Universitat de Girona

MULTI-SCALE INVESTIGATION OF OCCURRENCE, FATE, REMOVAL AND BIODEGRADATION OF PHARMACEUTICAL CONTAMINATION IN WASTEWATER TREATMENT AND RIVERS

Neus COLLADO ALSINA

PhD Thesis

Multi-scale investigation of occurrence, fate, removal and biodegradation of pharmaceutical contaminants in wastewater treatment and river systems

Neus Collado Alsina

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Supervisors: Dr. Gianluigi Buttiglieri and Prof. Joaquim Comas Matas

Dr. GIANLUIGI BUTTIGLIERI i Prof. JOAQUIM COMAS MATAS

Investigador Post-doc de l'Institut Català de Recerca de l'Aigua (ICRA) i Professor del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària de la Universitat de Girona i investigador de l'Institut de Medi Ambient, respectivament.

Declarem

Que la llicenciada en Ciències Ambientals per la Facultat de Ciències de la Universitat de Girona **Neus Collado Alsina** ha realitzat, sota la nostra direcció, el treball que amb el títol "**Multi-scale investigation of occurrence, fate, removal and biodegradation of pharmaceutical contaminants in wastewater treatment and river systems**", que es presenta en aquesta memòria la qual constitueix la seva Tesi per optar al Grau de Doctor per la Universitat de Girona.

I perquè en prengueu coneixement i tingui els efectes que corresponguin, presentem davant la Facultat de Ciències de la Universitat de Girona l'esmentada Tesi, signant aquesta declaració a

Girona, Juny de 2013

Gianluigi Buttiglieri   Joaquim Comas Matas

A l'Alena,

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RESUM
SUMMARY

Els compostos farmacèutics d'ús humà i animal s'han identificat com a microcontaminants aquàtics omnipresents amb possibles efectes nocius sobre el medi ambient i fins i tot sobre la salut humana. La major part d'aquests compostos, un cop consumits i excretats, van a parar a estacions depuradores d'aigües residuals (EDARs), que no estan dissenyades per eliminar-los completament. En conseqüència, els efluents d'EDARs esdevenen un flux, poc concentrat però constant, de compostos farmacèutics cap al medi aquàtic. La conscienciació creixent i la preocupació que genera actualment la presència de microcontaminants a les aigües receptores obliga a adoptar nous enfocaments pel que fa al tractament i la gestióde les aigües residuals.

Per això esdevé clau la investigació duta a terme al llarg d'aquesta tesi doctoral amb el títol de: "*Multi-scale investigation of occurrence, fate, removal and biodegradation of pharmaceutical contaminants in wastewater treatment and river systems*", la qual pretén abastar el problema dels compostos farmacèutics de manera multidisciplinar (mitjançant diferents enfocaments analítics i experimentals) i treballant a diferents escales. D'aquesta manera, l'objectiu principal d'aquest treball és el d'adquirir i proporcionar coneixement pel que fa tant a la presència d'aquests compostos farmacèutics a les aigües residuals, com a la seva degradació en EDARs i la seva descàrrega al medi receptor.

Per aquest motiu, la recerca realitzada s'ha estructurat de manera que permetés lligar conceptes tant a escala real, com serien campanyes de mostreig i diagnosi d'un escenari actual, com experiments a escala de laboratori que permeten treballar controladament aspectes concrets sobre la biodegradació de certs compostos, juntament amb l'aplicació de tècniques microscòpiques i proteòmiques que permeten abordar des d'una altra perspectiva el paper que hi juguen els microorganismes i el seu metabolisme, respectivament, durant el procés de biodegradació dels fàrmacs.

Els resultats obtinguts demostren la variabilitat en l'eficàcia dels tractaments convencionals d'aigües residuals i la contribució dels processos avançats a l'hora d'eliminar els compostos farmacèutics. Tanmateix, es corrobora l'entrada al medi ambient de molts d'aquests compostos i s'investiga el seu impacte en les aigües receptores. D'altra banda, s'ha aconseguit també modelar el comportament d'un fàrmac i dels seus productes de transformació. La tesi també aborda l'impacte d'un antibiòtic sobre la comunitat bacteriana a nivell microbiològic i el fenomen dels gens de resistència antibiòtica. I, finalment, el paper de tècniques proteòmiques a l'hora de buscar possibles proteïnes involucrades en el procés de biodegradació de certs compostos farmacèutics, ha permès demostrar fins a quin punt l'utilitat d'aquesta tècnica s'adequa a l'objectiu final.

SUMMARY
RESUM

Human and veterinary pharmaceuticals have been recognized as ubiquitous water microcontaminants with potential subtle detrimental effects on aquatic organisms and possibly also on human health. The majority of pharmaceutical compounds, after being consumed and excreted, end up in municipal wastewater treatment plants (WWTPs), which are not typically designed for complete pharmaceutical removal. Therefore, effluents from WWTPs constitute a low concentration, but continuous source of pharmaceutically active compounds to the aquatic environment. Nevertheless, an increased awareness of the presence of pharmaceuticals in the receiving waters requires new approaches in wastewater treatment and management.

Thus, it is crucial to conduct research studies as the carried out within this thesis entitled: "*Multi-scale investigation of occurrence, fate, removal and biodegradation of pharmaceutical contaminants in wastewater treatment and river systems*", which intent to tackle the pharmaceuticals issue under a multidisciplinary focusing (by means of different analytical and experimental approaches) and at different scales. Hence, the main objective of the thesis is to acquire and provide with knowledge not only on the occurrence of pharmaceutical compounds in wastewater, but on their biodegradation within WWTPs and their discharge into the receiving media.

According to the aforementioned goal, the present study has been structured in a way that will allow the link up of real scale concepts, sampling campaigns and diagnosis of actual scenarios, experiments on more specific aspects of pharmaceutical biodegradation at lab scale, the application of microscopic and proteomic techniques, and to undertake from another perspective the role of the microorganisms and their metabolism involved in the pharmaceutical biodegradation process.

The obtained results demonstrate conventional wastewater treatment's variable efficiency when removing the pharmaceutical compounds load and the advance treatment's contribution to their overall removal. Furthermore, pharmaceutical compounds release into the environment is corroborated and their subsequent impact into the receiving media has been investigated. On the other side, it has been possible to model the behaviour of a selected pharmaceutical and its transformation products. The thesis also includes investigation of an antibiotic's impact on the bacterial community performance, and the antibiotic resistance genes phenomenon. Finally, the role and usefulness of proteomics when investigating the possible proteins involved on the biodegradation pathways of certain pharmaceutical compounds has been elucidated in accordance to the final goal.

Table of CONTENTS

List of Publications

The following list contains the publications presented as chapters of this PhD thesis, together with the contributions of the PhD candidate in each paper:

1. **Collado, N**., Buttiglieri, G., Kolvenbach, B.A., Comas, J., Corvini, P.F.X and Rodríguez-Roda, I. (2013).''Exploring the potential of applying proteomics for tracking bisphenol A and nonylphenol degradation in activated sludge''. Chemosphere. **80**(8): 2309-2314.

   Author's Contribution: Design of the experimental study and analysis of the results. Writing the paper with special contribution from Núria Canela (URV) on the proteomics analytical aspects.

2. **Collado, N.,** Buttiglieri, G., Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., Comas, J. and Rodriguez-Roda, I. (2012). "Removal of Ibuprofen and its Transformation Products: Experimental and Simulation Studies". Science of the Total Environment. **433**, 296-301.

   Author's Contribution: Experimental design and operation of the batch reactors. Analysis of the results. Writing the paper with contributions from the other authors.

3. *Collado, N*., Rodriguez-Mozaz, S., Gros, M., Rubirola, A., Barceló, D., Comas, J., Rodriguez-Roda, I. and Buttiglieri, G. (2013). "Pharmaceuticals occurrence in a WWTP with significant industrial contribution and its impact into the river system". *Submitted to International Pollution*

   Author's Contribution: Design and planning of the different sampling campaigns. Execution of the sampling campaigns with Gianluigi Buttiglieri and UdG students support. Analysis and interpretation of the results. Writing the paper, with contributions from the other authors.

4. *Collado, N*., Buttiglieri, G., Marti, E., Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., Comas, J. and I. Rodriguez-Roda. (2013). "Effects on activated sludge bacterial community exposed to sulfamethoxazole ". *DOI: http://dx.doi.org/10.1016/j.chemosphere.2013.04.094*

   Author's Contribution: All the experimental study and data analysis. Writing the paper with contributions from Alex Sánchez (ICRA SCT) on the microbiological analysis and the other authors.

5. Buttiglieri, G., **Collado, N.,** Comas, J. and Rodriguez-Roda, I. (2013). "Proteomics reliability for micropollutants degradation insight into activated sludge systems". *Submitted to Environmental Science and Pollution Research.*

   Author's Contribution: All the experimental study (design and execution) and data analysis. Writing the paper with Gianluigi Buttiglieri**.**

6. **Collado, N.,** Buttiglieri, G., Ricken, B., Comas, J. and Rodriguez-Roda, I. (2013). "Specific bacterial strain role: Sulfamethoxazole biodegradation experiments and proteome analysis". *Submitted to Environmental Pollution.*

   Author's Contribution: All the experimental design and study and data analysis. Writing the paper, with contributions from the other authors.

Additional and relevant publications not included as chapters in the present thesis:

7. Ferrando-Climent, L., **Collado, N**., Buttiglieri, G., Gros, M., Rodriguez-Roda, I., Rodriguez-Mozaz, S., Barceló, D. (2012). "Comprehensive study of ibuprofen and its metabolites in activated sludge batch experiments and aquatic environment". Science of the Total Environment. **438**, 404-413.

Author's Contribution: All the experimental design and study and data analysis. Contribution on the writing.

8. Sipma J., Osuna B., **Collado N.**, Monclús H., Ferrero G., Comas J., Rodriguez-Roda I. (2009). "Comparison of removal of pharmaceuticals in MBR and activated sludge systems". Desalination **250**(2), 653-659.

Author's Contribution: Support on the data collection.

List of ACRONYMS

ARG	*antibiotic resistance genes*
ASM	*activated sludge model*
BNR	*biological nutrient removal*
BPA	*bisphenol A*
COD	*chemical oxygen demand*
DGGE	*denaturing Gradient Gel Electrophoresis*
DIGE	*difference gel electrophoresis*
F/M	*food to microorganisms ratio*
HPLC	*high-performance liquid chromatography*
HQDO	*hydroquinone dioxygenase*
HRT	*hydraulic retention time*
I.E.	*inhabitants equivalents*
IBU	*ibuprofen*
Kbiol	*biodegradation rate constant*
LOD	*limit of detection*
LOQ	*limit of quantification*
MBR	*membrane bioreactor*
MRM	*multiple reaction monitoring*
MW	*molecular weight*
N	*nitrogen*
NP	*nonylphenol*
NSAID	*nonsteroidal anti-inflammatory drug*
OD	*oxygen dissolved*
P	*phosphorous*
PCA	*principal component analysis*
PCR	*polymerase chain reaction*
PhAC	*pharmaceutically active compound*
qPCR	*quantitative polymerase chain reaction*
SBR	*sequencing batch reactor*
SFX	*sulfamethoxazole*
SRT	*solids retention time*
TN	*total nitrogen*
TP	*transformation products*
TSS	*total suspended solids*
UPLC	*ultra performance liquid chromatography*
UV	*ultra violet*
WWTP	*wastewater treatment plant*

Chapter 1
Problem statement

Over the past century, scientific evolution has led to major breakthroughs in pharmaceutical developments that have consequently spread and greatly improved human life. Their presence in our water bodies has been a fact ever since humans began taking drugs. However, coupled with a growing human population and the proliferation of drugs have meant that the loading of pharmaceutical compounds into the environment, which acts as a final pharmaceuticals sink, has significantly increased.

Pharmaceutical compounds are a structurally diverse class of emerging contaminants that have been detected worldwide, particularly in surface waters, groundwater, and drinking water (Petrovic *et al*., 2010). The enigma of pharmaceuticals appearing in drinking water has especially alarmed society, pharmaceutical company stakeholders and industry regulators, despite the fact that relatively few pharmaceuticals have actually been detected and then, only at much smaller concentrations than actual therapeutic doses (Benotti *et al*.,2009; Houtman *et al*., 2010).

Although adverse effects from trace levels of pharmaceuticals in tap water on human health are highly unlikely, the resulting consequences of such concentrations in treated wastewater on the aquatic ecosystems still remain vague. What differentiates aquatic organisms from humans in terms of adverse reactions and affects, is that aquatic organisms experience a continued exposure to contaminated aqueous media, compared to human exposure which is a more limited contact.

Furthermore, the synergy instigated by the large amount of compounds mixed together in the aquatic environment intensifies their harmful effects. There is great uncertainty regarding the modes of action of many of these compounds in aquatic organisms, and thus, without this knowledge of how compounds act (individually but also as a mixture), it becomes highly complicated to determine whether acute toxicity tests really provide the best assessment for adverse effects or not (Boxall *et al.,* 2004). Also the biomagnification of these compounds by the aquatic species play an important role since it increases chronic ecological risk and could also be a human health risk through the food chain. Likewise, the infinite interactions in ecosystems represent an additional drawback when working out reliable toxicological tests.

Pharmaceutical compounds can enter the environment via a number of pathways. Once there, their concentrations are attenuated by several processes such as dilution, adsorption to solids, microbial degradation, photolysis, or other abiotic transformations (Kunkel *et al*., 2012).

Common routes for these compounds to reach the environment are emissions from manufacturing sites, disposal of unused medicines in landfills, veterinary medicines, and disposal of carcasses of treated animals (Daughton and Ruhoy 2009; Heberer *et al.,* 2002). In addition, aquaculture facilities, run off from farmed fields, as well as releases into to soils when manure is spread, and irrigation with wastewater, are also important pharmaceuticals entering points into the environment. Nonetheless, in terms of magnitude, the foremost source of substances is households (excretion and disposal as detailed in Figure 1). The figure shows how, by direct disposal to trash and sewage, pharmaceuticals can reach surface and groundwater through both landfill and wastewater effluent.
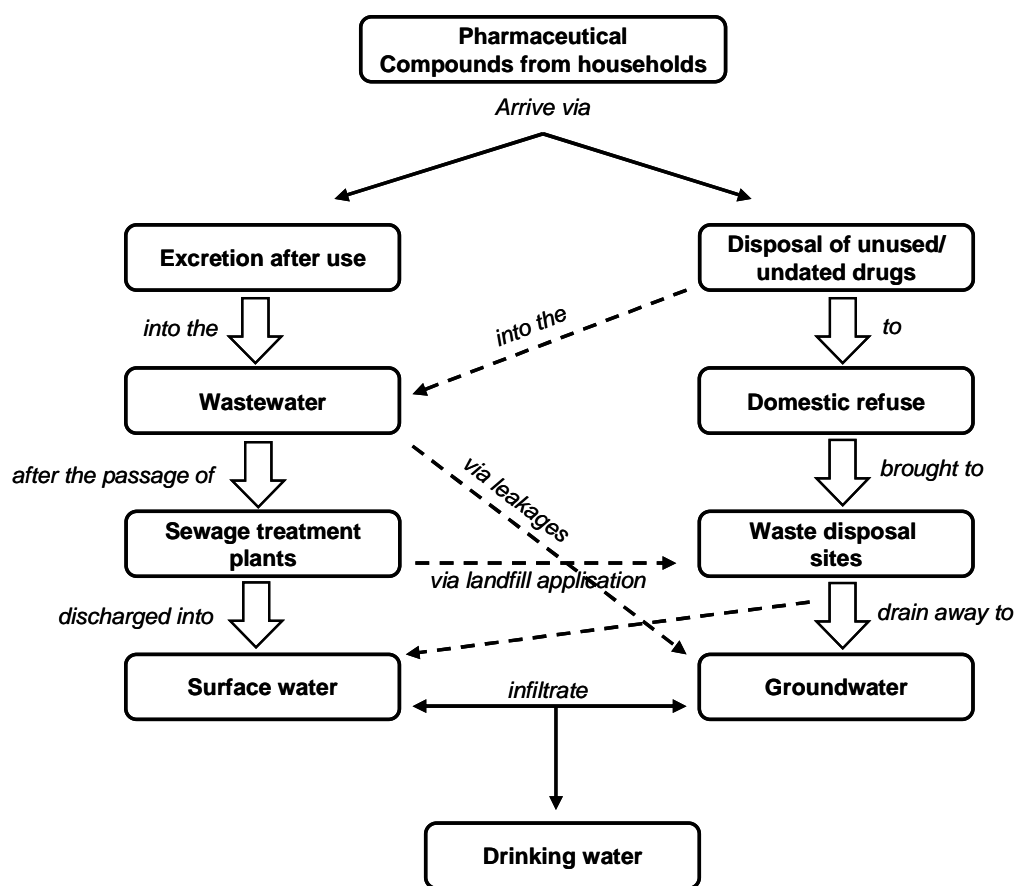
Figure 1: Representative sources and fate of pharmaceuticals in the environment (adapted from Ternes 2000)

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Conventional wastewater treatment processes can, and do, reduce the concentrations of pharmaceuticals in water. However, whether these compounds are amendable to treatment relies on their physicochemical properties and the key underlying removal mechanisms of a particular treatment process. The treatment efficiency is significantly affected by several operational factors such as the sludge retention time, the hydraulic retention time and environmental conditions, and also the temperature and light intensity (Fatta-Kassinos *et al.,* 2011; Le-Minh *et al.,* 2010; Castiglioni *et al.,* 2006).

Moreover, and given the wide range of properties represented by these substances, there is no single treatment process that works as an absolute barrier to pharmaceuticals. Focused on the aim to minimize their presence in wastewater, research studies have demonstrated that different treatment processes working as a whole system are needed to tackle this heterogeneity of compounds (Verlicchi *et al.,* 2012). In most cases, it can be achieved by advanced or tertiary treatment systems, which combine different processes such as biological processes coupled with chemical oxidation or activated carbon adsorption, physical separation followed by chemical oxidation, etc. Nonetheless, in most countries, only a small percentage of sewage treatment facilities have had these treatment upgrades. Also, as pharmaceuticals are either transformed, separated or mineralized during treatment, every process has some degree of secondary effect, such as the generation of transformation products.

There is an obvious potential for biological degradation of pharmaceutical compounds leading to a reduction of the parent compounds and/or their transformation products during wastewater treatment. It should be noted that the mere disappearance of the parent compound cannot be considered as a complete removal of the pharmaceutical, since the loss of the parent compound only indicates a certain degree of biotransformation and not necessarily the compound's mineralization. Only monitoring these resulting transformation products or end products of mineralization can provide useful information about the extent of biotransformation and its pathways (Deegan et al., 2011).

Some biodegradation may also occur during in-pipe transport to the sewage treatment plants, but most will probably occur in the secondary treatment when the compound is exposed to large concentrations of activated sludge microorganisms. Although there is a lack of data on the behaviour of pharmaceuticals, their fate is likely to be dependent on their physicochemical properties (e.g., chemical structure, aqueous solubility, octanol/water partition coefficient and Henry's law constants).

Estimation of degradation rates is a rather challenging process, especially for biodegradability, since biological processes and living organisms are involved (Struijs et al., 1995). Biodegradation studies and projects considering combinations of biodegradation and other removal processes have been conducted over a wide range of compound categories and therapeutic classes, as well as across different systems and scales of study (Onesios et al., 2008). Their behaviour during wastewater treatment will therefore comply with the biodegradation rates outlined by Joss et al., 2006 and there have been attempts to model the fate and behaviour of certain pharmaceutical compounds in the literature (Plósz et al., 2010; Benedetti et al., 2010).

Moreover, source control strategies should always be considered to efficiently reduce the burden on the environment when unknown or questionable occurrence in effluents is predicted or observed. Hence, prevention becomes a long-term control imperative (Kummerer et al., 2009; Lubrick et al., 2008).

While initial regulations were established to protect consumers from identified chemicals and microbial risks with evident toxicological endpoints (priority substances), substances known as "emerging contaminants" with less toxicology and occurrence data available are usually left out of the regulation process until adequate evidence is accumulated. Nevertheless, and even though there are currently no regulations limiting the levels of pharmaceuticals in wastewater or drinking water, the United States Environmental Protection Agency has added some pharmaceuticals to the most recent contaminant candidate list (CCL 3). However, only four of the compounds on the list are exclusively used as human pharmaceuticals: three birth control substances and one antibiotic, erythromycin (U.S: EPA, 2009a). In a European context, the WFD daughter Directive 2008/105/EC on environmental quality standards of water established the EU List of Priority Substances of 33 pollutants. In January 2012, the EU Commission proposed adding 15 chemicals more to the list, even though it was later rejected. Among those chemicals, and for the first time ever, three pharmaceutical compounds were considered, i.e. 17 alpha-ethinylestradiol (EE2), 17 beta-

estradiol (E2) and diclofenac. The proposal does not bring into question the therapeutic value of these pharmaceuticals; rather it addresses the potential harmful effects of their presence in the aquatic environment. Another international water research alliance, Global Research Coalition (GWRC), made up of 12 world leading research organisations, defined the group of pharmaceuticals: carbamazepine, sulfamethoxazole, diclofenac, ibuprofen, naproxen, bezafibrate, atenolol, ciprofloxacin, erythromycin and gemfibrozil, as a high priority level.

The application of ultra-sensitive analytical technologies to detect anthropogenic substances in water at one trillionth of a gram or less per litre will undoubtedly reveal that nearly every compound known to man will be detectable. The question is not whether these compounds occur, as they certainly will, but rather whether they pose a risk of harm to humans and wildlife that are exposed, giving some knowledge-based evidence that regulation is urgently needed (SOCWA).

Since the pharmaceuticals issue in the aquatic media is recognized as an environmental concern all over the world, it has opened an extensive area of research, including, among others, their chemical identification and quantification; elucidation of transformation pathways when present in wastewater treatment plants or in environmental matrices; assessment of their potential biological effects; and development and application of advanced treatment processes for their removal and/or mineralization. Over the last decade, the scientific community has embraced research in this specific field and the outcome has been immense (Fatta-Kassinos *et al.,* 2011).

Despite this, a number of unanswered questions exist and still there is much room for development and work towards a more solid understanding of the pharmaceutical removal, biodegradation and effects on the overall wastewater treatment system. Therefore, biodegradability studies become of high importance.

OUTLINE

- ➢ Large amounts of pharmaceuticals are used in human and veterinary medicine and are expected to continue to increase as the population burgeons.

- ➢ These compounds reach the aquatic environment, where their concentrations can reach microgram per litre levels, mainly through wastewater treatment systems via many different sources.

- ➢ There is still little experimental evidence showing their harmful effects once in the aquatic ecosystem, which becomes of concern particularly in view of the increasing use of pharmaceutical compounds and the importance of freshwater resources.

- ➢ Although some predictions can be made based on their physico-chemical properties, pharmaceuticals display a variety of removal efficiencies during wastewater treatment and their fate and behaviour are not yet sufficiently clear.

Chapter 2
Objectives

The main objective of this thesis was to study, throughout a multi-scale approach, the occurrence, removal and impact of pharmaceutical contamination in wastewater treatment plants and river systems.

To accomplish this main goal, several sub-objectives were differentiated as follows:

- To diagnose a real local scenario in terms of pharmaceutically active compounds (PhACs) levels encountered, not only their removal within a WWTP but also their impact into the receiving river waters. And to gain a deeper insight into:

    - How the WWTP operation and different treatment steps work towards the removal of these substances.

    - How the river is affected by the WWTP discharge and its dilution capacity role.

- To understand how a single compound is biodegraded in lab-scale activated sludge systems, in particular on:

    - The estimation of the kinetics involved, according to the biodegradation rates.

    - The quantification of the transformation products formed and their later degradation.

    - The modelling of the parent compound and the transformation products' behaviour.

- To study how an activated sludge pilot-plant system is affected by long-term exposure to a specific PhAC, and so obtaining some specific knowledge on the:

    - Microbial community changes.

    - Antibiotic resistance gene phenomenon.

    - Antibiotic removal efficiencies and side effects in the biological nutrient removal performance.

- To evaluate whether proteomics can be used when tracking proteins related to micropollutant degradation in wastewater, expressly focusing on:

    - How reliable the technique applied in activated sludge as a matrix is.

    - Detecting any possible protein hypothetically involved in the biodegradation process of PhACs.

    - Elucidate, if possible, the biodegradation pathway of the target compound, via the proteins identified as potentially responsible for this process.

Chapter 3
Research approach

To accomplish the previously stated objectives, the research approach of the present thesis has been divided into three different blocks, as follows (see also the illustration Figure 2):

BLOCK I: Real scenario diagnosis

Chapter 4: **"Pharmaceutical occurrence in a full-scale WWTP with significant industrial contribution and its impact on the river system"**

- *What pattern of pharmaceutical levels do we find in an industrialised area? How are they removed within the WWTP? What is the impact of this effluent discharged on the nearby aquatic environment?*

The main goal of this block was to reach a deeper understanding of the occurrence and fate of a wide range of pharmaceutical compounds in a local WWTP facility context. The initial characterization of the influent together with the evaluation of the different wastewater unit processes (including the tertiary treatment) lead to a proper removal efficiencies study, which was then complemented with the assessment of the impact of this discharge on the river water. Thus, a holistic work was carried out over three different sampling campaigns, which covered different seasons throughout the year, and enabled a perspective picture, from which the most representative pharmaceuticals were chosen for further detailed study.

BLOCK II: Lab and pilot-plant scale experiments with targeted compounds

Chapter 5: " **Removal of ibuprofen and its transformation products: Experimental and simulation studies"**

- *How ibuprofen is degraded in activated sludge systems, combining different concentrations of total suspended solids and ibuprofen, and which were the main transformation products formed? Which kinetics could be adjusted to the obtained degradation rates? Which model could be proposed to encompass the parent compound and the transformation products' behaviours?*

The main goal of this chapter was firstly, to focus on a single compound and its transformation products (in this particular chapter ibuprofen was chosen because of its well known biodegradation within the biological treatment and also its identified TPs), and secondly, to perform several batch studies in order to investigate its degradation in activated sludge systems and the dynamics of its main transformation products. The innovative aspect of this work was the assessment of the kinetics involved, not only of the parental compound but also of the transformation products. Moreover, a model was constructed that demonstrated the different behaviour when comparing the parental compound with the resultant TPs.

Chapter 6: **"Effects on activated sludge bacterial community exposed to sulfamethoxazole"**

- *Does the microbial community change after continuous antibiotic exposure? How do the resistance genes develop if they do so? Is there any side effect on the BNR performance?*

The main goal of the chapter was to gain a deeper insight into the microbiological changes in a pilot-plant scale reactor, which was started up and conditioned with the presence of the antibiotic sulfamethoxazole. In this case the antibiotic was chosen according to its variable biodegradation and the concern about the antibiotic's resistance genes proliferation. The idea embraces different scales as not only was the microbial community studied, but so was the BNR performance and the antibiotic degradation profiles.

BLOCK III: Proteomics approach experiments

- *Is proteomics a useful tool to investigate proteins related to the pharmaceuticals biodegradation mechanisms/pathways? How reliable is this technique in tracking proteins and how does it work with activated sludge as a matrix and with pure strain cultures?*

The main goal of this block was to evaluate the feasibility of the use of proteomics for the investigation of the biodegradation of pharmaceuticals in environmentally relevant conditions, i.e., in the low ppb range. Successful visualization of the differences in protein expression, when analyzed for individual pharmaceuticals, is presumed to aid the clarification of the biodegradation pathways, as this whole proteome contains the proteins responsible for the target PhAC biodegradation process. The idea behind is that, once the specific proteins involved in the biodegradation are known, biodegradation pathways may be able to be elucidated.

Chapter 7: **"Exploring the potential of applying proteomics for tracking bisphenol A and nonylphenol degradation in activated sludge"**

This first chapter tried to prove the feasibility of the adapted methodology protocol in activated sludge samples when tracking certain enzymes responsible for two specific micropollutant biodegradation (bisphenol A and nonylphenol). In this case, the target compounds were chosen for their well known biodegradation pathway and the role of the two enzymes involved in it. From this point, the technique's capability to work with activated sludge as a matrix and seek proteins of interest is demonstrated. Thus, further experiments could be carried out with pharmaceutical compounds to finally come up with potential proteins responsible for their biodegradation.

Chapter 8: **"Proteomics reliability for micropollutant degradation insight into activated sludge systems"**

Within this chapter, the use of a single compound (ibuprofen) allowed the technique to be self-proven when tracking specific proteins in activated sludge which are subjected to experience any overexpression during the pharmaceutical biodegradation process. The challenge here was to work with a whole bacterial community as a matrix and a single PhAC exposure, since activated sludge implies a huge amount of different bacteria and thus, a major degree of uncertainty when analysing protein profiles and interesting patterns.

Chapter 9: **"Specific bacterial strain role: Sulfamethoxazole biodegradation experiments and proteome analysis"**

As a last important experiment, this chapter refers to a close-fitting context, in terms of reducing uncertainties from the proteomic approach. The main difference in this study, when compared with the others, was the use of a pure strain instead of the whole bacterial community from activated sludge. But also, the use of another target pharmaceutical (in this case sulfamethoxazole), since its biodegradation, and more specifically, the ability of certain bacterial cultures, has recently been investigated.

Therefore, the main goal of this chapter was to evaluate the usefulness of proteomics to work with a pure strain and a single target compound, and finally to discuss its relative role when achieving the final aim of gaining insights into pharmaceutical biodegradation pathways, as an alternative methodology.

A multi-scale approach has been needed to deal with the previously stated blocks. To better visualize the route followed and the different experiments performed, see the following scheme (Figure 2).
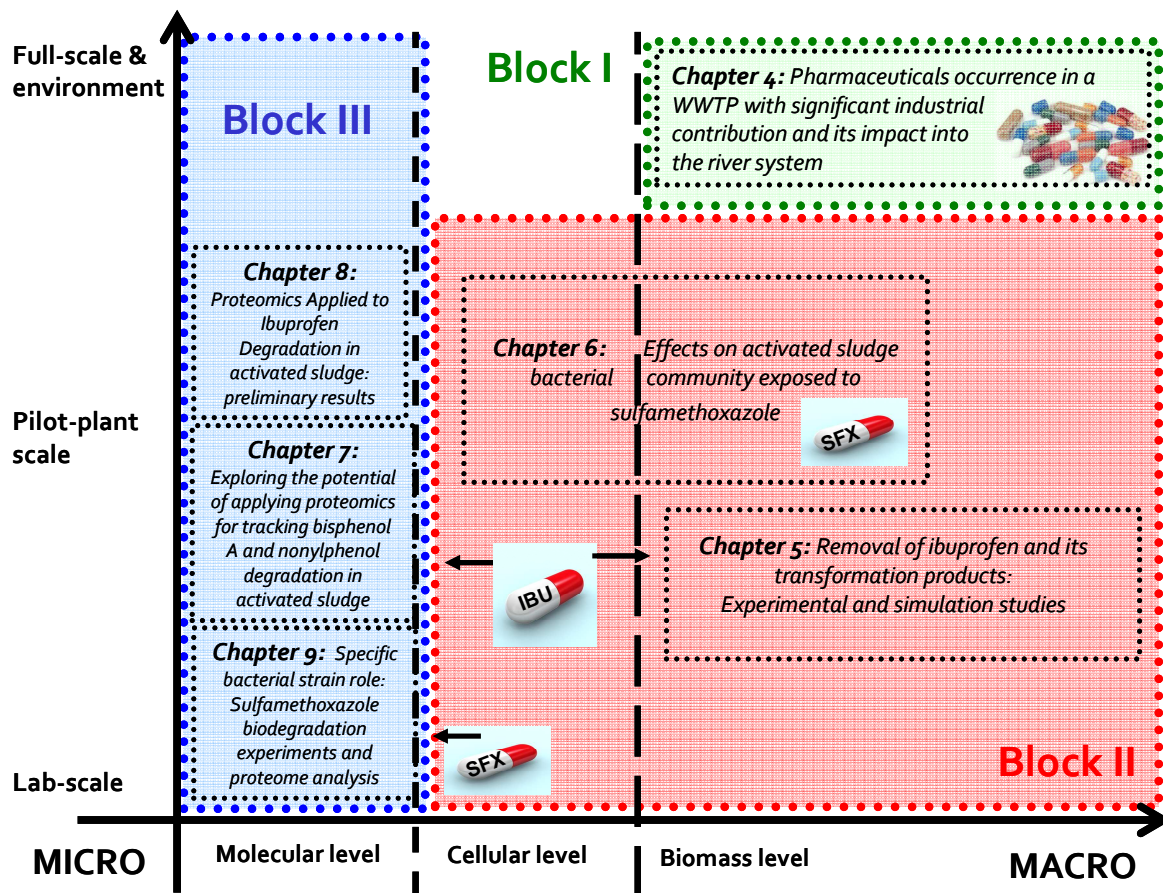

Figure 2: Scheme of the approach applied within the thesis and the chapters' distribution.

The different blocks and chapters of the thesis are placed depending on two main parameters. On the horizontal axis, the experiments are split into their micro or macro scale. The micro approach goes from molecular levels (proteins) to cellular level (specific bacterial communities), while aggregate biomass levels belong to the macro approach.

On this axis under the micro scale approach, the key point was that since the microbial functionality can be characterized either by the analysis of transcripts (bacterial community) and/or proteins (proteomics), in the present work it would be interesting to consider both, when working towards the accomplishment of a common goal. This combination of molecular and biochemical tools, targeting functional genes related to pharmaceutical biodegradation, allows the dynamics of the functions, and the associated communities in activated sludge systems to be followed. Furthermore, the choice of proteomic technique potential, was taken under the assumption that proteins present the advantage that they can confer insight into actual functionality, and hence are better markers for microbiological activity than DNA or even RNA.

With the macro scale view, the aim pursued was to deal with all the aspects concerning the operation in the wastewater treatment, rather than the microbiology involved. This covers the study of the biodegradation rates under different conditions and the transformation products dynamics, as well as, the kinetics involved in the biodegradation process, and the way to reflect this data in predictive models.

On the other hand, on the vertical axis the different experiments are located according to their scale and degree of specificity in terms of bacterial communities or pure culture strains, i.e. moving from lab-scale experiments to full scale WWTP and river sampling campaigns in real scenarios. Moreover, the transition between the mixture of compounds encountered in the real scenario and the single compounds studied in different chapters is also emphasized in the figure. This relies on the need to work with single pharmaceuticals at any one time in order to focus on specific aspects, diminish uncertainty and increase the accuracy of the results obtained.

As can be appreciated from the scheme, many different disciplines have been applied in this thesis in order to gain a wider perspective of the problem. Thus, the intrinsic idea was to cover a wide range of experiments which led to a global picture of the problem (from a macro to a micro point of view), to finally be able to acquire consistent knowledge and keep on adding small parts in the larger puzzle of the pharmaceuticals issue.

Block I

REAL SCENARIO DIAGNOSIS

Chapter 4

Pharmaceuticals occurrence in a WWTP with significant industrial contribution and its impact into the river system

Pharmaceuticals occurrence in a WWTP with significant industrial contribution and its impact into the river system

N. Collado[a], S. Rodriguez-Mozaz[b], M. Gros[b], A. Rubirola[b], D. Barceló[b,c], J. Comas[a], I. Rodriguez-Roda[a,b], G. Buttiglieri[b,*]

[a] LEQUiA, Institute of the Environment, University of Girona, Campus Montilivi, E-17071 Girona, Catalonia, Spain.

[b] Catalan Institute for Water Research (ICRA), H2O Building , Scientific and Technological Park of the University of Girona, Emili Grahit 101, 17003, Girona, Spain.

[c] Water and Soil Quality Research Group, Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona 18-26, 08034, Barcelona, Spain

* Correponding author: gbuttiglieri@icra.cat

ABSTRACT

Occurrence and removal of 81 representative Pharmaceutical Active Compounds (PhACs) were assessed in a municipal wastewater treatment plant (WWTP) located in a highly industrialized area, with partial water reuse after UV tertiary treatment and discharge to a typical Mediterranean regime river. Water monitoring was performed in an integrated way at different points in the WWTP and river along three seasons.

Significant differences between PhACs therapeutic classes were observed in terms of influent concentration, removal efficiencies and seasonal variation. Conventional (primary and secondary) treatment was unable to completely remove numerous compounds and UV-based tertiary treatment played a complementary role for a significant number of them. Industrial activity influence was highlighted in terms of PhACs presence and seasonal distribution.
Even if global WWTP effluent impact on the studied river appeared to be minor, PhACs resulted widespread pollutants in river waters. Contamination can be particularly critical in summer, when water flow decreases significantly as typical of water scarcity area.

Keywords: micropollutants; WWTP; pharmaceuticals; water scarcity area; UV treatment; river waters.

1.   Introduction

In the European Union around 3,000 different pharmaceutical active compounds (PhACs) belonging to different therapeutic classes are used in medicine. In particular Spain occupied fifth position in the European PhACs market in 2010 (IMS Health; www.farmaindustria.es).

Within the vast array of anthropogenic contaminants reaching the water bodies, PhACs, are among the ones with the major input into the environment due to their high consumption and some international organizations have shown their concern on regulating the presence of these compounds. Until now, PhACs are not included in the list of 33 priority substances regulated by the European Parliament Directive 2008/105/EC, which defines maximal tolerated concentration in inland and other surface water (EC, 2008) and thus, no environmental quality standards are stipulated for them. However, substances discharged into a basin should be controlled, as the same directive clearly establishes. United States Environmental Protection Agency has added some pharmaceuticals (erythromycin, nitroglycerin, quinoline, ethinyl estradiol, and other hormones used in drug formulations) are now on the Drinking Water Contaminant Candidate List (U.S. EPA, 2012). Also the Global Water Research Coalition, in an attempt to develop a common list of pharmaceuticals relevant to the water cycle, has included carbamazepine, sulfamethoxazole, diclofenac, ibuprofen, naproxen, bezafibrate, atenolol, erythromycin and gemfibrozil as Class 1 compounds, corresponding to high priority pharmaceuticals (GWRC, 2008).

The main route of PhACs into the environment is through ingestion, further excretion and load into urban wastewater systems via sewers and discharge into the aquatic media, mainly as a consequence of their incomplete removal in wastewater treatment plants (WWTPs) (Buttiglieri and Knepper, 2008). Other PhACs exposure pathways are pharmaceutical industries and hospital effluents, disposal of unused medicines both directly into the domestic sewage system and via burial in landfills and land disposal of sewage sludge (Daughton and Ternes, 1999).

WWTPs in Spain usually comprise only primary and secondary treatments, with the latter based on conventional activated sludge, whereas tertiary treatments are seldom applied (Gros et al., 2010). On the contrary, most of the European WWTPs do include tertiary treatment. Advanced oxidation technologies have been in some cases investigated for the elimination of PhACs by the use of ozone (De Witte et al., 2009),

different chemical oxidants (Sharma 2008) and sonolyis (Hartmann et al., 2008). Some studies have considered the effect of UV radiation in the removal of some of them (mainly analgesics, antiarrhythmia agents and antibiotics) and concluded that UV was not effective enough for the quantitative removal of the studied PhACs from water (Nakada et al., 2005, Benotti et al., 2009, Kim and Tanaka et al., 2009 and Yuan et al., 2009). The effectiveness of UV processes for a larger number of pharmaceuticals should therefore be investigated together to their coupling with biological treatments.

Levels of PhACs in river water depend not only on the loads from WWTPs but on river flow, which will thus lead to a different degree of dilution of pharmaceutical contamination. These compounds are then transported by river water and can suffer different attenuation processes such as biodegradation, photodegradation and adsorb to suspended particulate matter or accumulate in sediments (Ferreira da Silva et al., 2011). Mediterranean river basins management urges more attention due to their particular hydrology (low winter and summer discharges and periodically floods in spring and autumn) as well as to the continuous human pressure on resources and the ecosystem. Characterized by low flows during normal conditions and extraordinary peak events that periodically reset the system dynamics they are, in fact, more prone and sensible to environmental stress. It is also important to stress that wastewaters deriving from pharmaceutical industry can have a significant impact on the pollution of water bodies, and thus, investigations focused on that areas become of high interest.

On this context, the occurrence and removal of 81 representative PhACs were assessed in an integrated way in a municipal WWTP (located in a highly industrialized area and which included a UV tertiary treatment to reuse part of the effluent) and in the Ter River, a water body with typical Mediterranean regime. Both, removal rates in WWTP and attenuation factors in river were evaluated for different PhACs at different sampling points within the different season campaigns to assess the impact into the receiving water bodies.

The innovative aspects of this work is the study on the biological process coupled with the UV tertiary treatment and the subsequent integrated balance of the obtained removal efficiencies for a great number of pharmaceutical compounds in a WWTP with significant industrial contribution. Additionally, although occurrence of some PhACs in the Ter River was already studied before by Calderón-Preciado et al., (2011), our study is the first one focusing in seasonal attenuation of PhACs compounds along the river as

well as the first to provide extensive and integrated WWTP-river data on the occurrence of such a large number of them.


2.   Materials and methods

2.1.   Sampling site and sample collection

Wastewater samples were collected from the municipal WWTP at Celrà (Catalonia, Spain) (20 000 equivalent inhabitants), with an hydraulic retention time (HRT) of 48 hours and a sludge retention time (SRT) of 20 to 22 days. Average removal for COD and nitrogen was around 90 and 80% respectively. Celrà WWTP has 80% flow industrial contribution from a nearby industrialized area with several pharmaceutical industries, which have their own wastewater treatment process before discharging their effluents to the WWTP of study. This facility consists of a conventional primary and secondary biological treatment followed by a UV-based tertiary treatment, which is able to provide high quality water for internal reuse and other agricultural purposes. While the UV dose required for typical disinfection is 40-140 mJcm^{-2} (Pereira et al., 2007) the studied WWTP used 40 mJcm^{-2} from a medium pressure UV lamp emitting light at 254 nm.

WWTP sampling points are indicated in Fig. 1. 48-hours samples were collected both, at the WWTP inlet, before the primary treatments, and at the outlet of the secondary treatment, with an interval of 1h, by means of an auto-sampler, and a composite and flow proportional sample was finally obtained.

9-hours composite samples were manually taken, before and after the UV, treatment, since tertiary treatment was only applied during working hours. River water samples were taken from the Ter River (yearly average flow rate of approximately 25 m^{3}s^{-1}), which receives along its watershed discharges from metallurgic, textile, tannery, food, chemical and pharmaceutical industries, as well as raw sewage inputs from small adjacent communities. 3-hours composite samples were manually taken in the three different sites along the river: 100 m upstream (A), at the WWTP discharge point (B) and 1 km downstream (C) (Fig. 1). Between sites B and C no other discharge is present and at site C an homogeneous mixture of effluent water with river water was expected.
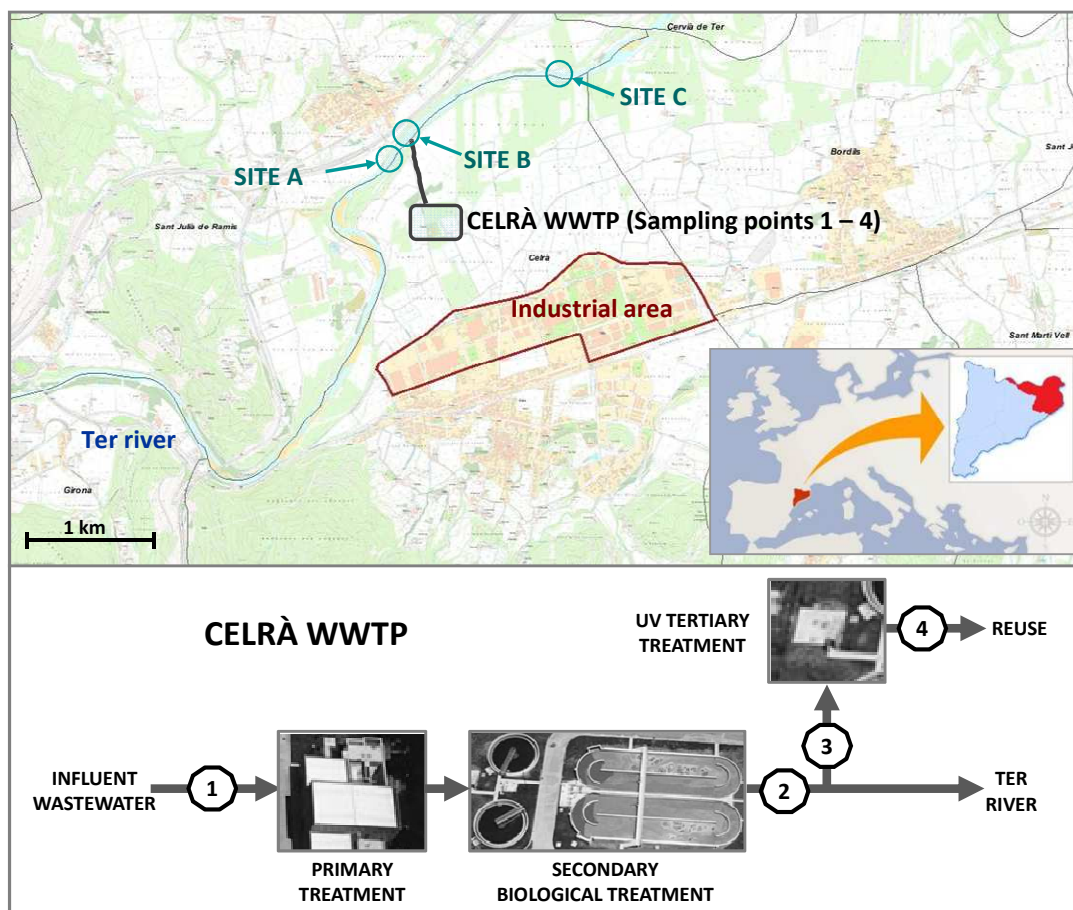
Fig. 1 – Ter River sampling sites (indicated with letter A, B and C) and Celrà WWTP location on the top part (image modified and adapted from **http://www.ddgi.cat**). Celrà WWTP scheme and sampling points (indicated with the number 1, 2, 3 and 4) in the bottom part.

Amber glass bottles pre-rinsed with ultrapure water were used for sample collection. Wastewater and river samples were filtered through 1 µm glass fibre filters followed by 0.45µm nylon membrane filters (Whatman, U.K.) and frozen until analysis at -20°C.

2.2. Chemicals and reagents

All pharmaceutical standards were of high purity grade (>90%). Compounds were purchased or either from Sigma–Aldrich, from the US (USP), from the European (EP) Pharmacopeia and from Toronto Research Chemicals (TRC). Some substances were purchased as hydrochloride salts, as sodium salts, as calcium salt, as hydrobromide salt, as tartrate, as besylate, as potassium salt, as hydrogen sulfate and as hemisulfate. Both individual stock standard and isotopically labelled internal standard and surrogate solutions were prepared on a weight basis in methanol (at a concentration of 1000 mgL⁻

[1]), except ofloxacin and ciprofloxacin, which were dissolved in methanol adding 100 μL of NaOH 1 M, as described by Ibanez et al., 2009 and cefalexin, which was solved in HPLC grade water, as indicated by Kantiani et al., 2009 since these substances are slightly soluble or insoluble in pure methanol. After preparation, standards were stored at −20 ∘C. Special precautions have to be taken into account for tetracycline, which has to be stored in the dark in order to avoid its exposure to the light, since it has been demonstrated that tetracycline antibiotics are liable to photodegradation (Eichhorn et al., 2004). Fresh stock antibiotic solutions were prepared every three months while fluoroquinolone antibiotics were prepared monthly due to their limited stability. Stock solutions for the rest of substances were renewed every six months. Working standard solutions, containing all pharmaceuticals, were also prepared in methanol/water (10:90, v/v) and were renewed before each analytical run by mixing appropriate amounts of the intermediate solutions. Separate mixtures of isotopically labelled internal standards, used for internal standard calibration, and surrogates, were prepared in methanol and further dilutions were also prepared in a methanol/water (10:90, v/v) mixture.

The cartridges used for solid phase extraction were Oasis HLB (60 mg, 3 mL), Oasis HLB (200 mg, 6 mL), from Waters Corporation (Milford, MA, USA). Glass fibre filters (1 μm) and nylon membrane filters (0.45 μm) were purchased from Whatman (U.K.). HPLC grade methanol, acetonitrile, water (Lichrosolv), HPLC grade methanol, acetonitrile, water (Lichrosolv) and formic acid 98% were supplied by Merck (Darmstadt, Germany). Ammonium hydroxyde, hydrochloric acid and ethylenediaminetetraacetic acid disodium salt solution (Na2EDTA) at 0.1 molL^{-1} were from Panreac. Nitrogen for drying was from AbellóLinde S.A. (Spain) and it was of 99.9990% purity. A Milli-Q-Advantage system from Millipore Ibérica S.A. (Spain) was used to obtain HPLC-grade water.

2.3. Analytical method for pharmaceuticals

Target pharmaceuticals were selected based on their occurrence and ubiquity in the aquatic environment, according to the scientific literature, as well as their high consumption in Spain. They included different therapeutic classes, namely, analgesics and anti-inflammatories (NSAIDs) (14), lipid regulators and cholesterol lowering statin drugs (5), psychiatric drugs (15), histamine H1 and H2 receptor antagonists (5), ß-blocking agents (6), diuretics (3), antidiabetics (1), antihypertensives (4), antiplatelet

agent (1), prostatic hyperplasia (1), to treat asthma (1), anticoagulant (1), X-ray contrast agent (1), antihelmintics (3), synthetic glucorticoid (1), for sedation and muscle relaxation (1), tranquilizers (2), antibiotics (13) and calcium channel blockers (3). A multiresidue analytical method was previously developed to measure the 81 pharmaceuticals selected in both surface and wastewaters, as described elsewhere (Gros et al., 2012). Briefly, after filtration, a suitable volume of a Na_2EDTA solution, having a concentration of 0.1 M, was added to the different types of water to achieve a final concentration of 0.1% (g solute/g solution). Water samples were automatically extracted by a GX-271 ASPEC^{TM} system (Gilson, Villiers le Bel, France) using Oasis HLB (60 mg, 3 mL) cartridges for all the matrices. SPE cartridges were conditioned with 5 mL of methanol followed by 5 mL of HPLC-grade water at a flow rate of 2 mLmin^{-1}. 25 mL of influent and 50 mL of effluent wastewater were loaded onto the cartridge at a flow rate of 1 mLmin^{-1}, while 100 mL of river were loaded at 2 mLmin^{-1}. After sample pre-concentration, cartridges were rinsed with 6 mL of HPLC grade water, at a flow rate of 2 mLmin^{-1}, and were dried with air for 5 min, to remove excess of water. Finally, analytes were eluted with 6 mL of pure methanol at a flow rate of 1 mLmin^{-1}. Extracts were evaporated to dryness under a gentle nitrogen stream and reconstituted with 1 mL of methanol/water (10:90, v/v). Finally, 10 μL of a 1 ngμL^{-1} standard mixture containing all isotopically labelled standards were added in the extract as internal standards.

Chromatographic separations were carried out with a Waters Acquity Ultra-PerformanceTM liquid chromatograph system, equipped with two binary pumps systems (Milford, MA, USA) using an Acquity HSS T3 column (50 mm × 2.1 mm i.d., 1.8 μm particle size) for the compounds analyzed under positive electrospray ionization (PI) and an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm particle size) for the ones analyzed under negative electrospray ionization (NI), both purchased from Waters Corporation. The UPLC instrument was coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. Two transitions, between the precursor ion and the most abundant fragment ions, were recorded for each compound by using the Scheduled MRM^{TM} algorithm, without losing sensitivity and achieving reproducible chromatographic peaks. All data were acquired and processed using Analyst 1.5.1

software. Further details about the method and method performance can be found elsewhere (Gros et al., 2012).

3. Results and discussion

3.1. Characterisation of the influent and effluent wastewater

40 different pharmaceuticals out of the 81 monitored were detected in the sampling campaigns, but only 21 were present in all the influent samples in the three sampling campaigns (Table 1). Additional 14 compounds out of the 40 investigated were detected in at least two of the three sampling campaigns, and five in only one campaign. Concerning therapeutic groups, non-steroidal anti-inflammatory drugs (NSAIDs) were the most ubiquitous compounds, in terms of both individual concentration and frequency of detection, throughout the three monitoring campaigns. The highest value was found in wastewater influent for acetaminophen (individual concentrations up to almost 13 μgL^{-1} in influent wastewater) followed by ibuprofen, naproxen and salicylic acid. Lower but still significant levels were found for meloxicam, ketoprofen, tenoxicam piroxicam and diclofenac in influent wastewaters, ranging from 288 up to 916 ngL^{-1}. Conversely, NSAIDs concentrations in the outlets decreased considerably (particularly for the compounds at higher levels), with values from 10 ngL^{-1} for codeine up to 325 ngL^{-1} for meloxicam.

Table 1 – PhACs concentration in the WWTP influent, secondary effluent and for the inlet and outlet of the UV treatment. For the calculations, the non-detected compounds were considered as zero.

Therapeutic group	Compound	WWTP influent [ngL^{-1}]	WWTP effluent [ngL^{-1}]	UV influent [ngL^{-1}]	UV effluent [ngL^{-1}]
Analgesic and anti-inflammatories	Acetaminophen	12,955±11,225	40±61 [a]	29±41 [a]	23±31 [a]
	Ibuprofen	10,751±1,227	28±48 [a;b]	nd[a;b;c]	nd [a;b;c]
	Naproxen	7,661±2,418	247±132 [2]	227±129 [2]	157±100 [b]
	Salicylic acid	6,593±6,332	65±59	107±97	6±6
	Meloxicam	916±1,391 [c]	325±375 [c]	318±394 [c]	125±111 [c]
	Ketoprofen	506±558 [c]	146±220 [c]	144±237 [c]	110±164 [c]
	Tenoxicam	325±556 [c]	238±396 [c]	216±358 [c]	169±290 [c]
	Piroxicam	325±177	225±154	212±137	177±114
	Diclofenac	288±252 [b]	309±111	286±73	154±114
	Codeine	81±90	60±49	63±36	49±35
	Indomethacine	45±78 [a;b]	61±53 [a]	61±53 [a]	47±41 [a]
	Phenazone	26±44 [a;b]	10±11 [b]	13±11 [b]	7±9 [b]
Antibiotics	Ciprofloxacin	392±218	176±170	178±141	137±120
	Azithromycin	129±80	143±131	153±110	115±93
	Ofloxacin	128±75	118±74	164±40	99±45
	Clarithromycin	100±50	99±121	87±91	77±94
	Sulfamethoxazol	70±99 [a;b]	10±13 [a;b]	11±16 [a;b]	12±16 [a;b]

	Trimethoprim	54±56 [b]	7±6 [b]	5±5 [b]	1±2 [a;b]
	Erithromycin	15±18 [b]	18±18	17±17 [b]	15±20
Psychiatric drugs	Venlafaxine	4,108±4,902	2,659±2,668	2,488±2,351	1,806±1,737
	Paroxetine	592±918 [c]	179±195 [c]	190±228 [c]	62±54 [c]
	Citalopram	95±85 [b]	50±16	43±19	36±18
	Trazodone	75±115 [b]	39±64 [b]	41±68 [b]	24±39 [b]
	Carbamazepine	27±24 [b]	49±71 [b]	54±71 [b]	47±70 [b]
B-blocking agents	Atenolol	2,224±1,416	274±240	282±238	184±181
	Metoprolol	393±168	169±58	184±39	110±55
	Nadolol	27±45 [c]	18±20 [c]	5±8 [b;c]	nd [a;b;b]
Lipid regulators and cholesterol lowering statin	Gemfibrozil	1,009±90	184±111	173±97	115±98
	Bezafibrate	121±108 [b]	3±4 [c]	8±8 [c]	12±12
	Atorvastatin	97±84 [b]	9±16 [b;c]	10±18 [b;c]	3±5 [b;c]
Anti-hypertensive	Valsartan	1,511±703	99±46	92±29	62±41
	Irbesartan	281±175	246±246	230±187	205±208
	Losartan	211±89	162±189	151±152	128±148
Diuretic	Furosemide	1,901±855	288±94	358±118	271±109
	HCTZ	1,370±579	1,036±705	1,075±689	622±352
Antihelmintics	Levamisole	24±24 [b]	45±20	50±14	41±23
	Thiabendazole	nd [a;b;c]	4±7 [b;c]	4±8 [b;c]	1±2 [b;c]
Others	Ranitidine	1,165±1,250	176±74	170±34	113±78
	Iopromide	62±107 [b;c]	195±328 [c]	489±848 [b;c]	106±158 [c]
	Xylazine	58±100 [b;c]	31±53 [b;c]	30±51 [b;c]	7±13 [b;c]

[a] not present in the spring sampling campaign
[b] not present in the winter sampling campaign
[c] not present in the summer sampling campaign

Other groups showing considerably high total average concentrations were the antihypertensive valsartan, the β-blockers atenolol and metoprolol, the psychiatric drugs paroxetine and venlafaxine and the diuretic furosemide. While all of them presented similar individual concentrations (from around 0.5 to 2 μgL^{-1}), nadolol, citalopram and trazodone were found, generally, at levels one order of magnitude lower (from 27 up to 95 ngL^{-1}).

Other significant and ubiquitous groups were lipid regulators, cholesterol lowering statin drugs and antibiotics. Concerning the antibiotics, sulfamethoxazole, ofloxacin, ciprofloxacin, clarithromycin, azithromycin and trimethoprim were the compounds with major significance (up to 392 ngL^{-1}). Psychiatric drugs (except for venlafaxine and paroxetine with levels at the μgL^{-1} range), were found at much lower levels, especially carbamazepine, at 27 ngL^{-1}. Finally, X-ray contrast agents and antihelmintics were observed at lower levels.

The influent characterization of the studied WWTP does not illustrate a significant overall higher concentration of PhACs with respect to previously published studies of

WWTP (Gros et al., 2010, Jelic et al., 2011, Verlicchi et al., 2012) with mainly domestic wastewater. Even so, paroxetine was detected (while usually it is not) and venlafaxine was found at considerable higher concentration (around 4,000 ngL^{-1}) than usual. It seems reasonable to attribute this, to the discharge of the industrial activity in the studied area, which may explain the abnormal high levels found for these compounds. These results are in good agreement with the finding by Gasser et al 2012, who detected levels of venlafaxine up to 4,700 ngL^{-1} in a Jerusalem WWTP located close to a large industrial plant, compared to average levels of 200 ngL^{-1} from domestic wastewater.

Taking into account the three sampling campaigns separately (

Table 2) a lower total PhACs concentration in winter sampling campaign than spring and summer was noticed, in contrast with previous results (Castiglioni et al., 2006). Additionally, considering the therapeutic class distribution in the influent wastewater (

Table 2) a much lower contribution of NSAIDs, psychiatric drugs and H1 and H2 receptor antagonists was measured in winter time. As to NSAIDs, a higher concentration was expected in winter (e.g. due to flu epidemics) (Vieno et al., 2005) but this was not the case. Consequently their presence in influent wastewater might be linked not only to municipal contribution but also to industrial production in periods other than winter.

Table 2 – Global PhACs influent and secondary effluent concentration (ngL^{-1}) in the three sampling campaigns separated by therapeutic class.

Therapeutic group	ngL^{-1}					
	January		**May**		**August**	
	influent	effluent	influent	effluent	influent	effluent
NSAID	22 365	2,575	50 894	1,298	48 154	1,390
Psychiatric drugs	9,833	300	2,455	212	2,403	76
Histamine H1 and H2 receptor antagonist	2,597	5,803	608	819	290	2,303
Lipid regulators	1,000	259	1,283	118	1,398	150
ß-blocking agents	1,706	803	4,145	196	2,080	382
Diuretic	3,658	2,083	3,466	539	2,689	1,349
Anti-hypertensives	1,808	1,055	1,379	164	2,823	304
Antibiotics	730	11	677	573	1,185	0
X-ray contrast agent	nd	68	186	46	nd	33
Anti-helmintics	nd	0	48	92	23	0
Sedation - muscle relaxation	nd	1,062	174	335	nd	307
Total	43 697	14,019	65 315	4,391	61 045	6,294

3.2. Pharmaceuticals removal during conventional wastewater treatment

Different removal rates due to primary and secondary treatment in the WWTP were observed for each therapeutic group (Fig. 2):

Low removal (0-30%) or increasing concentration: Levels of some compounds increased during the treatment and were, thus, higher in effluent wastewaters in at least one of the sampling campaigns, while some other few compounds exhibited low removals. In some cases (e.g. thiabendazole, erythromycin, levamisole), a low removal might be related to low ngL^{-1} detected concentrations, more susceptible to sampling and analytical error. In other cases it might be due to persistence, recalcitrance, conjugation–deconjugation effects (Zorita et al., 2009; e.g. carbamazepine; Lequerq et al., 2009) or high removal variability, affecting average removal rates, at different operational or seasonal conditions (e.g. diclofenac; Buttiglieri and Knepper, 2008; Tambosi et al., 2010). HCTZ, was the only compound with an influent concentration higher than 1,000 ngL^{-1} (1,370 ngL^{-1}) that was not removed from wastewater in winter monitoring, (see Fig. 3). A similar behaviour was observed for tenoxicam and losartan with influent concentrations higher than 200 ngL^{-1}. Finally, irbersartan was only efficiently removed (62%) in the spring sampling campaign, while in the other two it was only partially (22%) or not removed at all. All the compounds in this category are not likely to be comprehensively eliminated by a conventional treatment, and additional treatment would be necessary to enhance their removal.

Medium removal efficiency (30-70%): The psychiatric drugs (venlafaxine, citalopram, trazodone and paroxetine), the analgesic (piroxicam, phenazone and meloxicam), the β-blocking agent (nadolol and metoprolol), the antibiotics ciprofloxacin and the sedation xylazine were partially degraded, presenting average removal efficiencies between 30 and 70%. Even though average removal near 70% can be considered in some cases satisfactory, pharmaceuticals included in this category generally showed, in the present work, an overall irregular removal during the three sampling campaigns. Moreover, in the case of some pharmaceuticals present at very high concentration in influent water, medium removal resulted anyhow in a incomplete elimination and a remarkable effluent concentrations. This was the case, for example, of venlafaxine (35 % average removal)

with an average concentration of 4,108 and 2,659 ngL^{-1} in influent and secondary effluent respectively.

High removal efficiency (>70%): A few NSAIDs (ketoprofen, naproxen, salicylic acid, acetaminophen and ibuprofen), some lipid regulators and cholesterol lowering statin drugs (gemfibrozil, atorvastatin and bezafibrate), the antibiotics sulfamethoxazole and trimethoprim, the β-blocking agent atenolol, the diuretic furosemide, the histamine receptor antagonist ranitidine and the antihypertensive valsartan, were highly removed in the system. As a consequence, a primary treatment followed by a conventional activated sludge system can be considered sufficient to obtain a satisfactory removal for these compounds and, in most of the cases, confirm the literature results (Verlicchi et al., 2012; Buttiglieri and Knepper, 2008). Note that this category included all the compounds with an influent concentration above 1,000 ngL^{-1} (with the exceptions of venlafaxine and HCTZ), resulting in a substantial total PhACs load reduction in the secondary effluent. In some other studies, a lower and more variable removal was observed for instance for ranitidine and bezafibrate with removal ranging from 10 to 75% (Jelic et al., 2011).

Also trimethoprim and atenolol were unusually highly removed, while in most of the published literature they are ranked in a moderate to low removal category (Gros et al., 2010). A possible explanation, already observed by Suárez et al., 2010, can be found in the role of the plant operational parameters like SRT and HRT. In this case study, while SRT was comparable to most WWTPs, the HRT of 48 hours was much higher than conventional WWTP and this could have induced a greater removal of atenolol. On the other hand, trimethoprim appears to be more influenced by the SRT than by the HRT (Suárez et al., 2010) and the high removal observed in our study cannot be explained by means of these parameters.
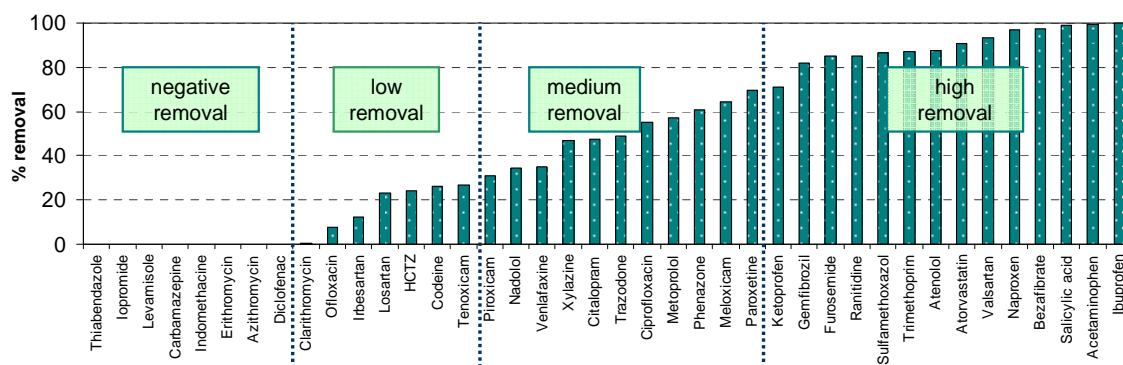
Fig. 2 - Average removal in the WWTP by primary and secondary treatment.

As observed in this study, several factors regarding WWTP operational parameters may influence in a great extent the PhACs biological treatment removal such as HRT, SRT, food to mass (F/M ratio), redox conditions and temperature (Suárez et al., 2008). HRT and SRT were maintained constant in the whole study. Nonetheless differences in the removal rates, in some cases very relevant, were observed within the three sampling campaigns (Fig. 3). F/M were 0.094, 0.041 and 0.018 $gCODgTSS^{-1}d^{-1}$ in winter, spring and summer respectively. A reduced F/M ratio, as it is the case of spring and summer time, may force microorganisms to metabolize also poorly degradable compounds and positively affect the elimination of compounds undergoing cometabolism (Gobel et al., 2007, Sipma et al., 2010). Nonetheless these effects are compound dependent and need more specific investigations. Taking into account that the sampling campaigns were carried out at different seasons, the temperature of operation can be considered a significant factor (Yu et al., 2013; Vieno et al., 2005).

A global and lower removal of 67.9% was calculated in January compared to 93.3% of May and 89.7% of August. For most of the compounds, a higher removal was observed in May and in August compared to January. Only for a few of them (tenoxicam, venlafaxine, nadolol and ranitidine) a decreasing removal with a temperature increase was observed (Fig. 3). Finally, for thiabendazole, iopromide, valsartan, naproxen, salicylic acid and ibuprofen no significant difference was observed (due to a negative removal or, conversely, to a very high removal).
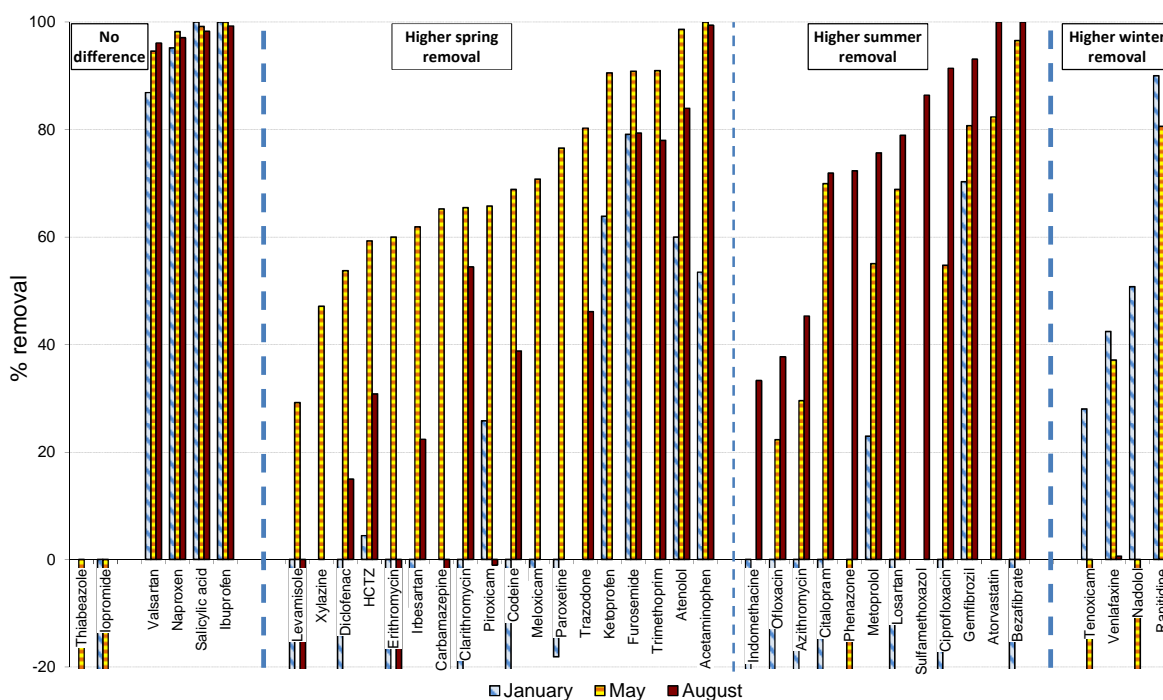
Fig. 3 – Primary and secondary treatment removal in three sampling campaigns. No difference in percentage removal was considered if the difference was below 10%.

Therefore, it can be stated that usually higher removal efficiencies were observed in summer periods in comparison with colder periods, as also concluded by Castiglioni et al. 2006. Biodegradation and sorption can be considered main pharmaceuticals elimination processes in WWTPs, the latter one to be considered minor, at least on first approximation, for most of PhACs (Urase and Kikuta, 2005). Biodegradation works with a minor efficiency at lower water temperature (Vieno et al., 2005). Nitrification is in some cases associated with high cometabolic degradation of emerging micropollutants (Fernandez-Fontaina et al., 2012; Clara et al., 2004). Nonetheless, in terms of nitrifying activity no seasonal influence was observed in the present study, with effluent ammonia lower than 2 mgNL^{-1} in the three sampling campaigns.

### 3.3. Contribution of UV-based tertiary treatment on pharmaceuticals' removal

The UV role after the biological treatment on the removal on the target PhACs was also evaluated in the three sampling campaigns. Taking only into account the UV step (inlet and outlet), the highest removal by UV radiation was detected for the following 14 PhACs: phenazone, thiabendazole, nadolol, tenoxicam, erythromycin, clarithromycin,

trimethoprim, xylazine, iopromide, metoprolol, diclofenac, paroxetine, atenolol and salicylic acid. All of them showed a removal higher than 80% by this photodegradation step in at least one of the three sampling campaigns. Thereby, the most UV degraded compounds were NSAIDs but also some antibiotics and ß-blocking agents. These findings are in good agreement with Salgado et al., 2012 who investigated the removal mechanisms of PhACs in a Portugal WWTP with UV radiation, throughout a 2-week sampling campaign.

Medium removal by UV radiation (meaning an average of 40-50%) was observed for trazodone, azithoromycin, codeine, carbamazepine, losartan, irbesartan, ofloxacin, ciprofloxacin, venlafaxacine, ranitidine, HCTZ, gemfibrozil, valsartan, meloxicam, furosemide and naproxen. And finally, the compounds poor removed by UV-based treatment were indomethacine, piroxicam, ketoprofen and acetaminophen, with removal percentages under 25%. These results are in agreement with Kim and Tanaka, 2009, which also revealed low UV photodegradabilities of some of these compounds in accordance with their quantum yields, with the exception of ketoprofen, poorly removed in our system.

The contribution of the tertiary treatment to the global removal of the WWTP was evaluated. The joint primary and secondary treatments efficiency was compared to the PhACs removal efficiency of all steps together, i.e. primary, secondary and tertiary treatments, and results are presented in Fig. 4. The further the compounds are from the symmetry axis, the more significant is the effect of the UV treatment on the global PhAC removal along the wastewater process.
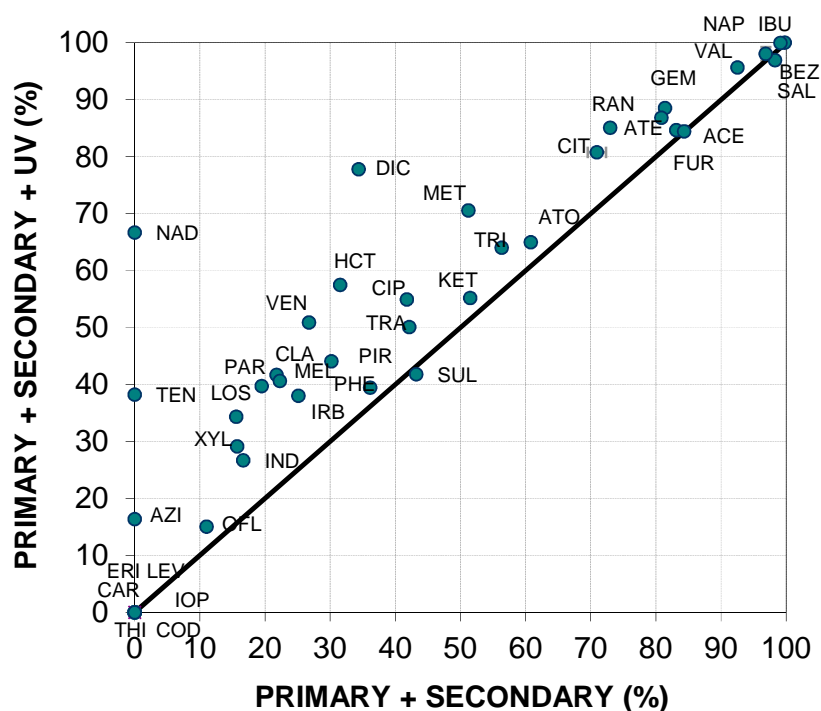
Fig. 4 – Removal efficiencies of the studied pharmaceutical compounds by joint primary and secondary treatment (x axis) and also taking into account UV treatment (y axis). The acronyms stand for the first three letters of PhACs name.

Some compounds (e.g. codeine, levamisole, iopromide, thiabendazole, erythromycin and carbamazepine) were not removed nor in the primary and secondary treatment nor in the UV treatment (bottom left corner in Fig. 4). Consequently, these compounds are to be considered recalcitrant and likely to be removed with other technologies than the here presented. On the other opposite, secondary treatment was enough to get a very high removal for a few other compounds (e.g. ibuprofen, naproxen, bezafibrate, salicylic acid, valsartan) and no information can be gained on their UV removal efficiency (upper right corner in Fig. 4).

For several compounds a partial removal up to the secondary treatment joint to a limited or no UV effect was observed (e.g. ofloxacin, sulfamethoxazole, furosemide, acetaminophen, ketoprofen, atovarstatin, phenazone). Results are in contrast for ketoprofen with medium removal obtained by Salgado et al., 2012 and, for sulfamethoxazole, with easy UV degradability reported by Kim and Tanaka, 2009. In this context, adjustments in the UV dose or contact time may be investigated to get higher removal.

A removal improvement by the UV treatment was observed for a few compounds (venlafaxine, tenoxicam, nadolol, meloxicam, HCTZ, diclofenac, metoprolol, paroxetine, clarithromycin, xilazine, etc.) and UV treatment can be considered relevant for their overall removal. There is little information on the removal of PhACs using UV treatment at real scale (Kim et al., 2009). Among these, it was observed that several PhACs with amide bonds ($RCONR_2$) in their chemical structures cannot be photolyzed easily with UV (Kim and Tanaka, 2009). Nonetheless, in contrast with this assumption, a high photodegradability for clarithromycin and diclofenac was observed, even in presence of an amide bond. Consequently, in accordance with Kim and Tanaka, 2009, the amide bond is not always the main site affected by UV energy during PhACs photodegradation.

These results suggest that, for the case study, the UV process coupled to primary and secondary treatment is not sufficient for the complete removal of several PhACs. Despite that, a significant additional removal was obtained for specific compounds which were not previously biologically removed. Thus, UV may represent and important polishing step for these pollutants (Salgado et al., 2012). More research is required to evaluate the influence of operative UV treatment parameters on one side and, on the other side, substances chemical characteristics and susceptibility to UV.

3.4. Entry of pharmaceuticals into the water cycle: occurrence in the Ter River and dilution mass balance

The PhACs presence in the river, the effect of the WWTP on the river water quality and the PhACs attenuation along the river (due to dilution, biodegradation, photodegradation, sorption processes and other possible mechanisms) were evaluated.

The total PhACs concentrations detected in the river waters, within the three different seasons on the different sampled points and the river flow, are presented in Fig. 5.
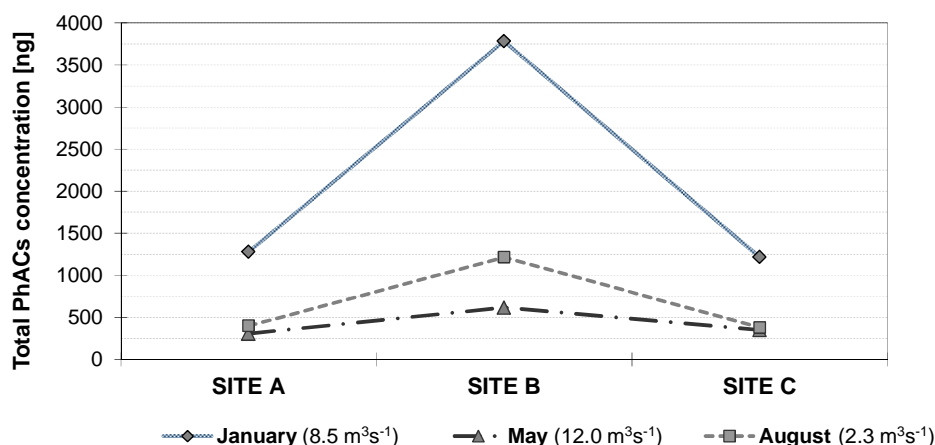
Fig. 5 – Comparison between mean monthly flow (m^3s^{-1}) with the sum up of all concentration levels of the pharmaceuticals detected in the three samples sites along the three sampling campaigns. Site A was 100 m upstream, site B at the WWTP discharge point and site C 1 km downstream. River flows are indicated in the x-axis.

The highest values of pharmaceuticals were observed in January in the three sites sampled in river Ter. These results were consistent with former research on WWTPs performance under seasonal variation with increased concentrations of pharmaceuticals in wintertime (Lacey et al., 2012; Sui et al., 2011; Vieno et al., 2005).

In Table 3 the concentration of PhACs in river water are individually presented for those compounds detected at concentrations above 20 ngL^{-1} at the WWTP discharge point (site B). The wide spectrum of pharmaceuticals found in river perfectly matched with the consumption patterns of the society. As a matter of fact, NSAIDs, cholesterol lowering stating drugs, antibiotics, antihistamines, antidepressants and antihypertensives are of major consumption in Spain (according to the National Health System). NSAIDs, even though highly removed within the wastewater treatment process, are anyway detected at significant concentrations in river waters due to extremely high influent concentrations and confirming them as pseudo-persistent pollutants in the environment.

A high variability, not only in concentration but also in the detection itself was observed. Seasonal related human consumption of specific PhACs and inconstancy in WWTP operation efficiency may contribute to this result. In May no more than nine compounds were detected at significant concentrations in the three sampling sites, while 18 and 19 compounds in the other campaigns (carried out in winter and summer respectively). Only three compounds (venlafaxine, HCTZ and gemfibrozil) were always detected in three sampling campaigns.

Just six PhACs compounds, out of the 81 of the present study, were measured in Ter River in previous studies (Calderón-Preciado et al., 2011). Ketoprofen and acetaminophen were under the respective LOQs in the river water (while in the present study up to 102 and 36 ngL^{-1} respectively). On the contrary, for the other common compounds higher concentration was always observed in Calderón-Preciado et al., 2011. In particular, ibuprofen was measured up to 303 ngL^{-1} (not detected here). Finally, diclofenac, carbamazepine and naproxen concentrations were up to 168, 274 and 444 ngL^{-1} respectively, up to one order of magnitude higher than those found in this work (Table 3).

PhACs were anyway considerably diluted when they enter river waters if compared to usual effluent wastewater concentrations. Average concentrations were between the low ngL^{-1} range and the high ngL^{-1} for all the samples in agreement to previous studies at rivers downstream WWTPs (Daneshvar et al., 2010; Radke et al., 2010). The highest levels were detected for venlafaxine, HCTZ and gemfibrozil (1,654, 841 and 159 ngL^{-1} respectively) even if only at site B, the closer to the WWTP. As regards to HCTZ, other authors detected levels from 47 up to 1,660 ngL^{-1} (Gasser et al 2012) and of 202 ngL^{-1} for gemfibrozil (Ferreira da Silva et al., 2011). Iopromide, irbesartan, piroxicam, ketoprofen, valsartan and naproxen were detected at levels around 100 ngL^{-1} and the remaining compounds under this level.

Table 3 – Highest detected compounds in river waters expressed in ngL^{-1}, emphasized in bold the common PhACs found in all three sampling campaigns. C$_{theor}$ corresponds to the individual theoretical concentration at site C taking into account the dilution mass balance calculations.

Compounds	January					May					August				
	A	B	C	Effluent WWTP	C$_{theor}$	A	B	C	Effluent WWTP	C$_{theor}$	A	B	C	Effluent WWTP	C$_{theor}$
Acetaminophen	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	26	36	33	111	27
Atenolol	46	92	38	529	48	nd	nd	nd	nd	nd	36	74	36	239	37
Azithromycin	0	29	0	287	1	nd	nd	nd	nd	nd	29	43	27	111	29
Carbamazepine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	5	24	5	130	6
Ciprofloxacin	0	24	0	370	1	nd	nd	nd	nd	nd	9	36	9	55	9
Codeine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	6.3	30	6	112	7
Diclofenac	7	30	19	347	9	nd	nd	nd	nd	nd	25	83	24	397	27
Furosemide	46	72	28	395	47	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Gemfibrozil	**99**	**159**	**97**	**297**	**100**	**19**	**26**	**22**	**178**	**19**	**15**	**20**	**18**	**76**	**15**
HCTZ	**596**	**841**	**552**	**1688**	**600**	**22**	**40**	**28**	**287**	**23**	**74**	**174**	**76**	**1132**	**80**
Iopromide	98	27	85	11	98	10	115	14	573	11	nd	nd	nd	nd	nd
Irbesartan	64	100	55	526	66	nd	nd	nd	nd	nd	3.1	33	2	149	4
Ketoprofen	66	102	51	399	67	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Losartan	26	58	21	380	27	nd	nd	nd	nd	nd	6	25	6	65	6
Meloxicam	nd	nd	nd	nd	nd	0	74	0	735	1	nd	nd	nd	nd	nd
Metoprolol	0	46	0	235	1	nd	nd	nd	nd	nd	0	36	0	143	1
Naproxen	36	102	31	347	37	nd	nd	nd	nd	nd	20	59	18	297	22
Ofloxacin	nd	nd	nd	nd	nd	19	33	25	157	19	nd	nd	nd	nd	nd
Paroxetine	0	30	0	150	1	0	40	0	386	1	nd	nd	nd	nd	nd
Piroxicam	0	134	59	391	1	nd	nd	nd	nd	nd	0	43	0	197	1
Ranitidine	29	68	27	259	30	nd	nd	nd	nd	nd	20	49	21	150	21
Salicylic acid	nd	nd	nd	nd	nd	54	48	76	114	54	19	20	15	82	20
Trazodone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	29	30	22	112	29
Valsartan	141	133	133	149	141	nd	nd	nd	nd	nd	11	24	11	90	11
Venlafaxine	**0**	**1654**	**0**	**5587**	**19**	**10**	**45**	**13**	**364**	**11**	**15**	**309**	**16**	**2027**	**27**
Xylazine	nd	nd	nd	nd	nd	21	20	20	92	21	nd	nd	nd	nd	nd

All site B concentrations (being B the site at the WWTP discharge point) were higher than sites A and C, demonstrating that the WWTP was generally contributing to river water contamination at least at local scale. This may be due to a non-complete mixing between the effluent wastewater and the river water at this point. The maximum level corresponding to venlafaxine, as previously discussed, cannot be attributed to the season influence, but more likely to the pharmaceutical industry production. A similar conclusion can be reached for paroxetine, which was found at levels much higher than usually detected in river water (Gasser et al., 2012; Gros et al., 2010). The only exception in this context was iopromide, that in the winter monitoring was measured at lower concentration in the WWTP effluent than in river (27 and 98 ngL^{-1} respectively). Nonetheless, in general an increase in pharmaceutical concentrations in the river due to WWTP discharge was observed at local scale. This may lead to a higher risk for the aquatic ecosystem in case of water droughts and water scarcity, which is common in Mediterranean rivers.

An increase for site C compared to site A was observed for a few compounds (acetaminophen, diclofenac, gemfibrozil, HCTZ, iopromide, ofloxacin, piroxicam, ranitidine, salicylic acid and venlafaxine). For these compounds a direct influence of the WWTP discharge can be assumed (as no other discharges are present in the area). For all the remaining compounds the same level or a decrease was measured.

River water flow fluctuations are proposed to be one of the most relevant governing factors of variability in the occurrence of PhACs in the river. WWTP effluent flow was 0.029, 0.023 and 0.014 m^{3}s^{-1} in January, May and August respectively whereas river flow was 8.5, 12.0 and 2.3 m^{3}s^{-1} respectively. Resulting dilution factors (ratio river flow on WWTP effluent flow) were 294, 515 and 166 in winter, spring and summer respectively. Consequently, for example, even though the pharmaceutical levels discharged into the river were globally the highest in winter time (Table 2) there was also a higher dilution effect than in summer and therefore, a mitigation effect can be foreseen.

Dilution mass balance calculations were hence performed in order to quantitatively evaluate the contribution of dilution and of other natural process to the total attenuation of contaminants along the river. Theoretical concentration was calculated for each

compound (C_{theor}) in river water after the WWTP discharge applying the following balance equation:

$$C_{theor} = \frac{C_A Q_A + C_{ef} Q_{ef}}{Q_A + Q_{ef}}$$

Where C_A and C_{ef} (ngL^{-1}) are the compound concentrations upstream and at the WWTP effluent respectively (Table 3), and Q_A and Q_{ef} are the flow (m^{3}s^{-1}) from the river and the WWTP effluent.

C_{theor} should thus coincide with measured PhACs concentration at site C when dilution is the only factor contributing to attenuation of contaminant, i.e. recalcitrant compounds. Only in few cases, a slightly lower C_{theor} was observed in a few cases (diclofenac in January; gemfibrozil, HCTZ, iopromide, ofloxacin and venlafacine in May, acetaminophen and gemfibrozil in August) and a more pronounced difference for piroxicam in January and salycilic acid in May. This can be related to the fact that, substantially, only dilution may be considered and, hence, that these PhACs are recalcitrant, at least locally, to other attenuation factors.

However, for most of the compounds C_{theor} levels were higher than at point C, which can be attributed to other attenuation factors different from dilution, namely photodegradation, sorption processes, biodegradation (taking into account the short time span between B and C), etc. Several governing mechanisms may be involved in the fate study in the natural environment and dilution capacity cannot be considered as the unique factor governing the concentration levels of PhACs downstream off the receiving river waters, confirming previous studies (Gros et al., 2011). Other sources of variability could be changes in temperature, sediment remobilization, and analytical error among others which must be taken into account as contributors to the overall uncertainty (Petrovic et al., 2011).

4.   Conclusions

The occurrence and removal of 81 pharmaceutical compounds was evaluated in a municipal WWTP with significant industrial contribution and in the receiving Ter River waters. 40 compounds were detected at least in one of the three sampling campaigns and 21 were detected (from few ngL^{-1} up to 13 ugL^{-1}) within the compounds in all of them, within three different seasons.

The WWTP was unable to provide a complete removal for most of the compounds, being the analgesics the most efficiently eliminated group of substances. UV-based tertiary treatment did play a complementary role with significant additional removal for single compounds. Moreover, seasonal variation revealed a higher spring-summer removal compared to winter results.

The overall observed PhACs load and distribution were comparable to domestic wastewater. Even so, paroxetine and venlafaxine levels, together with an unusual therapeutic class distribution throughout the year (with a lower NSAIDs concentration in winter than spring and summer) suggested certain industrial pollution pressure.

PhACs were confirmed as widespread pollutants along the Ter river, characterized by a typical water scarcity regime and suffering from frequent flow fluctuations. PhACs were detected also before WWTP discharge point, meaning a diffuse river contamination due to upstream wastewater discharge and other diffuse sources. The global WWTP contribution to river pollution appeared to be minor, being the river attenuation capacity an important factor. More severe contamination phenomena can be predicted at local scale or in drought period.

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LAB AND PILOT-PLANT SCALE EXPERIMENTS WITH TARGET COMPOUNDS

Chapter 5

Removal of ibuprofen and its transformation products: Experimental and simulation studies

Abstract

Pharmaceutically active compounds (PhACs) deserve attention because of their effect on ecosystems and human health, as well as their continuous introduction into the aquatic environment. Classification schemes are suggested to characterise their biological degradation, e.g., based on pseudo-first-order kinetics, but these schemes can vary significantly, presumably due to pharmaceutical loads, sludge characteristics and experimental conditions. Degradation data for PhAC transformation products (TPs) are largely lacking.

The present work focuses not only on the biodegradation of the pharmaceutical compound ibuprofen but also on its best-known TPs (i.e., carboxyl ibuprofen and both hydroxyl ibuprofen isomers). Ibuprofen is one of the most commonly consumed PhACs and can be found in different environmental compartments.

The experiment performed consisted of a set of aerated batch tests with different suspended solid and ibuprofen concentrations to determine the influence of these parameters on the calculated biodegradation constant (Kbiol). Sampling of the liquid phase at different scheduled times was assessed, removal efficiencies were calculated and pseudo-first-order kinetics were adjusted to obtain experimental Kbiol values for the parent compound and its TPs.

The experimental data were successfully fitted to ASM-based models, with Kbiol values for the target compounds ranging from almost 1 to 17 L gSST^{-1} d^{-1}, depending on the concentrations of the biomass and ibuprofen. This work provides innovative knowledge not only regarding the removal of TPs but also the formation kinetics of these TPs.

Keywords

Chapter 6

**Effects on activated sludge bacterial community
exposed to sulfamethoxazole**

Abstract

The bacterial community shift on a lab scale Sequencing Batch Reactor (SBR) fed with synthetic wastewater and exposed to 50 µg L−1 of sulfamethoxazole (SFX) for 2 months was investigated in this study. The impact on biological nutrient removal performance and SFX removal efficiencies were also studied. Satisfactory biological nutrient removal was observed as regards to COD and Nitrogen. SFX removal efficiencies ranged between 20% and 50% throughout the experimental period, enhanced within the aerobic phases of the SBR cycle, with no evident signs of biomass acclimation. Nevertheless, denaturing gradient gel electrophoresis (DGGE) analysis showed significant variance leading to not only the fading, but also the emergence of new species in the bioreactor bacterial community after SFX dosage. According to the phylogenetic analysis, bacteria belonging to Betaproteobacteria and Gammaproteobacteria classes were the dominant species, among them, the Thiotrix spp. (Gammaproteobacteria) cell number increased due to its tolerance to the antibiotic. On the other hand, the classes Sphingobacteria, Actinobacteria, Chloroflexi and Chlorobi were found to be more vulnerable to the antibiotic load and disappeared. The sulphonamide resistance gene sulI was also quantified and discussed, as there are very few studies on bacterial resistance in lab-scale treatment reactors.

Keywords

Block III

PROTEOMICS APPROACH EXPERIMENTS

Chapter 7

Exploring the potential of applying proteomics for tracking bisphenol A and nonylphenol degradation in activated sludge

Abstract

A significant percentage of bisphenol A and nonylphenol removal in municipal wastewater treatment plants relies on biodegradation. Nonetheless, incomplete information is available concerning their degradation pathways performed by microbial communities in activated sludge systems. Hydroquinone dioxygenase (HQDO) is a specific degradation marker enzyme, involved in bisphenol A and nonylphenol biodegradation, and it can be produced by axenic cultures of the bacterium Sphingomonas sp. strain TTNP3. Proteomics, a technique based on the analysis of microbial community proteins, was applied to this strain. The bacterium proteome map was obtained and a HQDO subunit was successfully identified. Additionally, the reliability of the applied proteomics protocol was evaluated in activated sludge samples. Proteins belonging to Sphingomonas were searched at decreasing biomass ratios, i.e. serially diluting the bacterium in activated sludge. The protein patterns were compared and Sphingomonas proteins were discriminated against the ones from sludge itself on 2D-gels. The detection limit of the applied protocol was defined as $10-3$ g TTNP3 $g-1$ total suspended solids (TSSs). The results proved that proteomics can be a promising methodology to assess the presence of specific enzymes in activated sludge samples, however improvements of its sensitivity are still needed.

Keywords

Chapter 8

Proteomics reliability for micropollutants degradation insight into activated sludge systems

Abstract

Pharmaceutical compounds discharged into the sewer system may arrive to the environment producing deleterious effects. A significant removal percentage relies on biodegradation but little information is available to define degradation pathways. The present work evaluates the potential of using the proteomics approach to extract information about activated sludge microbial metabolism in degrading trace concentration of a pharmaceutical compound. Ibuprofen is one of the most consumed pharmaceuticals and it is thus found in wastewater at very high concentrations. Despite its high removal rates in wastewater treatment plants, it can still be found in different environmental compartments, so it can be considered a pollutant of emerging concern. First objective was to apply proteomics to evaluate profile variations of proteins belonging to such complex matrix like activated sludge. The second one was to determine, at different concentrations of a contaminant, which proteins followed specific and plausible trends along the time in terms of presence and intensity changes. Aerated and completely mixed activated sludge batch tests were spiked with ibuprofen (10 and 1000 μgL-1). The solid phase was analysed for proteomics purposes. Proteins expressions were compared over the time and between the two tested ibuprofen concentrations. The resulting statistical and comparative study permitted to find proteins following the expected trends. The liquid phase was sampled to determine ibuprofen concentrations, removal efficiencies and kinetics estimations.

Chapter 9

Specific bacterial strain role: Sulfamethoxazole biodegradation experiments and proteome analysis

Abstract

Antibiotic compounds, as in the case of sulfamethoxazole (SFX), are of concern because, as they undergo only partial removal in wastewater treatment, their presence in the environment is on-going. As a consequence, SFX is often detected at µg/L levels in treated effluents and receiving water bodies. Little information on the biodegradation of this antibiotic is available in the literature. Thus, the objective of this research was to check the ability of a pure strain (Microbacterium sp.) present in activated sludge, to break down, and then, to further analyse its overall proteome while looking for potential candidate proteins involved in its biodegradation process. The hypothesis followed here focuses on the variability of the protein expression profiles while the SFX biodegradation occurs. Biodegradation experiment results showed an almost complete removal of SFX within 24h by the pure strain. Protein patterns were subsequently analysed by difference gel electrophoresis (DIGE), leading to detailed statistical analysis which revealed certain homogeneity of protein profiles throughout the experimental period. Different time patterns were compared with the blank proteomes in order to identify any potential change in the proteins' expression levels, and the results obtained suggested either a plausible SFX co-metabolism pathway or a major housekeeping proteins role, as no over expressed proteins were significantly detected.

Keywords

Chapter 10
Key results and discussion

The various studies developed throughout this thesis relate to several aspects of the pharmaceuticals in wastewater issue and are listed below:

- Real scenario diagnosis (**chapter 4**)

- Lab-scale and pilot-plant batch experiments with target pharmaceutical compounds;
    - Kinetics and modelling (**chapter 5**)
    - Microbiological studies (**chapter 6**)

- Proteomics approach experiments (**chapters 7, chapter 8 and chapter 9**)

Each study contributed to gaining a wider picture of the problem from diverse perspectives and by means of different disciplines working towards the same goal. In the following sections, the main results achieved in each chapter are summarized and properly discussed.

Real scenario diagnosis

The main goal of chapter 4 was to acquire knowledge, by carrying out several sampling campaigns, on the occurrence and fate of a wide range of pharmaceutical compounds in a local WWTP facility context, whilst assessing the impact of the WWTP discharge into the receiving aquatic environment.

Three sampling campaigns carried out over different seasons meant a reliable diagnosis of a real context in terms of influent characterization. 81 pharmaceutical compounds were analysed and monitored during the primary and secondary treatment stages of a WWTP facility with significant industrial contribution and until they reached the nearby river waters. The UV-based tertiary treatment contribution to the overall pharmaceutical removal was also quantified, and showed a significant decrease of a few compounds concentration, which were not removed during the conventional activated sludge process.

The results of the study show clear differences between various therapeutic classes of PhACs, in terms of presence in the WWTP influent, along with removal efficiencies within secondary and tertiary treatments, where analgesics were the group of compounds that were most efficiently eliminated from wastewater. Also seasonal variation revealed a higher spring-summer removal compared to winter efficiencies. Moreover, the results obtained also demonstrated the industrial contribution from the nearby pharmaceutical industries to the discharge going into the river, giving evidence of the magnification of the problem in terms of the increase of some specific compounds detected in the WWTP and the receiving aquatic environment. Thus, the global WWTP contribution to river pollution appeared to be minor, since river dilution capacity was evaluated and shown as an important factor to consider when discussing pharmaceutical pollution in river waters.

Future research may also consider adsorption into sludge in WWTPs, photodegradation and all the other attenuation factors in surface waters affecting the overall

pharmaceutical removal. To successfully reduce the environmental risk posed by pharmaceutical pollution, the influence of UV treatment should be considered.

Specific input into the overall thesis: diagnosis of what kind of pharmaceutical profiles are found in a real scenario, together with the removal efficiencies within wastewater treatment processes; so as to later critically choose which compounds may be most representative for further detailed study in lab-scale experiments.

Lab-scale and pilot-plant batch experiments

The main goal of **chapters 5 and 6** was to focus on a single compound and try to gain a deeper insight into the specific degradation processes, embracing not only the transformation products' formation but also the development of the bacterial community involved.

In **chapter 5,** ibuprofen degradation batch studies were carried out with the aim of studying the kinetics of the TPs formation and their subsequent degradation, which were later used to develop a model on the overall ibuprofen biodegradation process.

Ibuprofen removal was successfully adjusted to a pseudo-first-order kinetic equation at different concentrations of suspended solids and initial pharmaceutical loads, obtaining consistent biodegradation rates (K_{biol}). Finally, a novel model which encompassed not only the formation, but also the degradation of the TPs was suggested, and the formation yields and biodegradation rates for the three quantified TPs were calculated and evaluated.

Further investigation into the modelling of pharmaceuticals and TPs is required in order to better assess their fate and removal in wastewater treatment plants, and which would help to estimate the final levels in the receiving water bodies.

The aim of **chapter 6** was to study the bacterial community shifts of a lab-scale SBR which was continuously spiked with 50 µg/L of the antibiotic sulfamethoxazole. Furthermore, the side effects of the addition of SFX were assessed in terms of BNR performance and antibiotic removal efficiency.

The results acquired demonstrated that the presence of sulfamethoxazole in the bioreactor studied did alter the distribution of the bacterial community, significantly decreasing its diversity with time. On the other hand, no affectation of BNR performance or SFX removal efficiency could be attributed to these microbiological changes due to the continuous SFX exposure. However, the antibiotic resistance gene *SulI* could be detected and subsequently identified, and its prevalence throughout the experimental period was proven.

Further investigation on specific bacterial groups is required for better knowledge of community shifts in response to pharmaceutical exposure in activated sludge systems. Since microorganisms represent the key components in wastewater treatment systems as a whole, this research is crucial for future predictions of the pharmaceuticals' effects on WWTP processes and on their release into the environment.

Specific input into the overall thesis: the attainment of specific degradation aspects related to single pharmaceutical compounds and effects on the activated sludge system involved. Furthermore, these in-depth investigations accounted for the point between field studies and going deeper into the micro analysis of the proteins hypothetically involved in the pharmaceuticals degradation process.

Proteomics approach experiments

In **chapters 7, 8** and **9,** the main objective was to prove the proteomics technique's usefulness and reliability, when dealing with an activated sludge bacterial community and pharmaceutical degradation pathways.

The preliminary step to accomplish this is reflected in **chapter 7**, where an adapted protocol for activated sludge as a matrix was successfully developed and thus, applied to a specific pure strain with known enzymes responsible for the micropollutants Bisphenol A and Nonylphenol biodegradation. The two enzymes were searched for on the *Sphingomonas TTNP3* strain proteome and afterwards, on spiked activated sludge samples at decreasing biomass ratios, i.e. serially diluting the bacteria in activated sludge, in order to determine the technique's detection limit, and finally prove that proteomics can be a promising methodology to assess the presence of target enzymes in activated sludge samples. However, improvements to its sensitivity are still needed.

Once the technique was validated in the matrix of interest, the next step was to involve a target pharmaceutical biodegradation process. Thus, **chapter 8** focused on working with a well-degradedable compound, ibuprofen, even though not much information was available in terms of possible metabolic pathways. The challenge here was to investigate if proteomics could be used to detect any specific protein which would become overexpressed while ibuprofen was being biodegraded. Preliminary results showed that no newly expressed proteins were found. However, the comparative and statistical analysis proved that there were proteins which followed the expected trend (being active or overexpressed within the target compound biodegradation) in terms of presence and spot intensity in the 2D gels, and which could be investigated further.

As a last step, and due to the continuos improvements and findings on the working topic, **chapter 9** represents the most suitable approach to deal with the role of proteomics in this work context. Hence, the identification of hypothetical proteins involved in the target pharmaceutical biodegradation process was, in this chapter, attempted under more narrowed conditions, meaning the use of a pure strain instead of activated sludge as a matrix. These working conditions significantly decreased the proteome variability uncertainties and allowed a more precise interpretation of the results. So, the information obtained in this chapter led to the conclusion that, even though proteomics have been proven to be suitable when working as a tool for the proposed aim, perhaps some other technique would be useful and complementary to finally being able to give some hints on the pharmaceutical biodegradation pathways.

According to the results obtained, seeking unknown proteins became a huge challenge when tackling the initial aim and thus, two main reasons could explain our findings. The first speculation is that possibly the use of proteomic techniques should be applied based on some already acknowledged metabolic pathway data. This way, it would be used as a suitable tool to give some extra verification on specific proteins behaviour which are already known to be taking part in the target process. On the other hand, a second potential explanaition is that, there are no specific proteins in charge of the pharmaceutical biodegradation process studied, and consequently, the results obtained were not in accordance to our expectations. Hence, housekeeping proteins or even co-metabolism could explain the results obtained.

Therefore, future investigations should focus on metabolic pharmaceutical pathway studies and also the implementation of complementary tools to work towards this same goal. Much more effort should be invested in individual pharmaceutical compound biodegradation to demonstrate/explore the proteomics' potential.

Specific input into the overall thesis: acquisition of valuable data regarding the changing proteome of bacterial communities exposed to a pharmaceutical compound. The results obtained give some clues to the biodegradation pathways engaged and/or protein profile variations, which lead to the validation or rejection of the initial hypothesis.

As a discussion taking into account each chapter contribution, the presented work accomplished with the overall goal and specific objectives. As a first objective and within the macro scale approach, a wide variety of substances were investigated at full scale in terms of presence, in waste and natural waters, jointly to their removal efficiency in biological and UV treatments. Among them, two compounds were chosen (ibuprofen and sulfamethoxazole) to be studied in more detail both at macro and micro scale. Pilot-plant experiments (including removal efficiencies, degradation and TPs formation kinetics, modelling studies, affectation on the bacterial community) were performed. According to those results, both target compounds were chosen as suitable to further work at protein level to investigate the metabolic implications within the pharmaceuticals biodegradation process, and thus, proteomic techniques were applied as an alternative tool to provide hints on the role of potential proteins. All the experimental designs, either being micro or macro scale, were focused on revealing some new insights on the pharmaceuticals biodegradation enhancement, in order to contribute with knowledge-based outputs to minimise these compounds release into the environment. Stress out that each chapter of the present work was thought to investigate different aspects on the pharmaceuticals contamination, from, for example, monitoring the transformation products formation till evaluating the bacterial community changes and antibiotic resistance genes phenomenon after a pharmaceutical exposure. Thus, even maintaining the final and general goal as common for each experiment, the multidisciplinarity was strongly present along the thesis and the different scale approaches were used towards the aforementioned objective.

Chapter 11

Conclusions and personal view

Conclusions

The results of this thesis contribute to a better understanding of the occurrence, removal and impact of pharmaceutical contamination in activated sludge and river systems. Thus, the main conclusions acquired in the present work can be listed as follows:

➢ The occurrence of pharmaceuticals in the environment is wide spread and WWTPs are not sufficient to act as a barrier to these compounds before they enter the waterways.

➢ The efficiency study of wastewater treatment in removing these compounds from wastewater, has led to an understanding of the disparity of behaviours of each compound, according to the treatment process applied and their physico-chemocal structures.

➢ The study and modelling of the degradation behaviour of specific pharmaceuticals and their main transformation products, led to valuable information which can be further used as a potential tool to increase their removal and reduce their release to the environment.

➢ The role and subsequent affectation of the bacterial communities exposed to pharmaceutical loads, become highly important in determining how these compounds can disturb the operational conditions required in wastewater treatment systems.

➢ The antibiotic resistant genes phenomenon has been demonstrated as a prevalent and serious concern.

➢ At the proteome level, pure culture studies allowed the acquisition of valuable data that can uncover certain detailed aspects about the pharmaceuticals' biodegradation process.

➢ Proteomics resulted as a possible methodology to be used when analysing activated sludge samples and pure strain cultures; and potentially applicable to the study of the pharmaceutical biodegradation phenomenon. However, a more protein-target study and used as a complementary technique, would enhance its potential and minimise the uncertainties encountered here.

In summary, the present work aimed to bridge the gap between different scales, while testing an alternative methodology to gain a deeper insight into the pharmaceutical biodegradation process in wastewater treatment. And thus, the above-mentioned conclusions reflect the knowledge acquired throughout the whole study.

Personal view

In this section a personal opinion on the pharmaceuticals issue is given, beyond the scope of the present work, to express the author's own view from the whole and contextualised picture.

"As a personal overall conclusion, I must recognise that after three-years of researching the pharmaceuticals (PhACs) issue, it is difficult to tackle its complex multidisciplinarity. It is important to note that PhAC contamination encompasses many more aspects than those you can initially think of. Thus, different expertises may contribute together to guide us through the intrinsic uncertainties regarding this issue. Moreover, the fact that more than 4,000 pharmaceuticals are currently in use, makes it impossible to experimentally assess the hazards and risks of all of these substances in a timely manner, as Boxall et al., (2012) stated. Thus, prioritization approaches should be used to focus monitoring, testing, and identifying those PhACs that are likely to pose the greatest risk in a particular context.

Once those potential PhACs which pose an unacceptable risk to the environment have been identified, there are several options to minimise or even remove their emissions from the environment, including a) substitution of the compound with a more environmentally friendly one; b) development of better drug delivery system so that smaller doses are needed; c) improve package size to extend shelf life and reduce the amount of product that expires and must be discarded unused; d) change prescription and husbandry practices; and e) introduce improved wastewater options. However, the efficiency and practicality of many of these solutions is poorly understood.

Apart from framing the whole spectrum of possibilities to deal with the pharmaceutical contamination issue, I would also like to give an example of how solutions can easily turn into other problems, as is the case of encapsulation of antimicrobial pharmaceuticals in nanoparticle systems, which has emerged as an innovative and promising alternative that enhances therapeutic effectiveness and minimizes undesirable side effects of these compounds and a considerable minimization of the prescribed doses (Zhang et al., 2010). As already known, nanosized particles have always been present in nature, but the accelerated penetration of engineered nanoparticles (ENP) in the market is raising serious concerns over their potential impact on the environment, which leads to a similar problem as with the PhACs.

To justify and stimulate the strategies listed above, it is necessary to achieve robust knowledge-based data that substantiate the harmful effects of these compounds on the aquatic environment, which can then subsequently act as a base to regulate them. Some of the handicaps which can be encountered on the way to reaching this goal are, for instance, the difficulty in assessing an ecotoxicological experiment under low-dose and long-term exposure conditions, to obtain reliable chronic effect data. Also the complexity of the ecosystems and the synergic effect of the pharmaceutical mixture make it even more difficult to handle. Another dilemma that researchers have to confront is the transformation product formation which results from the degradation of the parent compounds and which may have similar or even more harmful effects to the aquatic media. There is still a significant knowledge gap to be filled here.

Both, end-of-pipe measures and at-source strategy controls must be applied simultaneously and involve all different parties, from pharmaceutical industries to society in general, in order to accomplish this goal and provide a collaborative platform.

Moreover, it is also important to highlight that the current economic situation does not help in terms of investing in wastewater treatment processes to remove pharmaceuticals, which are indeed costly right now, and even more so if these compounds are not yet subjected to any regulation. This may lead to choosing creative and cost-efffective options which allow the problem to be solved, 'low-cost'.

Along similar lines, another matter related to the near future of water management and pharmaceuticals, comes from the so called "smart cities". Cities in search of a self-sufficient water supply lead to closing the water cycle on a regional scale, for instance by reusing wastewater for other purposes. This could lead to certain pharmaceutical accumulation, since reclaimed water would represent a more dynamic and closed water cycle. As a consequence, some persistent pharmaceuticals or those not completely removed, even at trace levels, could worsen the problem.

After those few examples on the up-coming challenges for water management and pharmaceuticals, I would like to stress the importance of developing multidisciplinary and multi-scale studies which follow one path in the same direction based on similar and common criteria. This would mean scientifically relevant data being achieved and being directly applied by those stakeholders in charge of PhAC regulation. Understanding the respective contribution and reciprocal feed-back of each part (in a common integrated picture), constitutes a tremendous scientific challenge in which the concurring interdisciplinary efforts of environmental chemists, biologists, hydrologists, engineers and even social scientists are needed. And this thesis undertakes part of this commitment to contribute to the cause".

Chapter 12
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Chapter 13
Annex

Curriculum vitae

PERSONAL INFORMATION

NEUS COLLADO ALSINA

Date of birth:	**26th October 1986**
Nationality	Catalan (Spanish)

Institute of the Environment (LEQUIA-UdG)
ICRA, Catalan Institute for Water Research
17003, Girona (Spain)

Land line phone:	0034 972 18 33 80
Fax:	0034 972 18 32 48
Mobile:	0034 636 177 078

Email addresses:	**ncollado@icra.cat** (work)
	neuscoal@gmail.com (personal)

Driving license	B1 since 2006

LANGUAGE SKILLS*

Native language: Catalan

	Understanding		Speaking		Writing
	Listening	Reading	Spoken interaction	Spoken production	
Spanish	C2	C2	C2	C1	C2
English	C1	C1	C1	C1	C1
French	B1	B1	B2	B1	B2

(*)Common European Framework of Reference for Languages

EDUCATION & TRAINING

Education and qualifications

Current thesis in the Environmental PhD program at the University of Girona (2010-2013)

Title dissertation: "Multi-scale investigation of occurrence, fate, removal and biodegradation of pharmaceutical contaminants in wastewater treatment and river systems". Supervisors: J. Comas; G. Buttiglieri.

Master thesis on Water Science and Technology by the University of Girona (2008-2010)

Title dissertation: "Biodegradation of selected pharmaceuticals in activated sludge systems".
<u>Supervisor</u>: J. Sipma (February, 2010).

Bachelor in Environmental Science at Girona University (2004-2008)

Final project dissertation: "Proposta de gestió de les aigües pluvials en el municipi de Begur".
<u>Supervisor</u>: J. Comas (June, 2007).

Fellowships

Collaboration fellowship with the Institute of the Environment (LEQUIA) UdG. (April-June 2013)

Predoctoral fellowshipassigned to LEQUIA, according to the UdG Research Grant Programme 2007-2010.
Ref number BR09/09. (April 2010-April 2013)

ERASMUS scholarship assigned as an AGAUR grant by the Generalitat de Catalunya (February-June 2008)

Collaboration scholarship at Torroella de Montgrí Wastewater Treatment Plant (Jul-Aug 2006) as a technical assistant in the laboratory with Consorci d'Aigües de la Costa Brava (Torroella de Montrgí, Spain)

Courses

Cross Border Doctorials 2012 by the University of Girona (UdG).
Caldes de Malavella, Girona, May 2012.

Specific course on safety and risks in the laboratory and emergency plan (Cod: 1732581-E11/2204).
ICRA, Girona, November 2011.

SCARCE-Consolider CSD-2009-00065 Advanced course, "Analysis, fate and risks of organic contaminants in river basins under water scarcity.
Valencia, February 2011.

Hands-on course on sample treatment for mass-spectrometry-based proteomics.
Ourense, March 2010.

The MBR Short Course by Simon Judd.
ICRA, Girona, July 2010.

Laboratory management course at the University of Girona.
Girona, November 2009. Organised by Labour Health Office from UdG.

SCIENTIFIC OR TECHNOLOGICAL ACTIVITY

Participation in R&D&I projects funded by public bodies

Mecapharm. Study of biodegradation mechanisms of target pharmaceuticals in wastewater treatment by peptide profiling **(June-Desember 2010).**
Financial entity: ICRA (Catalan Institute for Water Research)
Main researcher of the project: Prof. Ignasi Rodríguez-Roda

Grant amount: 28.000 euros

COLMATAR+: New developments, implementations and validation at different scales of a SAD for the MBR control and operation. From the basic research to the optimal operation **(January 2010-Desember 2012).** Ref.:CTM2009-14742-C02-01
Financial entity: Environmental Institute
Main researcher of the project: Prof. Joaquim Comas Matas
Grant amount: 198.924,01 euros

SANITAS: Sustainable and integrated urban water system management **(Desember 2011-Desember 2013).** Ref.: FP7–PEOPLE–2011–ITN
Financial entity: Environmental Institute
Main researcher of the project: Prof. Joaquim Comas Matas
Grant amount: 3.546.049 euros (128.194 euros at ICRA)

ITACA: Research of treatment, reuse and control of treatment technologies for a future sustenible wastewater treatment (**Desember 2011-Desember 2014).**
Financial entity: Programa INNPRONTA, CDTI (Centro para el Desarrollo Tecnológico Industrial) presented by ADASA Sistemas, S.A.
Main researcher of the project: Prof. Ignasi Rodriguez-Roda
Grant amount: 131.000 euros

Research stays abroad

Center: University of Amsterdam.   **Supervisor**: Prof. Pim de Vogt
City: Amsterdam   **Country:** Holand   **Year:** 2008   **Duration:** 5 months
Topic: Optional MSc subjects to finish the environmental science degree.

Center: FHNW (FachhochschuleNordwestschweiz), Institute of Ecopreneurship, Univeristy of Basel. **Supervisor**: Prof. Philippe Corvini
City: Basel   **Country:** Switzerland   **Year:** 2010   **Duration:** 3 months
Topic: Pharmaceuticals biodegradation studies and use of ^{14}C radiolabelled compounds.

Oral and poster communications to conferences

VII ANQUE'S International Congress, Integral water cycle: present and future "A Shared Commitment". Oviedo (Spain) 14-16th June 2010.

> *Collado N., Comas J., Rodriguez-Roda I, Canals, J., (2010). Biodegradation of pharmaceuticals during wastewater treatment and the evaluation of proteomics for metabolic pathway elucidation.*

1st IWA Spain National Young Water Professional Conference. Barcelona (Spain), 16-18th June 2010.

> *Collado N., Osuna, B., Comas J., Rodriguez-Roda, I., Sipma, J. (2010). Biodegradation of Pharmaceuticals during Wastewater Treatment and the Evaluation of Proteomics for Metabolic Pathway Elucidation*

"Emerging and priority pollutants: Bringing Science into River management Plans", KeyBioEffects. ICRA, Girona (Spain) 25-26th March 2010. *Poster*

IWA Conference on Sustainable Solutions for Small Water and Wastewater Systems (S2SMALL). Girona (Spain) 19-22nd April 2010. *Poster*

Micropol&Ecohazard 2011, the 7th IWA specialist conference on assessment and control of micropollutants/hazardous substances in water. Sydney, NSW, Australia 11-13th July 2011.

Collado N., Bouju H., Buttiglieri G., Comas J., Corvini P.F.X., Wintgens T., Rodriguez-Roda I. (2011). Evaluation of Proteomics for Pharmaceuticals Metabolic Pathway Elucidation in Wastewater Treatment.

CEST2011 12th International Conference on Environmental Science and Technology. Rhodes island, Greece 8-10th September 2011.

Collado N., Buttiglieri G., Kolvenbach B., Comas J., Corvini P., Rodriguez-Roda I. (2011). Is Proteomics Reliable to Investigate Pharmaceuticals Degradation in Wastewater?

ICCE 2011. EuCheMs International Conference on Chemistry and the Environment. Zurich (Switzerland) 11-15th September 2011.

Ferrando L., Collado N., Buttiglieri G., Rodriguez-Roda I., Rodriguez-Mozaz S., Barceló D. (2011). Elimination processes of Ibuprofen in activate sludge batch experiments. (presented by Laura Ferrando-Climent).

9th Edition International Symposium of Sanitary and Environmental Engineering. Milan (Italy) 26-29th June 2012.

Buttiglieri G., Collado N., Ferrando L., Rodriguez S., Comas J., Rodriguez-Roda I. (2012). Proteomics applied to Ibuprofen degradation in activated sludge: preliminary results. (presented by Gianluigi Buttiglieri).

International Young Water Professionals Conference 2012. Budapest, Hungary 10-13th July 2012.

Collado N., Buttiglieri G., Ferrando L., Rodriguez S., Comas J., Rodriguez-Roda I. (2012). Kinetics Exploration of Ibuprofen and its Metabolites.

ICCE 2013. 14th EuCheMS International Conference on Chemistry and the Environment. Barcelona (Spain), June 25 – 28th, 2013.

Collado N., Buttiglieri G., Marti E., Ferrando-Climent L., Rodriguez-Mozaz S.,Barceló D., Comas J., and Rodriguez-Roda I. Influence of sulfamethoxazole on activated sludge bacterial community and removal efficiencies.

Buttiglieri G., Collado N., Rodriguez-Caballero A., Rodriguez-Mozaz S., Ferrando-Climent L., Rodriguez-Roda I., Comas J., Barceló D. and Pijuan M. Evaluating partial nitrification capability to degrade Sulfamethoxazole and their greenhouse gases emissions. *Poster*

Micropol & Ecohazard 2013. Zurich (Switzerland),16 – 20th June 2013.

N. Collado, G. Buttiglieri, S. Rodriguez-Mozaz, A. Rubirola, M. Gros, D. Barceló, J. Comas, I. Rodriguez-Roda. Integrated study on pharmaceuticals occurrence in a WWTP with significant industrial contribution and the impact into the river system.

N. Collado, G. Buttiglieri, E. Marti, L. Ferrando-Climent, S. Rodriguez-Mozaz, J. Comas, and I. Rodriguez-Roda. Shifts in bacterial community and removal efficiencies on a SBR with sulfamethoxazole continuous feeding. *Poster*

Other conferences assistance

Water scarcity and management under Mediterranean Climate. Girona (Spain), 24-25th November 2008.

Medi ambient i societat: Pautes per a la gestió ambiental. Barcelona (Spain), 4th May 2010.

Journal publications

1. Sipma J., Osuna B., **Collado N.**, Monclús H., Ferrero G., Comas J., Rodriguez-Roda I. (2009). "Comparison of removal of pharmaceuticals in MBR and activated sludge systems". *Desalination*, 250 (2), 653-659.

2. **Collado N.,** Buttiglieri G., Ferrando-Climent L., Rodriguez-Mozaz S., Barceló D., Comas J., Rodriguez-Roda I. (2012). "Removal of Ibuprofen and its Transformation Products: Experimental and Simulation Studies". Science of the Total Environment. 433, 296-301.

3. Ferrando-Climent L., **Collado N.**, Buttiglieri G., Gros M., Rodriguez-Roda I., Rodriguez-Mozaz S., Barceló D. (2012). "Comprehensive study of ibuprofen and its metabolites in activated sludge batch experiments and aquatic environment". Science of the Total Environment. 438, 404-413.

4. **Collado N.**, Buttiglieri G., Kolvenbach B.A., Comas J., Corvini P.F.X and Rodríguez-Roda I. (2013). "Exploring the potential of applying proteomics for tracking bisphenol A and nonylphenol degradation in activated sludge". Chemosphere. 80 (8), 2309-2314.

5. **Collado N.**, Buttiglieri G., Marti E., Ferrando-Climent L., Rodriguez-Mozaz S., Barceló D., Comas J., and Rodriguez-Roda I. (2013). "Effects on activated sludge bacterial community exposed to sulfamethoxazole". DOI: http://dx.doi.org/10.1016/j.chemosphere.2013.04.094

6. **Collado N**., Rodriguez-Mozaz S., Gros M., Rubirola A., Barceló D., Comas J., Rodriguez-Roda I. and Buttiglieri G. (2013). "Pharmaceuticals occurrence in a WWTP with significant industrial contribution and its impact into the river system". Submitted to Environmental Pollution.

7. Buttiglieri G., **Collado N.**, Comas J. and Rodriguez-Roda I. (2013). "Proteomics reliability for micropollutants degradation insight into activated sludge systems". Submitted to Environmental Science and Pollution Research.

8. **Collado N.**, Buttiglieri G., Ricken B., Comas J. and Rodriguez-Roda I. (2013). "Specific bacterial strain role: Sulfamethoxazole biodegradation experiments and proteome analysis". Submitted to Environmental Pollution.

COLLECTION OF OTHER CREDITS

a. Description credits: Environmental Engineering practical lessons from 2nd year students of the Environmental Science degree. **Body conferring the credit and date:** Girona University (May 2012)

b. Description credits: Environmental Engineering practical lessons from 2nd year students of the Environmental Science degree. **Body conferring the credit and date:** Girona University (April 2013)

REFERENCES

Prof. Ignasi Rodríguez-Roda, Senior researcher at the Catalan Institute for Water Research. irodriguezroda@icra.cat

Prof. Joaquim Comas iMatas, Associate professor at the University of Girona. quim@lequia.udg.cat

Dr. Gianluigi Buttiglieri, postdoctoral researcher at the Catalan Institute for Water Research. gbuttiglieri@icra.cat