatalytic reaction presented an induction period of ca. 20–25 $kJ_{UV}\,L^{-1}$, characterized by a low mineralization rate and H_2O_2 consumption. This stage can be attributed to the dissolution of organic compounds retained in the foam and/or to the degradation of complex molecules into simpler ones, without achieving mineralization, as it can be exemplified by the increase of terbuthylazine–desethyl. Afterwards, a higher mineralization rate was observed, following a pseudo-first-order kinetic behavior (k = 0.031 ± 0.002 L kJ_{UV}^{-1}, r_0 = 4.9 ± 0.3 mg C kJ_{UV}^{-1}, R^2 = 0.988, S_R^2 = 28 $mg^2\,C\,L^{-2}$), up to a total mineralization of 76% and a 127 mM H_2O_2 overall consumption ($k_{H_2O_2}$ = 3.0 ± 0.1 mmol H_2O_2 kJ_{UV}^{-1}, R^2 = 0.988, S_R^2 = 22 mM^2), after 54 $kJ_{UV}\,L^{-1}$ irradiation. The low efficiency observed for the TiO_2/H_2O_2/UV system at neutral/alkaline wastewater normal pH can be possible attributed to the same reasons presented above for the TiO_2/UV system and also to the higher instability and self-decomposition of H_2O_2 [37], as indicated by Eq. (2). Other constraints that may occur include H_2O_2 scavenging of produced holes (Eq. (3)), its reaction with $HO^{\bullet}$ (Eq. (4)) and TiO_2 surface modification by H_2O_2 adsorption, forming peroxocompounds [3]. All these reactions may explain the better results achieved for the system with acidification.

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (2)$$

$$H_2O_2 \rightarrow 2h_{VB}^{+} \rightarrow O_2 + 2H^{+} \quad (3)$$

$$H_2O_2 + HO^{\bullet} \rightarrow H_2O + HO_2^{\bullet} \quad (4)$$

Concerning pesticide content (Fig. 4), the TiO_2/H_2O_2/UV-with acidification system showed the highest pesticide removal efficiency among all systems studied until now. After 28 $kJ_{UV}\,L^{-1}$, eighteen pesticides were degraded to levels below the respective quantification limit. Terbuthylazine–desethyl concentration increased 39 times relatively to its initial value after 22 $kJ_{UV}\,L^{-1}$ irradiation, attaining a maximum concentration of 1472 $\mu g\,L^{-1}$, and subsequently decreased to levels below the quantification limit after 41 $kJ_{UV}\,L^{-1}$. About 34% of the initial wastewater DOC was related to the pesticide content, though at the end of the reaction its contribution was nil. H_2O_2/UV and TiO_2/H_2O_2/UV-without acidification systems needed beyond 3 times more UV energy (85 $kJ_{UV}\,L^{-1}$) to achieve a similar overall degradation extent. For H_2O_2/UV and TiO_2/H_2O_2/UV-without acidification systems, respectively, terbuthylazine–desethyl concentration increased up to values 29 and 36 times above the initial value (maximum values of 1316 and

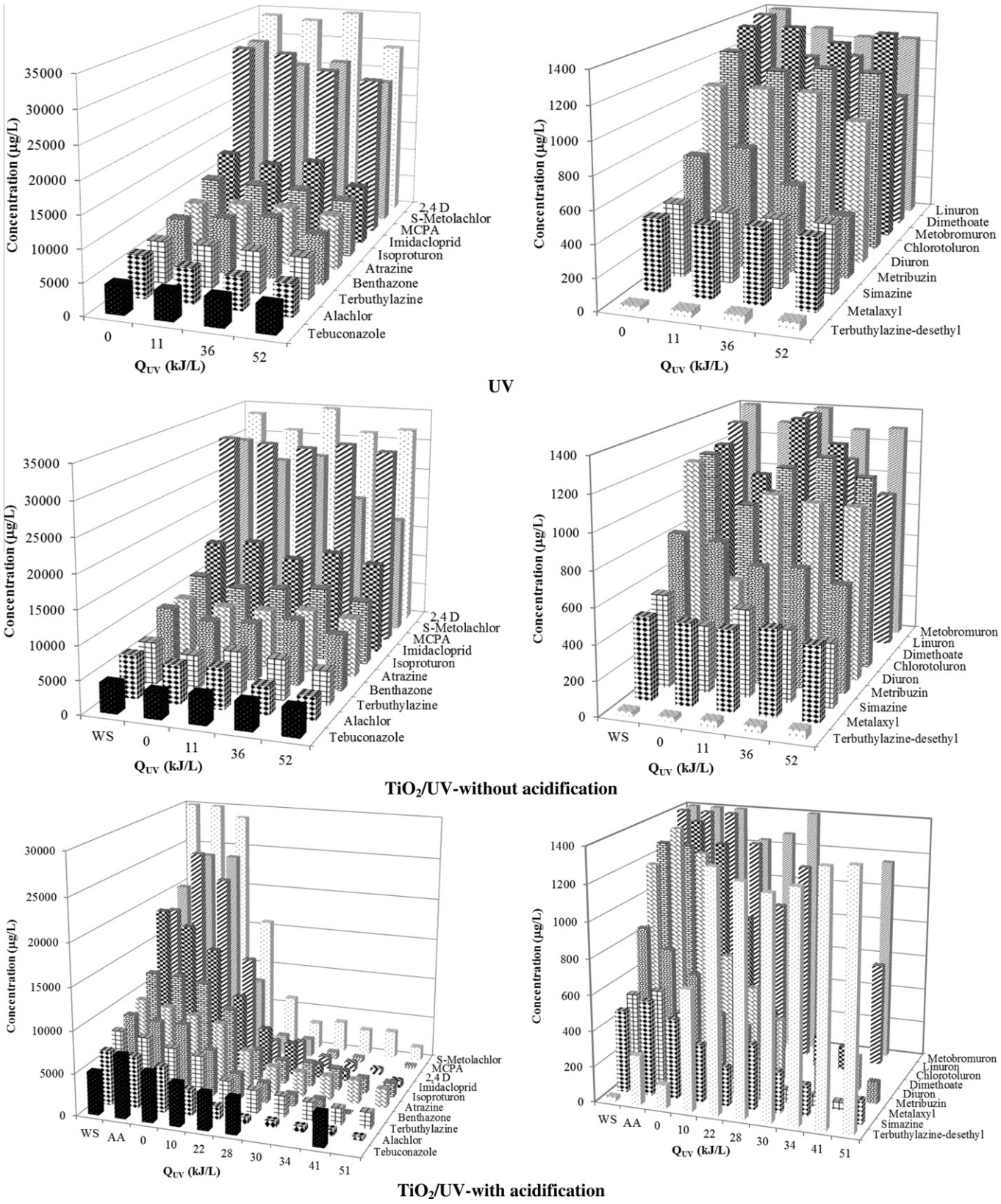

Fig. 2. Pesticide degradation profile as a function of the accumulated UV energy per liter of wastewater for the systems: UV, TiO_2/UV-without acidification and TiO_2/UV-with acidification at pH 2.8. WS – water sample; AA – wastewater after acidification.

1396 μg L^{-1}), which corresponds to terbuthylazine disappearance (i.e., concentration below the quantification limit), then starting to slightly decrease to values 21 and 30 times above the initial one. Evaluating the contribution of pesticide-carbon content to the

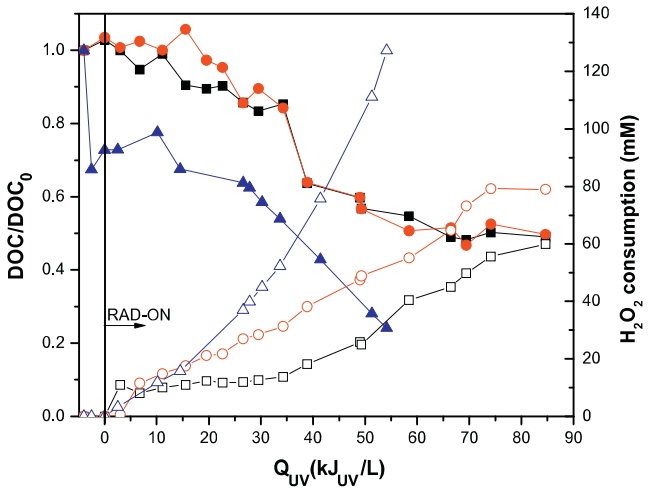

Fig. 3. DOC/DOC_0 variation and H_2O_2 consumption profiles for the following systems: ■, □ – UV/H_2O_2; ●, ○ – TiO_2/H_2O_2/UV-without acidification; ▲, △ – TiO_2/H_2O_2/UV-with acidification to pH 2.8, as a function of the accumulated UV energy per liter of wastewater. Solid symbols – DOC/DOC_0; open symbols – H_2O_2 consumption.

overall DOC, it initially represented 23% and 21% of the DOC and, at the end of the reaction, only 0.5% and 0.8%, for H_2O_2/UV and TiO_2/H_2O_2/UV-without acidification systems, respectively.

The combination of solar UV-light and/or TiO_2 with H_2O_2 showed higher mineralization rates than the same systems without H_2O_2, probably due to two main reasons: (a) H_2O_2 oxidant power; (b) prevention of the electron–hole recombination by H_2O_2 by accepting photogenerated electrons from the conduction band (Eq. (6)) and production of additional HO· through reactions (6) and (7). Production of HO· from photocatalytic cleavage of H_2O_2 (Eq. (5)) is not significant, due to the fact that radiation below 280 nm is needed for an effective H_2O_2 cleavage, and borosilicate glass tubes have a cut-off at 280 nm.

$$H_2O_2 + h\nu \rightarrow 2HO^{\cdot} \quad (5)$$

$$H_2O_2 + e^-_{CB} \rightarrow HO^{\cdot} + OH^- \quad (6)$$

$$H_2O_2 + O_2^{\cdot-} \rightarrow HO^{\cdot} + OH^- + O_2 \quad (7)$$

3.3.3. Photo-Fenton and Fenton systems

The photo-Fenton reaction, i.e., combination of Fe^{2+} with H_2O_2 and solar UV–Vis radiation (Fe^{2+}/H_2O_2/UV–Vis), was tested at four different initial iron concentrations: 20, 40, 140 and 160 mg Fe^{2+} L^{-1} (Fig. 5a and b). It was observed a DOC abatement (13–21%) after pH adjustment to 2.8 (Fig. 5a), similarly to what had already happened in the previously analyzed systems with pH correction. This pH value was selected for the photo-Fenton reaction not only because the predominant iron species in solution is $FeOH^{2+}$, which is the most photoactive ferric ion-water complex [38], but also because it avoids iron precipitation. Considering sulfate content after acidification and iron addition, Fe^{3+} speciation diagram (see Supporting Information) shows that at pH 2.8 the predominant iron species in solution are $FeSO_4^+$ (37%), $FeOH^{2+}$ (32%) and Fe^{3+} (22%), leading to the formation of $SO_4^{\cdot-}$ and HO·, respectively, according to Eqs. (8) and (9) [39]. For pH 3.4, the predominant iron species in solution are $FeOH^{2+}$ (50%), $Fe(OH)_2^+$ (25%), $FeSO_4^+$ (15%) and Fe^{3+} (9%). As the reactivity of HO· is higher than the one of $SO_4^{\cdot-}$, an increment of the oxidation rate can be expected increasing the pH until 3.4, although some precipitation of iron can occur.

$$FeSO_4^+ + h\nu \rightarrow Fe^{2+} + SO_4^{\cdot-} \quad (8)$$

$$FeOH^{2+} + h\nu \rightarrow Fe^{2+} + HO^{\cdot} \quad (9)$$

For the two experiments with the lowest initial iron doses (20 and 40 mg Fe^{2+} L^{-1}) and after the addition of H_2O_2, it can be perceived an induction period of ca. 12 kJ_{UV} L^{-1}, characterized by low mineralization and H_2O_2 consumption, associated to the almost complete disappearance of dissolved iron, relatively to the initial added iron dose (Fig. 5b). This is probably due to iron precipitation as iron phosphate, $FePO_4$, which is in accordance with the observed yellow precipitate formed and with the decrease of phosphates concentration from ca. 81 mg PO_4^{3-} L^{-1} to values lower than the detection limit (<0.1 mg L^{-1}). The addition of H_2O_2 to Fe^{2+} leads to the formation of Fe^{3+} (Eq. (10)) and consequent precipitation with phosphates.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\cdot} \quad (10)$$

Therefore, in the experiments using the lowest initial iron doses, 20 and 40 mg Fe^{2+} L^{-1}, it was necessary to add three more doses of 40 mg Fe^{2+} L^{-1} during the reaction, in order to precipitate all phosphates and further increase dissolved iron concentration to values similar to the initial ones.

Two more experiments were performed with an initial iron concentration of 140 and 160 mg Fe^{2+} L^{-1}, leading to complete phosphates precipitation, resulting in a dissolved iron concentration during the reaction in a range of 10–20 and 17–30 mg (Fe^{2+} + Fe^{3+}) L^{-1}, respectively.

Iron precipitation due to temperature increase along the reaction (temperature increased from morning start-up (18–20 °C) to maximum values between noon and 5 p.m. (32–38 °C) (Fig. 5b)) was not observed, since, according to Zapata el al. [40], iron precipitation due to the decrease of iron solubility only occurs for temperature values higher than 42 °C, considering maximum iron concentrations of 55 mg L^{-1}.

Mineralization profiles followed a pseudo-first-order kinetic model with $k = 0.16 \pm 0.01$, 0.18 ± 0.01, 0.13 ± 0.02 and 0.21 ± 0.04 L kJ_{UV}^{-1}, $r_0 = 14 \pm 1$, 15 ± 1, 25 ± 4 and 42 ± 7 mg C kJ_{UV}^{-1}, R^2 = 0.982, 0.997, 0.950 and 0.959, S_R^2 = 11, 1.9, 349 and 414 mg^2 C L^{-2}, for initial iron doses of 20, 40, 140 and 160 mg Fe^{2+} L^{-1}, respectively. H_2O_2 consumption profile showed a linear correlation with accumulated UV energy per unit volume of wastewater: $k_{H_2O_2}$ = 14 ± 2, 15 ± 2, 8.2 ± 0.1 and 9.6 ± 0.3 mM H_2O_2 kJ_{UV}^{-1}, R^2 = 0.918, 0.930, 0.996 and 0.987, S_R^2 = 141, 123, 16 and 95 mM^2, for initial iron doses of 20, 40, 140 and 160 mg Fe^{2+} L^{-1}, respectively. The kinetic constants and average dissolved iron concentration for the experiments with an initial iron concentration of 20 and 40 mg L^{-1} were calculated using only the data after the induction period (absence of iron). Kinetic constants increased with catalyst concentration, bearing in mind that average dissolved iron concentration during the considered period was of 21, 38, 14 and 22 mg (Fe^{2+} + Fe^{3+}) L^{-1}, for the experiments with initial iron doses of 20, 40, 140 and 160 mg Fe^{2+} L^{-1}, respectively. However, when increasing the dissolved iron concentration at values higher than 20 mg L^{-1}, no significant difference occurred, which was also observed by Zapata et al. [1] during the treatment of simulated wastewaters contaminated with five commercial pesticides. This can be easily justified since a 20 mg L^{-1} dissolved iron concentration is enough to absorb all the solar photons in a photoreactor with a 5-cm light path length [41]. This concentration value is also more advisable, as the effluent is more compatible with a subsequent biological treatment [42].

A dark Fenton reaction using an initial iron dose of 140 mg Fe^{2+} L^{-1} was also performed at pH 2.8. This process originated 56% mineralization after 7.9 h (475 min), consuming 34 mM of H_2O_2 (Fig. 6; see also supporting information). Comparing Fenton and photo-Fenton systems, both using an initial iron dose of

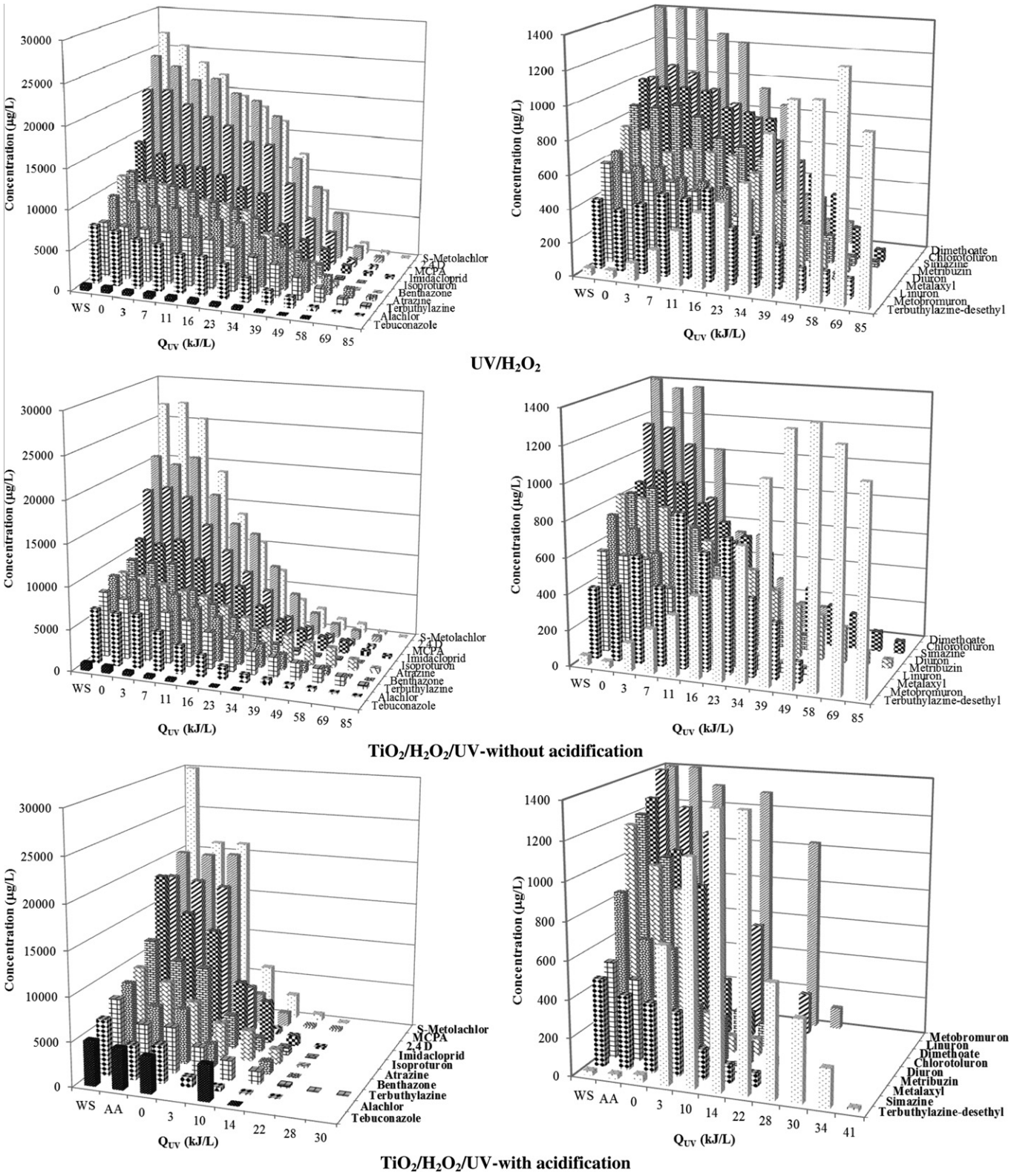

Fig. 4. Pesticide degradation profile as a function of accumulated UV energy per liter of wastewater for the systems: UV/H_2O_2, $TiO_2/H_2O_2/UV$-without acidification and $TiO_2/H_2O_2/UV$-with acidification at pH 2.8. WS – water sample; AA – wastewater after acidification.

140 mg $Fe^{2+}\,L^{-1}$, the photo-Fenton reaction required four times less exposure (120 min) and consumed only 39 mM H_2O_2 to reach the same mineralization degree (Fig. 6). The slower reaction rate observed in the Fenton system can be explained by the unavailability of radiation for the regeneration of ferrous ions (Eq. (9)). As the dissolved iron concentration decreased to values lower than 20 mg $Fe^{2+}\,L^{-1}$ in the Fenton reaction, a new addition of 140 mg $Fe^{2+}\,L^{-1}$ was performed and the dissolved iron concentration remained between 120 and 140 mg $Fe^{2+}\,L^{-1}$ until the end of the experiment. According to Fig. 6, the DOC profile remained very similar, which leads to the conclusion that the regeneration of ferrous ions is the limiting step.

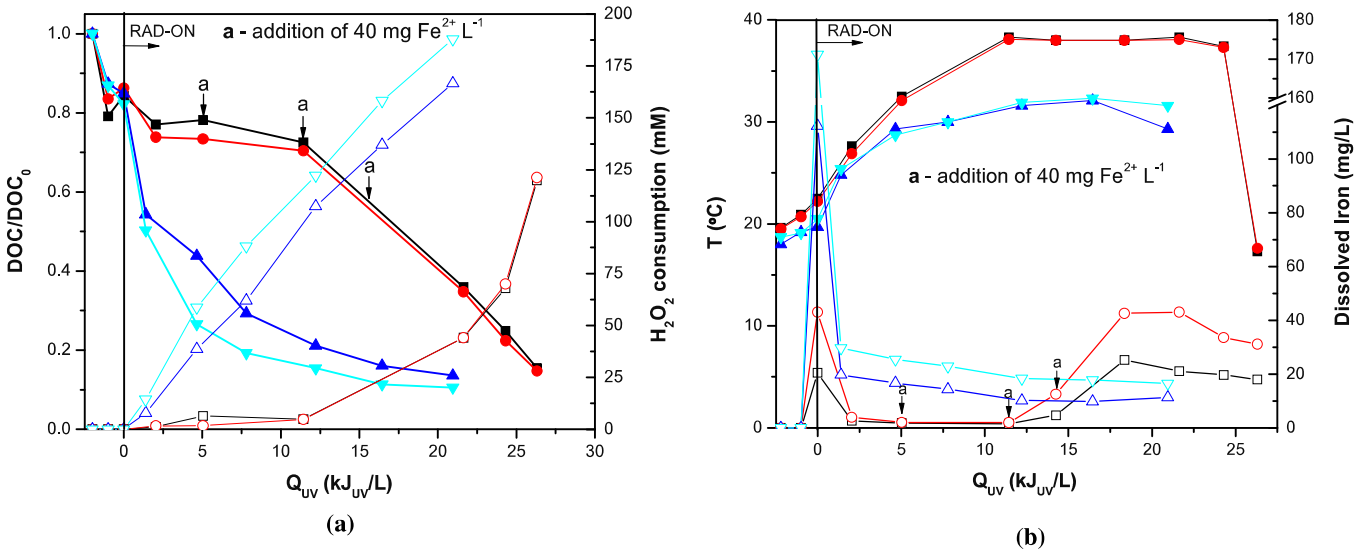

Fig. 5. DOC/DOC_0 variation and H_2O_2 consumption (a) and dissolved iron concentration and temperature (b), during the photo-Fenton reaction ($Fe^{2+}/H_2O_2/UV$; pH 2.8) as a function of the accumulated UV energy per liter of wastewater at different initial iron concentrations. Solid symbols – DOC/DOC_0 and temperature; open symbols – H_2O_2 consumption and dissolved iron concentration. ■, □ – $[Fe^{2+}] = 20\ mg\ L^{-1}$; ●, ○ – $[Fe^{2+}] = 40\ mg\ L^{-1}$; ▲, △ – $[Fe^{2+}] = 140\ mg\ L^{-1}$; ▼, ▽ – $[Fe^{2+}] = 160\ mg\ L^{-1}$.

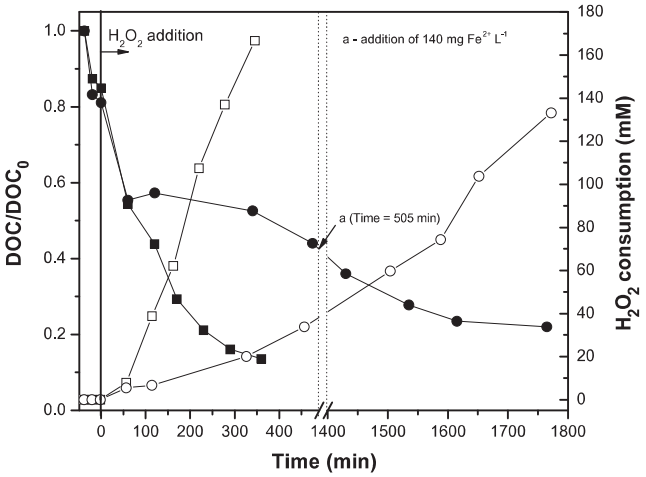

Fig. 6. DOC/DOC_0 variation and H_2O_2 consumption for the following systems: ■, □ – photo-Fenton reaction ($[Fe^{2+}] = 140\ mg\ L^{-1}$; pH 2.8); ●, ○ – Fenton reaction ($[Fe^{2+}] = 140\ mg\ L^{-1}$; pH 2.8), as a function of time, in minutes. Solid symbols – DOC/DOC_0; open symbols – H_2O_2 consumption.

Evaluating the pesticide content (Fig. 7), the photo-Fenton experiments performed with the two lowest iron doses (20 and 40 mg $Fe^{2+}\ L^{-1}$) led to similar results, i.e., eighteen pesticides were eliminated (residual concentrations below the respective quantification limit) after 26 $kJ_{UV}\ L^{-1}$. Terbuthylazine–desethyl concentration firstly raised in both situations, up to maximum values of 2200 and 2886 $\mu g\ L^{-1}$ for the reactions with 20 and 40 mg $Fe^{2+}\ L^{-1}$, respectively, and then diminished to values below the quantification limit after 26 $kJ_{UV}\ L^{-1}$. The photo-Fenton experiments performed with the two highest iron doses yielded equivalent results, i.e., after 8 $kJ_{UV}\ L^{-1}$ (t_{30W} = 115 min, considering a constant solar UV power of 30 W m^{-2}) nineteen pesticides were removed to levels below the respective quantification limit. For all photo-Fenton experiments, initially, pesticide content represented 21–32% of the DOC content and at the end of the reactions it was negligible. Nevertheless, when using 140 mg $Fe^{2+}\ L^{-1}$, pesticide content corresponded to 2.6 and 0.1 mg C L^{-1} (2.1 and 0.1% of total DOC) after 1.4 (t_{30W} = 9 min) and 5.0 (t_{30W} = 49 min) $kJ_{UV}\ L^{-1}$, respectively, whereas for 160 mg $Fe^{2+}\ L^{-1}$, 0.8 and 0.0 mg C L^{-1} (0.6 and 0.0% of total DOC) after 1.4 (t_{30W} = 9 min) and 5.0 (t_{30W} = 49 min) $kJ_{UV}\ L^{-1}$ were achieved, respectively.

Zapata et al. (2010) reported similar results in the treatment of a real pesticide-contaminated wastewater resulting from the washing of plastic pesticide containers from greenhouses in El Ejido, Almeria (southeastern Spain) for recycling. The containers were shredded and washed, producing rinse water contaminated with pesticides, with an initial DOC ranging between 200 and 500 mg L^{-1}, related to the amount of plastic washed in the same amount of water. In this effluent, it was also possible to detect and quantify twelve pesticides, with the highest individual concentration of about 1000 $\mu g\ L^{-1}$, being necessary 9 and 15 mM of H_2O_2, and no more than 2 and 4 h of illumination, respectively, to achieve almost complete elimination of the pesticides, using 20 mg $Fe^{2+}\ L^{-1}$. In this case, precipitation of iron with phosphates is not reported.

Regarding the Fenton reaction, eighteen pesticides were eliminated to levels inferior to the respective quantification limit, after 475 min and a H_2O_2 consumption of 34 mM (Fig. 7). The photo-Fenton reaction with 140 mg $Fe^{2+}\ L^{-1}$ consumed a similar H_2O_2 dose (39 mM) to attain an almost equal pesticide removal, although it only took 120 min. Maldonado et al. [43] also observed the degradation of a mixture of six commercial pesticides (alachlor, chlorfenvinphos, isoproturon, diuron, atrazine and isoproturon) using the Fenton reaction, although no degradation was observed for atrazine and only a slight degradation was observed for the remaining pesticides, using an initial iron concentration of 20 mg L^{-1} at pH 2.8.

Bearing in mind that either induction times, reaction rates or pesticide removal percentages presented no significant difference between photo-Fenton experiments at the two highest iron concentrations, and considering that a lower iron concentration brings on lower costs, the catalyst concentration of 140 mg $Fe^{2+}\ L^{-1}$ was selected as the optimum value for further studies.

3.3.4. Comparison between AOPs

UV and TiO_2/UV-without acidification systems revealed to be inefficient in terms of mineralization and pesticide removal, leading to very low mineralization rates and almost null pesticide

reduction, even after long reaction times. TiO_2/UV-with acidification process gave similar results in terms of mineralization, despite allowing better ones with respect to pesticide removal. The combination of TiO_2 with H_2O_2 as oxidant proved to be more efficient than both TiO_2 and H_2O_2 alone, using solar irradiation. UV/H_2O_2 and TiO_2/H_2O_2/UV-without acidification processes led to acceptable mineralization degrees and almost total pesticide removal, although requiring longer reaction times. Higher mineralization and pesticide degradation were reached in a shorter time using TiO_2/H_2O_2/UV-with acidification system. Acidification has demonstrated to always improve the treatment efficiency, either in terms of mineralization or pesticide removal. The photo-Fenton process using an initial iron concentration of 140 mg Fe^{2+} L^{-1} proved to be the most efficient among all studied solar AOPs, showing an ini-

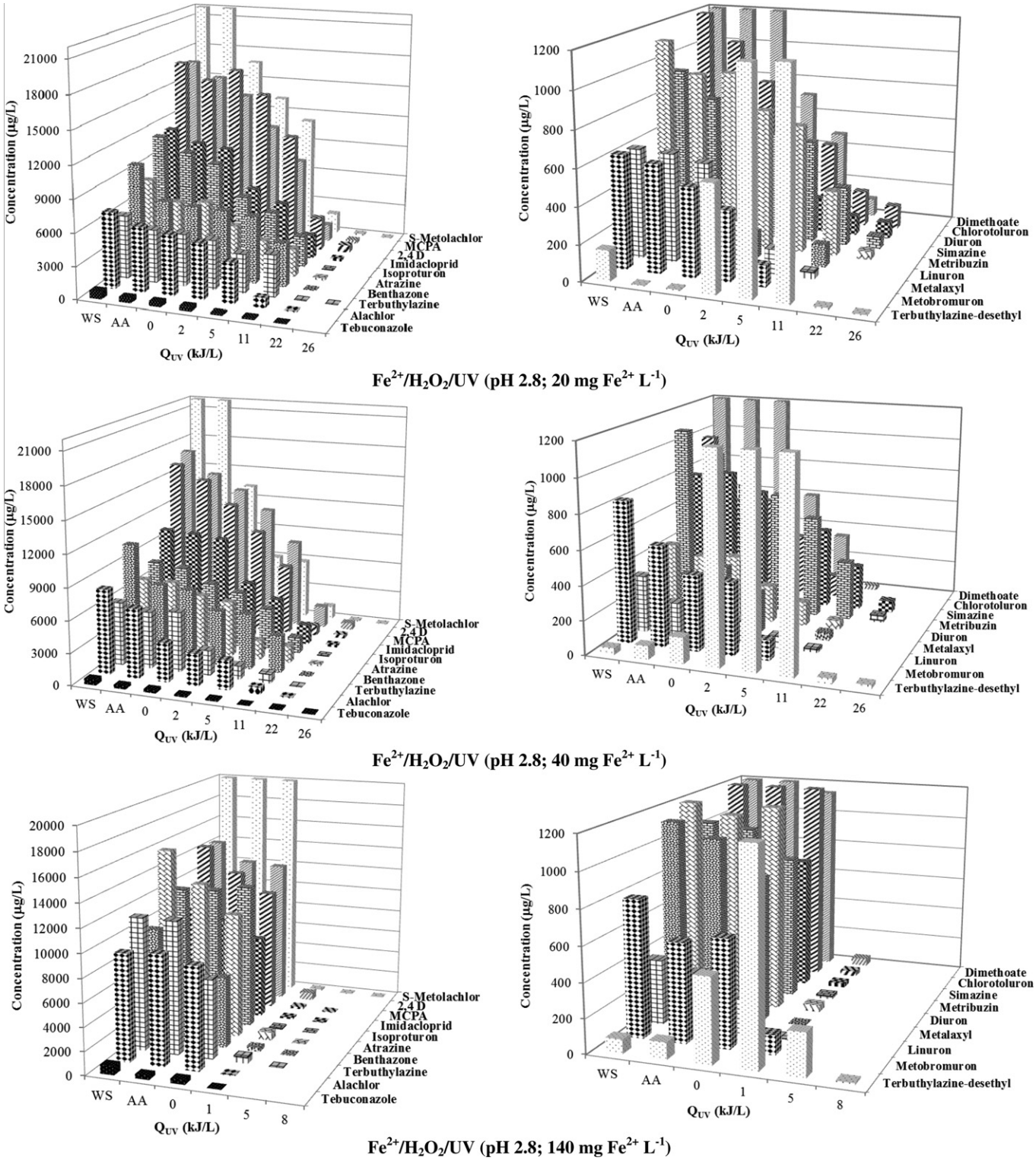

Fig. 7. Pesticide degradation profile as a function of H_2O_2 consumption (Fenton reaction) or the accumulated UV energy per liter of wastewater (photo-Fenton reaction at different initial irons concentrations). WS – water sample; AA – wastewater after acidification.

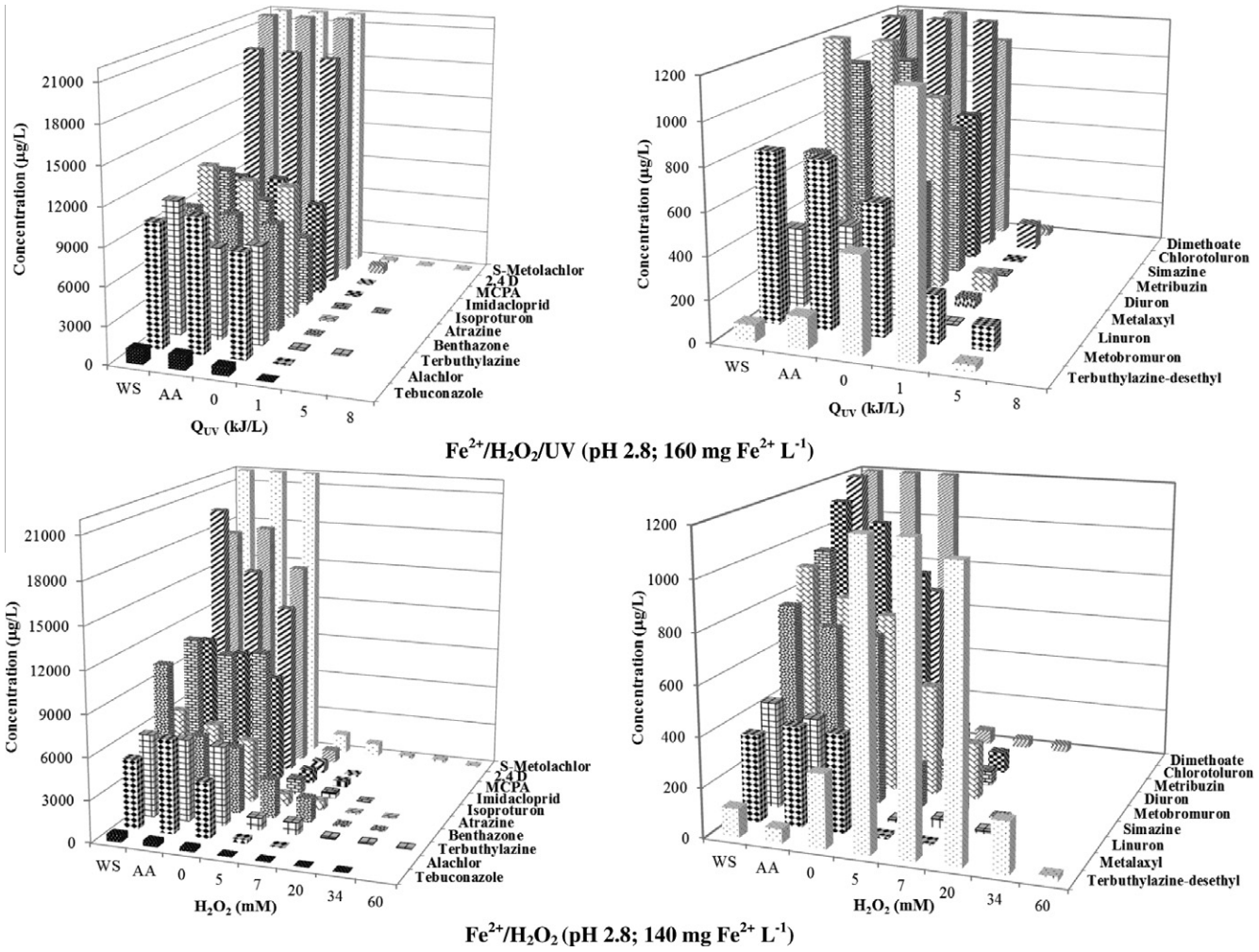

Fig. 7 *(continued)*

tial reaction rate 8.4, 8.7 and 5.1 times higher than for H_2O_2/UV, TiO_2/H_2O_2/UV-without and with acidification systems, respectively. The Fenton reaction needed much more time than photo-Fenton to achieve the same mineralization and pesticide removal percentages.

4. Conclusions

The wastewater resulted from the washing of phytopharmaceutical plastic containers presents moderate organic matter load and high biodegradability, where nineteen pesticides were detected, most of which at high concentrations (>10 mg L^{-1}). The high biodegradability induced to perform a biological treatment prior to AOPs. Although almost all biodegradable organic matter was removed, the bio-treated effluent still contained recalcitrant compounds, including pesticides, which supported the need for an additional AOPs step. Considering all AOPs tested, the photo-Fenton reaction achieved better results in terms of pesticides degradation and mineralization.

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