Occurrence and fate of pharmaceuticals in wastewater treatment processes

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per

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"Treat the Earth well:
it was not given to you by your parents,
it was loaned to you by your children.
We do not inherit the Earth from our Ancestors,
we borrow it from our Children."

Ancient Indian Proverb

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Marija, Jasna and sister-Ana to the welcome BGD crew! And my young uncle Dimitrije, he did a good job in raising my self-esteem to a higher level. I appreciate the business classes I had with my brothers Goran and Ivan. And from the south, I always enjoyed the positive vibes of my precious princesses – sister Danijela and her daughters Delfina and Tina. My "kuma" Ana probably expected me to follow up my early career of "human right defender", but... Look where I am! I have the fortune of having so many dear friends, my big Montenegrian family and my adored aunties! I received always love and support... (and chocolate!) They all know how much I love them!

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Aleksandra

In Barcelona, on a sunny November afternoon

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Preface

Why pharmaceuticals?

Pharmaceutical products have an important role in the treatment and prevention of disease in both humans and animals. They comprise a large and diverse group of compounds designed either to be highly active and interact with receptors in humans and animals or to be toxic for many infectious organisms. Hundreds of tons of pharmaceuticals is dispensed and consumed annually worldwide. The usage and consumption of pharmaceuticals have been increasing consistently due to the discoveries of new drugs, the expanding population and the inverting age structure in the general population, as well as due to expiration of patents with resulting availability of less expensive generics (1).

After the intake, the pharmaceutically active compounds undergo metabolic processes in organism. Significant fractions of the parent compound are excreted in unmetabolized form or as active/inactive metabolites into raw sewage and wastewater treatment systems. Municipal sewage treatment plant effluents are discharged to water bodies or reused for irrigation, and biosolids produced are reused in agriculture as soil amendment or disposed to landfill. Thus body metabolization and excretion followed by wastewater treatment is considered to be the primary pathway of pharmaceuticals to the environment (Figure I). Disposal of drug leftovers to sewage and trash is another source of entry, but its relative significance is unknown with respect to the overall levels of pharmaceuticals in the environment (2).

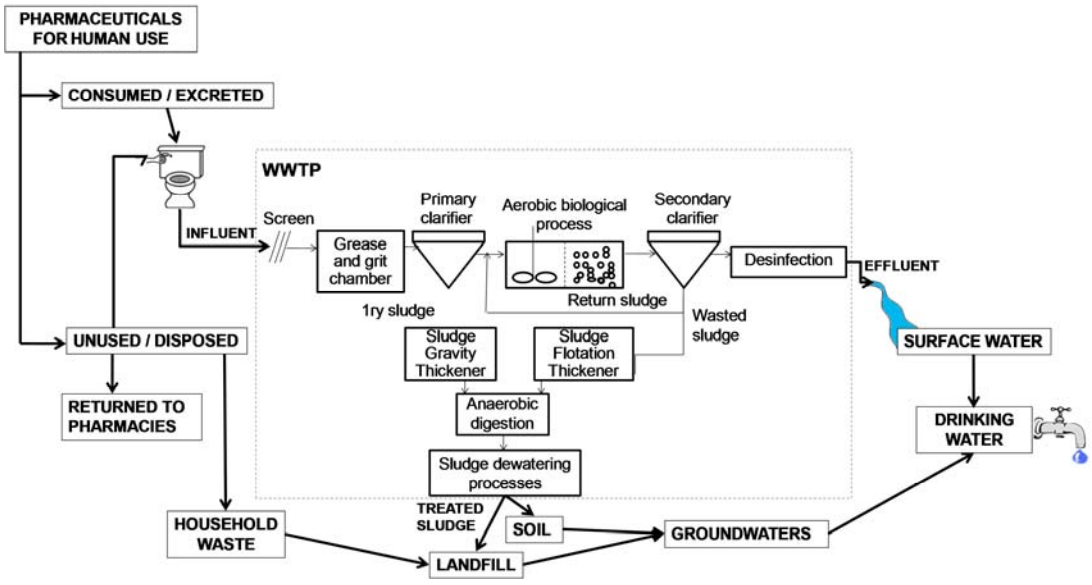


Figure I. Routes of release of pharmaceuticals for human use to the environment with a schematic diagram of a conventional WWTP

Recent development and continual improvement of the advanced instruments and analytical methodologies made possible detection of pharmaceuticals at low levels (i.e. ng to low µg per litter) in different environmental matrixes (3). Traces of pharmaceuticals and their metabolites have been found in surface water and aquatic sediment (4-6), but also in soil irrigated with reclaimed water (7-9) and soil that received biosolids from urban sewage treatment plants (10-11). These studies indicated that the applied wastewater treatments are not efficient enough to remove these micropollutants from wastewater and sludge, and as a result they find their way into the environment.

Once entered the environment, pharmaceutically active compounds can bioaccumulate and produce subtle effects on aquatic and terrestrial organisms, especially on the former since they are exposed to long-term continuous influx of wastewater effluents (12-14). Although the effects of the pharmaceuticals are investigated through safety and toxicology studies, the potential environmental impacts of their production and use are less understood and have recently become a topic of research interest (15). There have been only a few examples of adverse effects of pharmaceuticals to ecosystem. A widely used anti-inflammatory drug diclofenac caused the decline of Oriental white-backed vulture (*Gyps bengalensis*) in the Indian subcontinent (16). It was shown that the pharmaceutical, ingested *via* food, was responsible for renal disease in the scavenging birds. Similar effects of diclofenac on renal function of rainbow trout (*Oncorhynchus mykiss*) were observed after prolonged exposure (28 days) to an environmentally relevant concentration range (17). Till now, there is no evidence linking the presence of pharmaceuticals in the environment to human health risks, still complex mixtures may have long-term unseen effects, especially on tissues other than those on which the pharmaceuticals were designed to act.

For all this, the occurrence of pharmaceutical compounds in the environment and their potential effects on human and environmental health as well as the extent to which they can be eliminated during wastewater treatment have become active subject matter of actual research. The topic is still relatively new, and thus the pharmaceuticals in the environment are not yet covered by the currently existing regulation. But, it is important that the governmental and non-governmental researchers and water regulators, water suppliers and the public have become aware of the problem and concerned about the discharge of pharmaceuticals into the environment and about the potential effects to the human health via drinking water. By understanding the problem, we can try to find a solution that can, at least, diminish it, if not completely resolve it.

Objectives and structure of the thesis

The general objectives of the thesis were:

a) to assess the occurrence, removal and fate of selected pharmaceuticals during conventional wastewater treatment, and

b) to study the degradation of the anticonvulsant carbamazepine, and identify its transformation products formed during biological treatment using white rot fungus and during advanced oxidation processes (i.e. heterogeneous photocatalysis and sonophotocatalysis).

The thesis is divided into three major sections. In Chapter I, properties of pharmaceuticals and the mechanisms related to their environmental fate, their consumption and the existing legislation regarding the levels of pharmaceutical residues in the aquatic environment are discussed. This Chapter also provides a detailed literature review on the analysis of pharmaceuticals and their occurrence and removal in wastewater treatment plants. Chapter II is divided in two sections. In the first one, the methodologies applied for the chemical analysis of the selected pharmaceuticals in wastewater and sludge are explained. The method developed for the analysis of the selected pharmaceuticals in sludge is presented as a scientific publication, along with some additional comments on the topic. In the second part, the results of three studies regarding the occurrence and removal of the selected pharmaceuticals, performed at four biological full-scale wastewater treatment plants, were presented in three scientific publications. The issues related to the evaluation of the removal efficiencies, in general, were additionally discussed. Chapter III gives some basic concepts of degradation of organic contaminants by an eco-friendly, soft treatment, using a white rot fungus *Trametes versicolor*, and by advanced oxidation processes (AOP) with the accent on photocatalysis under UV-A and simulated solar irradiation, and under the combined use of ultrasonic sound waves and UV-A irradiation (sonophotocatalysis). The results of two studies on the degradation of the antiepileptic carbamazepine in lab-scale reactors under the above-mentioned technologies are presented as scientific papers. Finally, the results of the studies conducted during the thesis are outlined in the summary written in Spanish.

Chapter I

Literature review

Literature review

1.1. Consumption of pharmaceuticals

More than 10000 prescription and over-the-counter pharmaceuticals are registered and approved for usage today, with around 1300 unique active ingredients (Orange book, FDA). From 1995 until today, 765 medicinal products more have received marketing authorization in EU (Community register of medicinal products for human use (18)). While there is much information available about the pharmaceutical products, and their active ingredients, as they are evaluated and approved for use by official agencies/organizations of different countries or union of countries, the information on their consumption is more difficult to obtain. It is especially difficult to get precise information on the total worldwide use of pharmaceuticals, as the consumption varies considerably from country to country. The variations in the consumption may be due to many factors: the economic situation and the organization of the health system of a country (e.g. the proportion of people treated); drug prescription guidelines and behavior, e.g. average dose used and the duration of the treatments; the rising/lower prevalence of a disease in a certain moment and place, etc. The global consumption of human pharmaceuticals was predicted to reach 100,000 tons per year, which corresponds to a worldwide average consumption of 15 grams per capita per year (19). There is an almost consistent increase in the use of pharmaceuticals in any population over time, mostly due to growth in the population size, the ageing process, and changing attitudes towards the use of drugs (e.g. a lesser willingness to tolerate illness or pain). The increasing tendency towards greater drug use has also been influenced by commercial pressures (20).

The OECD (Organisation for Economic Co-operation and Development) policy study from 2008, *"Pharmaceutical Pricing Policies in a Global Market"* (21), reported that France and Spain had the greatest volume of pharmaceutical consumption per person in 2005, followed by the United States and Australia, while Mexico had the lowest volume of consumption per capita. Nine OECD countries account for about 80% of the value of global sales of pharmaceuticals.According to Intercontinental Marketing Services (IMS) Health Report from 2011, the top five pharmaceutical markets in the world are the US, Japan, Germany, France and China, with the US representing 38.1% of global prescription pharmaceutical sales. As the figure 1.1 shows, the global pharmaceutical market grew by 4.5% in 2011 (AstraZeneca Annual Report and Form 20-F Information 2011). Average revenue growth in US, EU, Japan, Canada, Australia and New Zelend was 2.8% while that in emerging markets (i.w. rest of the world, in Figure X) was over four times higher, at 12%. China, now the world's third largest pharmaceutical market, expanded at an astonishing 20 percent in 2009, targeting

substantial improvement which is projected to double the size of this pharmaceutical market by 2013.Much slower growth in the five major European markets (France, Germany, Italy, Spain and the UK), along with Canada, has been observed in the last years (IMS Health, 2011).

Consumption of pharmaceuticals is commonly expressed by Defined Daily Dose (DDD) unit, as recommended by the World Health Organization (WHO) Collaborating Center for Drug Statistics, rather than in terms of costs. *The DDD is the assumed average maintenance dose per day for a drug used for its main indication in adults.* Although it gives just a rough estimate of consumption, DDD is a fixed unit of measurement independent of price and dosage form and therefore enables the assessments of the trends in pharmaceutical consumption and the comparison between population groups (DDD, WHO(22)). Table 1.1 shows the average consumption of the selected pharmaceuticals belonging to the most representative therapeutic groups (e.g. anti-inflamatory drugs, antibiotics, antidiabetics etc) in Spain, expressed as DDD per 1000 Inhabitants per day (DDD $1000Inh^{-1}$ day^{-1}). The data were obtained from the technical reports from 2006. prepared by the Spanish Agency of Medicines and Helthcare Products (Agencia Española de Medicamentos y Productos Sanitarios) together with Spanish Drug Agency (Dirección General de Farmacia y Productos Sanitarios) (23).

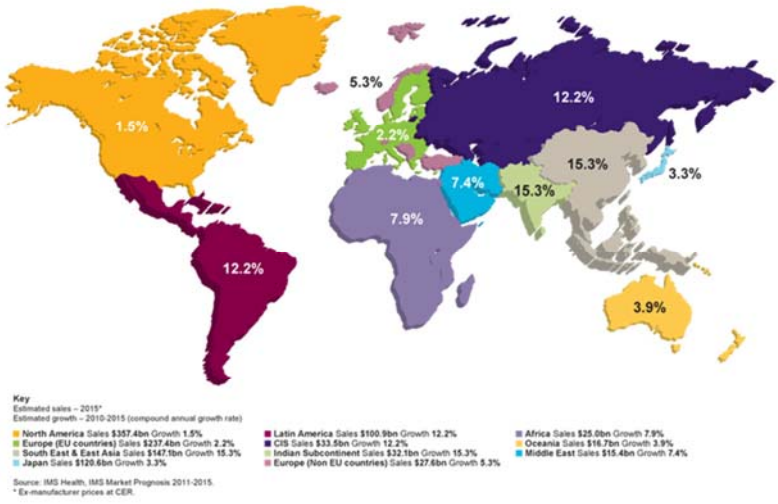


Figure 1.1. Estimated pharmaceutical market growth 2010-2015

Table 1.1. Average daily consumption of the selected pharmaceuticals given as Defined Daily Dose (DDD) per 1000 Inhabitants per day (DDD 1000Inh-1 day-1)

Therapeutic group	Total *per* group, DDD 1000 Inh^{-1} Day^{-1}	Selected compounds	DDD 1000Inh^{-1} Day^{-1}
M01 Anti-inflammatory and antirheumatic products	45.8	Ibuprofen	21.3
		Diclofenac	7.9
		Naproxen	5.2
NO2 Analgesics	18.7	Paracetamol	14.5
A10 Drugs used in diabetes	55.7	Insulin	13.5
		Metformine	13.3
		Glimepiride	10.3
		Glibenclamide	7.0
J01 Antibacterials	19.7	Amoxicilin (Peniciline)	4.4
		Amoxicilin in combinations	7.6
		Ciprofloxacin (Fluoroquinolones)	1.1
NO3 Antiepilpetics	10.8	Gabapentin	1.6
		Valproate sodium	1.6
		Carbamazepine	1.3
C02 Antihypertensives	8.7	Doxazosine	8.2
C03 Diuretics	40.2	Furosemide	10.7
		Hydrochlorothiazide	7.8
		Chlortalidone	5.2
		Torasemide	4.9
		Indapamide	4.9
C08 Calcium channel blockers	36.0	Diltiazem	4.3
		Amplidpine	16.2
		Nifedipine	4.7
		Verapamile	2.3
C09 Agents acting on the renin-angiotensin system	233.0	Enalapril	35.4
		Ramipril	9.1
		Valsartan	9.8
		Candesartan	9.3
C07 Beta blocking agents	19.6	Atenolol	7.6
		Cervedelol	2.5
		Bisoprolol	3.9
		Nebivolol	1.5
A02BA H2-receptor antagonists	4.3	Ranitidine	3.7
		Famotidine	0.6
A02BC Proton pump inhibitors	75.9	Omeprazole	59.9
		Pantoprazole	7.7
C10AA (Lipid modifying agents) HMG CoA reductase inhibitors	81.3	Atorvastatin	36.5
		Simvastatin	26.7
C10AB (Lipid modifying agents) Fibrates	4.1	Fenofibrate	2.1
		Gemfibrozil	1.6
N05 Psycholeptics (Anxiolytic)	69.9	Diazepam	5.4
		Potassium clorazepate	4.1
		Alprazolam	14.0
		Lorazepam	16.8
		Lormetazepam	13.3
		Zolpidem	6.0

1.2. EU Legislation on pharmaceutical residues in the aquatic environment

In European Union (EU), the environmental risk assessment of human and veterinary drugs has been established by EC Directives 93/39/EEC (24) and 81/851/EEC (25), respectively, that later have been replaced by EC Directives 2001/83/EC (26) on human medicine and 2001/82/EC (27) on veterinary medicine. According to both Directives, the application for marketing authorization should include the *"evaluation of the potential environmental risks posed by the medicinal product. This impact shall be assessed and, on a case-by-case basis, specific arrangements to limit it shall be envisaged"*. The important difference is that in the case of human medicine, the environmental impact should not constitute a criterion for refusal of a marketing authorization; while for the veterinary medicine: *"where urgent action is essential to protect human or animal health or the environment, until a definitive decision is adopted, a Member State may suspend the marketing and the use of the veterinary medicinal product concerned on its territory".*

In a recently introduced Directive 2010/84/EU (28) on pharmacovigilance, accompanied by Regulation 1235/2010/EU, is stated: *"The pollution of waters and soils with pharmaceutical residues is an emerging environmental problem. Member States should consider measures to monitor and evaluate the risk of environmental effects of such medicinal products, including those which may have an impact on public health. The Commission should, based, inter alia, on data received from the European Medicines Agency, the European Environment Agency and Member States, produce a report on the scale of the problem, along with an assessment on whether amendments to Union legislation on medicinal products or other relevant Union legislation are required."*

An environmental risk assessment (ERA) evaluating "*possible risks to the environment due to the use and/or disposal of the medicinal product*" is required for all new marketing authorization applications for a medicinal product. The Guideline of the European Agency for the Evaluation of Medicinal Products by the European Medicines Agency (EMEA, 2006), covers both the prediction of the environmental concentrations and the assessment of fate and effects in the aquatic and terrestrial compartments. The ERA procedure is divided into two phases:

Phase I – the estimation of the exposure of the environment to the drug substance, irrespective of its route of administration, pharmaceutical form, metabolism and excretion;

and the Phase II (Tier A and B) – analysis of the information about the environmental fate and effects.

EU legislation concerning the protection of aquatic environments is mainly represented by the Directive 2000/60/EC - Water Framework Directive (WFD) (29), accompanied by the Ground Water Directive (GWD) 2006/118/EC (30) for the protection of groundwater. The general objectives of the WFD is to reach better ecological and chemical status of European water bodies within 2015, through the adoption of measures aimed at reducing, limiting, and preventing pollution. The WFD daughter Directive 2008/105/EC (31) on environmental quality standards in the field of water, established the EU List of Priority Substances of 33 pollutants. In January 2012, the EU Commission proposed to add 15 chemicals to the List, selected according the levels found in surface water, production and use, and hazardousness. Among these newly proposed pollutants, there are, for the first time, three pharmaceutical compounds, i.e. 17 alpha-ethinylestradiol (EE2), 17 beta-estradiol (E2) and diclofenac. The proposal does not put into question the therapeutic value of these pharmaceuticals, just addresses the potential harmful effects of their presence in the aquatic environment.

Currently, there are only few regulatory bodies that recognize pharmaceuticals as a threat to the environment. The OSPAR Convention is a current legal instrument guiding international cooperation for the Protection of the marine Environment of the North-East Atlantic (http://www.ospar.org). They published the List of Chemicals for Priority Action (Revised 2011) that includes pharmaceutical clotrimazole (an antifungal agent) and diosgenin (steroid), while other 22 pharmaceuticals and 5 hormones are listed as Substances of Possible Concern.

Another international water research alliance, the Global Water Research Coalition (GWRC), is focused on urban water supply, wastewater issues, and renewable water resources. The organization defined a high priority level for a group of pharmaceuticals (belonging to different therapeutic classes): carbamazepine, sulfamethoxazole, diclofenac, ibuprofen, naproxen, bezafibrate, atenolol, ciprofloxacin, erythromycin and gemfibrozil.

In addition, national prioritisation procedures have also designated some pharmaceutical compounds according to the potential risk that they may pose to the aquatic environment. In the United Kingdom, 12 pharmaceuticals belonging to analgesics, antidepressants, antibiotics, and antineoplastics were prioritised for targeted monitoring based upon their predicted environmental concentrations, persistence, bioaccumulation and toxic properties. In the United States, in the EPA report CCL-3 (Contaminant Candidate List-3 from 2009.), erythromycin, 17α-ethinylestradiol, and nitroglycerin, among 104 chemicals, were prioritized for potential regulation and treatment (3).

There is not yet any legislation regarding pharmaceuticals in the terrestrial environment and biosolids from wastewater treatment.

1.3. Physicochemical properties of pharmaceuticals related to their environmental fate

Most pharmaceutically active compounds are small organic molecules (Mw<1000 Da), moderately hydrophilic but also lipophilic to be bioavailable and biologically active. Carbocyclic and heterocyclic systems are ubiquitous in pharmaceutical structure, with five- and six- membered aromatic (or pseudoaromatic) rings being the most common. Pharmaceuticals usually contain polar groups, such as ethers, amines, amides, esters, carboxylic acids, nitriles, halides, alcohols, thiols, N-oxides, sulfoxides, sulfonamides and others. Carboxylic acids, phenols, thiols, sulfonamides, amines and other nitrogen-containing functional groups (e.g. amidines) are capable of existing in both ionized and non-ionized forms which is very important for transfer of drugs in the body and the environment. Many pharmaceuticals are chiral and often administrated as racemic mixtures (32).

An important characteristic of pharmaceuticals is their pharmacological activity that can affect biological endocrine systems and result in effects on growth, development, or reproduction at much lower concentration levels than would be expected on the basis of their acute toxicity. Besides, some other characteristics of pharmaceuticals should be highlighted as they influence the fate of these micro-contaminants in the environment and make them different from conventional industrial chemical pollutants. These characteristics include: polymorphic crystal structures, introduction to the environment after human metabolism, a diversity of compounds under the class of pharmaceuticals, and chemically complex structure and ionizable nature with multiple ionization sites spread throughout the molecule (33).

Polymorphism is the ability of a substance to exist as two or more crystalline phases that have different arrangements and/or conformations of the molecules in the crystal lattice (34). The existence of different crystal structures of the various polymorphs of a drug often causes these solids to exhibit a variety of different physical properties, such as kinetic (e.g. stability, dissolution rate), surface (e.g. interfacial tension, surface free energy), thermodynamic properties (e.g. solubility, vapor pressure) (35) etc. Many pharmaceutically active compounds are solids with tendency to form polymorphs. The different crystal forms of pharmaceuticals can lead to their different physico-chemical properties, but also affect their processing, formulation and drug availability (35). The information on the solid forms of pharmaceuticals is appreciable for better understanding of their fate in the environment, as well as for the assessment of the environmental effects of these compounds. For instance, the polymorphism has important consequences on bioavailability or toxicity of pharmaceuticals if the bioavailability is mediated via dissolution (34).

Metabolism. Before being excreted from the human body, pharmaceutically active molecules undergo two main biotransformation steps: Phase I

(functionalization) reactions, which include oxidation, reduction and hydrolysis, followed by Phase II conjugation reactions to form readily excreted water-soluble polar metabolites (36). The most important Phase II reactions are glycosylation, sulfation, amino acid conjugation and acetylation. Conjugation is considered as a detoxication reaction, as the metabolites are usually less toxic than the parent compounds (37). In general, the purpose of biotransformation of pharmaceuticals into Phase II metabolites is to rendering the compounds more hydrophilic, less active and less toxic (38). However, there are some exceptions to this rule such as two classes of glucuronides with electrophilic reactivity, the *N*-*O*-glucuronides of hydroxamic acids (*N*-hydroxy-*N*-acetyl-arylamines) and the acyl glucuronides of carboxylic acids (39). The latter have been involved in a wide range of adverse drug effects, including drug hypersensitivity reactions and specific organ toxicities. Organic molecules containing hydroxyl, carbonyl, amino, tertiary amine and thiol groups undergo glucuronide conjugation; while sulfonate conjugates are formed with hydroxyl group (especially phenolic groups), and, less frequently, amino groups (aromatic amines) (40). Uridine diphophoglucuronic acid (UDPGA), generated from glucose, is a donor of glucuronic acid in glucuronidation (39, 41). Because of the general availability of glucose in biological systems, glucuronide formation is one of the most common Phase II reactions for pharmaceuticals, and may account for a major share of metabolites. An antidepressant lamotrigine and its conjugate 2-N-glucruonide were determined in wastewater and receiving surface water, indicating that this conjugate is passing WWTP without a major cleavage (42). Glucuronide and sulfate conjugates of oestrogens have been reported to occur in wastewater influent and effluent (43-44). Thus, the conjugates (especially the frequently occurred N- and O-glucuronide conjugates) should be included in monitoring programs to obtain more accurate information on the loads of pharmaceuticals present in wastewater and environmental matrixes. Although pharmaceuticals are extensively metabolized in the human body, the occurrence of the parent compounds indicates that the metabolites convert back to their active forms on the way to or within the WWTPs, increasing the concentrations and justifying the importance of metabolites. The cleavage of the conjugates is catalyzes by *β*-glucuridase enzyme that is produced by *Escherichia coli* present in wastewaters (45-46). The mechanism and the final result are still unclear. If a Phase II metabolite was formed from a Phase I metabolite, and not directly from the parent compound, then the deconjugation would convert the conjugate back to the Phase I metabolite, and not into the parent compound (47). As we deal here with the complex wastewater matrix, with a variety of microorganisms present, it is difficult to reach any firm conclusion. What is clear is that the glucuronides can be cleaved, and not necessarily completely cleaved/reconjugated (48), and thus for assessing the relevant environmental concentrations, both parent compounds and their conjugates/metabolites have to be included into mass balances.

Diversity of compounds. DIRECTIVE 2004/27/EC of the European Parliament and of the Council of 31 March 2004 defines medicinal product as:

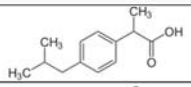
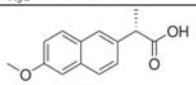
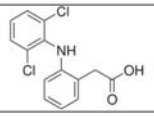
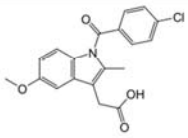
(a) Any substance or combination of substances presented as having properties for treating or preventing disease in human beings; or

(b) Any substance or combination of substances which may be used in or administered to human beings either with a view to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis.

Pharmaceuticals are a heterogeneous class of compounds designed to meet a wide range of medical needs. They differ in mode of action, chemical structure, physicochemical properties, metabolism, and eco-toxicological characteristics, and thus they will have their own specific fate in the environment. The classification of such a chemically and pharmacologically heterogeneous group of compounds is complex, as well. For simplicity, all the registered therapeutic drugs are divided into fourteen main groups according to the organ or system on which they act (anatomical) and their chemical, pharmacological and therapeutic properties - Anatomical Therapeutic Chemical Classification System (ATC system) (WHO Collaborating Centre for Drug Statistics Methodology - WHOCC). Due to the volume of prescription, the toxicity and the evidence for presence in the environment, nonsteroidal anti-inflammatory drugs (NSAIDs), antibiotics, beta-blockers, antiepileptics, blood lipid-lowering agents, antidepressants, hormones, and antihistamines have been the most studied pharmaceutical groups (49). For example, Table 1.2 shows few pharmaceuticals that belong to ATC MO1 group – *Anti-inflammatory and antirheumatic products* - a therapeutic subgroup of the anatomical group M-Musculo-skeletal system (Source: DrugBank). As shown, these four compounds are acids, and although they have similar therapeutic application, they have different solubility and partitioning properties that will influence their fate in the environment.

Polar and ionic compounds. Because of their chemically complex and multifunctional composition, many pharmaceuticals are ionizable and polar compounds. The degree of ionization of pharmaceuticals, which depends on pH, will affect their solubility, transport, sorption and bioavailability in the environment. Their ultimate fate in the environment does not depend only on pH, but also on the moisture content of sorbing system, the presence of exchangeable cations and electrolyte concentration (50). For such polar and ionizable compounds, the octanol/water partition coefficient (K_{ow} or P) does not describe adequately the environmental partitioning and their dynamics in the environment (33).

Table 1.2. Selected NSAIDs and their characteristic properties

Compound	IUPAC name	Structure	Mw	pKa	logP	Solubilty, mg/L
IBUPROFEN	(RS)-2-(4-(2-methylporpyl)phenyl)propanoic acid)		206.29	4.91	3.6	21
NAPROXEN	(+)-(S)-2-(6-methoxynaphtalen-2-yl)propanoic acid		230.27	4.15	3.18	15.9
DICLOFENAC	2-(2-(2,6-dichlorophenylamino]) phenyl)acetic acid		296.16	4.15	4.51	2.37
INDOMETACIN	2-{1-[(4-chlorophenyl)carbonyl]-5-methoxy-2-methyl-1H-indol-3-yl}acetic acid		357.79	4.5	4.27	0.937

For an ionizable compound, acidic or basic, which can exist as neutral or dissociated form, the partitioning depends on pH and pK_a of the compound, and so the pH-dependent *n*-octanol–water distribution (D_{ow}) appears to be more appropriate physicochemical parameter for understanding the behavior of pharmaceuticals in different environmental matrixes (38). Nevertheless, the charged groups within the molecules of pharmaceuticals can lead to ionic, ion paring and complexation reactions with the particulate matter and microorganisms present in biomass, sediment and soil, leading to errors in estimating of partitioning to these solids by $LogD_{ow}$ (33). Additionally, most of pharmaceuticals compound contain one or more aromatic or pseudo-aromatic rings that play an important role in the hydrophobic interaction. Therefore, the best would be to estimate experimentally the partitioning coefficients for each compound, matrix and pH. But, it would be also time and money consuming process. Sorption mechanisms will be discussed more throughout the chapter.

1.4. Source of pharmaceuticals to the environment

Pharmaceuticals find their way to the environment primarily via the discharge of raw and treated sewage from residential users or medical facilities, as a result of their intended use in therapy or for cosmetic purposes. Through the excretion via urine and feces, extensively metabolized drugs are released into the environment. For most pharmaceuticals, the fraction of parent, unchanged compound transported to the environment is attenuated as a result of metabolic processes in the body or transformation within a sewage system and treatment facility. But, the topically applied pharmaceuticals (when washed off) and the expired and unused ones, disposed directly to trash or sewage, pose a direct risk to the environment because they enter sewage in their unmetabolised and powerful form (2). By direct disposal to

trash and sewage, pharmaceuticals can reach surface and groundwater through landfill and wastewater effluents, respectively. The importance of the purposeful disposal of drugs is subject to speculations as it is unknown the percentage of any particular pharmaceutical in the environment from this type of disposal, but of any other as well. Although the production of drugs is governed by rigorous regulations, pharmaceuticals are frequently released with the waste from drug manufacturing plants (51-53). The contribution to the contamination of surface and groundwater during manufacturing is unknown. In order to design and implement effective pollution prevention strategy, it is important to understand the sources of disposal of pharmaceuticals along with the type and quantity of pharmaceuticals resulting from each source (2, 54).

1.5. Conventional activated sludge process for treatment of wastewater – basic concepts

Wastewater treatment involves different physical, chemical and biological processes to improve or upgrade the quality of wastewater for its safe and healthy reuse or discharge into receiving waters. A variety of treatment operation and processes have been designed, arranged in sequential series, and applied to remove contaminants from wastewater. The list of wastewater contaminants of primarily concern includes biodegradable organics (measured in terms of biochemical oxygen demand (BOD) and chemical oxygen demand (COD)), suspended solids, nutrients (i.e. nitrogen and phosphorus), pathogens, heavy metals, dissolved inorganics (Ca^{2+}, Na^{+}, SO_4^{2-}), priority pollutants and refractory organics (55). Wastewater treatment systems that use activated sludge processes have been employed extensively throughout the world for the treatment of municipal and biodegradable industrial wastewater, mostly because they produce effluents that meet required quality standards (suitable for disposal or recycling purposes), at reasonable operating and maintenance costs. Figure 1.2 shows a simplified flow diagram of a conventional wastewater treatment that employs activated sludge process.

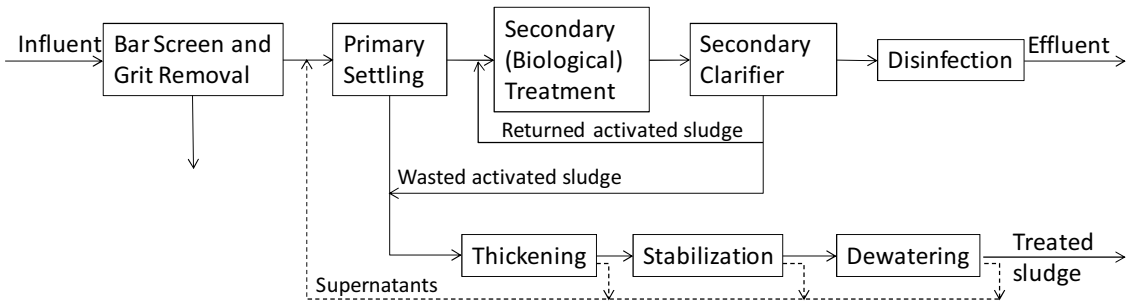


Figure 1.2. Flow diagram of a conventional wastewater treatment plant

All conventional WWTP design processes include preliminary treatment consisting of bar screen, grit chamber, and oil and grease removal unit (56), that are used to remove larger and heavier inorganic and organic solids (e.g. sand, gravel, etc.) in order to protect the process equipment (e.g. from abrasion, deposition in pipelines, etc) and prevent interferences with the treatment process. Subsequently, the settleable undissolved solids are separated in the primary gravity settling tank, to reduce the organic loading on the following processes. In the case of smaller treatment plants or those that receive low-loaded wastewater, and treat it in a biological nutrient removal process, the primary sedimentation is omitted in order to preserve particulate fraction of the influent COD necessary for the secondary process. The wastewater then enters into a biological treatment process – commonly an aerobic suspended-growth process (i.e. activated sludge process) – where mixed liquor, i.e. microorganisms responsible for the treatment, along with biodegradable and nonbiodegradable suspended, colloidal, and soluble organic and inorganic matter, is maintained in liquid suspension by appropriate mixing methods. During the aeration period, adsorption, flocculation, and oxidation of organic matter occur, and the remaining BOD and suspended solids are almost totally removed from the wastewater. Nitrogen and phosphorus are generally removed simultaneously, although nitrogen is easily targeted first. After enough time for appropriate biochemical reactions, mixed liquor is transferred to a settling reactor (clarifier) to allow gravity separation of the suspended solids, in form of floc particles, from the treated wastewater. The secondary wastewater effluent then flows though the filters and it is disinfected prior to discharge. In order to achieve better effluent water quality, some further treatment steps - tertiary treatment - can be added to the above outlined general process, e.g. activated carbon adsorption, additional nutrient removal, membrane filtration, etc.

Settled solids from the secondary clarification are returned to the biological reactor (i.e. return activated sludge) to maintain a concentrated biomass (i.e. microorganisms) at the required level for the treatment. Since the microorganisms are continuously synthesized in the process, some of suspended solids must be wasted from the system in order to maintain a selected biomass concentration in the system. Wasting is performed by diverting a portion of the solids from the biological reactor to solids-handling processes. The most common practice is to waste sludge from the return sludge line because return activated sludge is more concentrated and requires smaller waste sludge pumps. The waste sludge can be discharged to the primary sedimentation tanks for co-thickening, to thickening tanks, or to other sludge-thickening facilities, in order to increase the solid content of sludge by removing a portion of the liquid fraction. Through the subsequent processes such as sludge stabilization, dewatering, drying, and combustion, the water and organic content is considerably reduced, and the processed solids are suitable for reuse or final disposal. The sludge stabilization is a very important step in the whole treatment because the sludge generated during primary and secondary treatment contains a great deal of

concentrated wastewater macro-contaminant. Anaerobic digestion is the mostly applied sludge stabilization process, but also an essential part of WWTP, as it destroys most of pathogens present in sludge, and provides sludge volume reduction, nutrient recycling and renewable energy production (i.e. biogas production) while stabilizing the sludge. Depending on the quality of the treated sludge (e.g. amount, metals, pathogen content etc.), and defined by the official regulations, it will be disposed directly to land, landfills (i.e. safe disposal), composted (to reuse the nutrients) or incinerated.

Conventional WWTP have been primarily designed to remove pathogens and organic and inorganic suspended and flocculated matter, and not to specifically remove pharmaceuticals that may be present in sewage water. Nevertheless, they can and do remove pharmaceuticals to some degree, but never completely. Upgrading of the existing conventional wastewater treatment plants has been recognized as a strategy that could provide more efficient removal of pharmaceuticals. A range of new technologies have been developed, e.g. membrane bioreactors, micro-, ultra-, and nanofiltration, reverse osmosis, and advanced oxidation technologies, but they are mostly effective for the treatment of drinking water. The systems such as filtration and reverse osmosis systems foul quickly when used for wastewater because of the high organic matter content. The organic matter limits the capability of the advanced oxidation processes as well, and thus these processes could only be applied to highly treated wastewater to reduce the dissolved organic matter as much as possible. The high content of organic matter in wastewaters reduces the effectiveness of the advanced treatment and increases their costs, and therefore greatly limits them from being used commercially for wastewater treatments.

Another important strategy to effectively reduce the input of these contaminants to the environment is source control and source separation options. The prevention of contamination (e.g. source reduction/control) is a long-term control imperative (1). The source control activities are tied closely to the end users (consumers) and the issues associated to drug disposal rather than those that involve the control of the health care system and industry. Thus effective source control reduces the ecological exposure of drugs by reducing their consumed quantities. It can be implemented through the controlled pharmaceuticals consumption, i.e. targeted therapy, and the use of alternative therapeutic choices that are less bioaccumulative and less persistent drugs, as well as eco-labelling and product take-back programs guided by national regulations (57).

Even though the new treatment technologies have been developed to deal with health and environmental concerns associated with findings of nowadays research, the progress was not as enhanced as the one of the analytical detection capabilities and the pharmaceutical residues remain in output of WWTPs. This is the main reason that

we find a variety of pharmaceuticals in different environmental matrices including surface, groundwater and even drinking water. In the following paragraphs, we aim to summarize recent literature information on the occurrence of pharmaceuticals and their removal during wastewater treatment.

1.6. Occurrence of pharmaceuticals in wastewater

Numerous research publication*s* have reported on the occurrence, fate and effects of pharmaceuticals in the environment, but we have data on the occurrence of only 10% of the registered active compounds, and very few information on their effects in the environment. There is even less information regarding the occurrence and fate of the metabolites and transformation products of pharmaceuticals. Both the qualitative and the quantitative analysis of pharmaceuticals in the environmental matrices are definitely a starting point for the environmental risk assessment of pharmaceuticals and the establishment of new regulations regarding these micro-pollutants in the environment.

The occurrence of the pharmaceutical compounds in wastewater treatment plants has been investigated in many countries around the world (EU countries, Switzerland, USA, China, Australia). More than 150 pharmaceuticals belonging to different therapeutic groups have been detected in concentration ranging up to the µg/L-level in sewage water. Their environmental occurrence naturally depends on the rate of production, the dosage and frequency of administration and usage, the metabolism and environmental persistence, as well as on the removal efficiency of WWTPs. Figure 1.3 show the occurrence of the selected, most investigated pharmaceuticals in wastewater influent (A) and effluent (B), as compiled from international scientific papers (cited throughout the thesis) covering a period from 1999 until nowadays.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most used class of drugs for treatment of acute pain and inflammation. They are administered both orally and topically and available as prescription and over-the-counter (non-prescription) drugs. High consumption and way of administration of NSAIDs result in elevated concentration reported in the effluent from WWTPs. Among the most studied NSAIDs during wastewater treatments are: ibuprofen, diclofenac, naproxen, ketoprofen and mefenamic acid (58). The compounds usually detected in the highest concentrations in the influent of WWTPs are ibuprofen, naproxen and ketoprofen (in range of some µg/L) (59-61). Even though the concentrations of these compounds are markedly lowered at the effluent, they are far from negligible. Heberer et al (62) identified diclofenac as one of the most important pharmaceuticals in the water-cycle, with low

µg/L concentrations in both row and treated wastewater (3.0 and 2.5 µg/L at the influent and effluent, respectively).

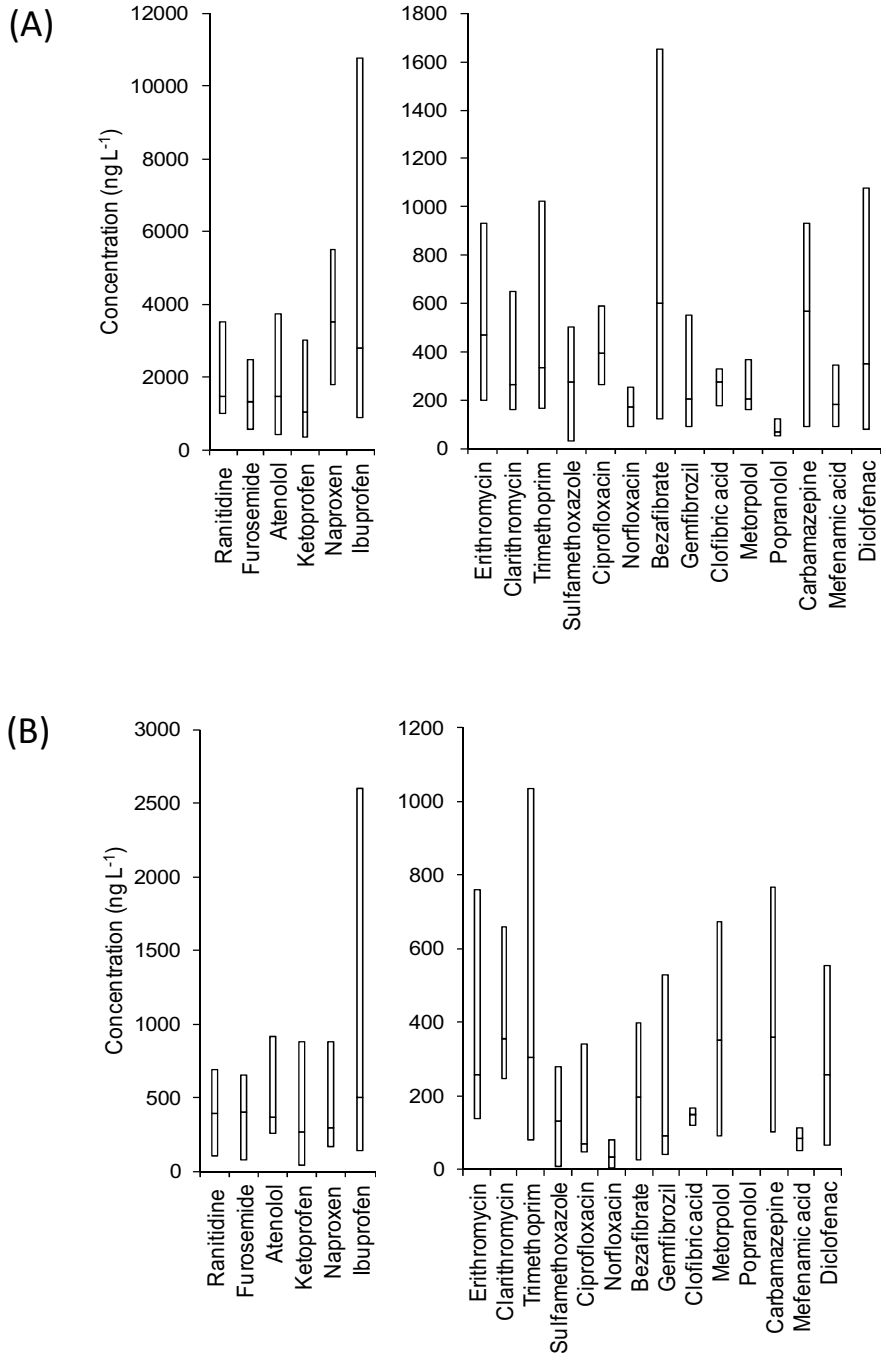


Figure 1.3. Concentrations of pharmaceuticals in wastewater influent (A) and effluent (B) (25^{th}, median and 75^{th} percentile, for the values found in the literature)

Beta-blockers are another very important class of prescription drugs. They are very effective in treating cardiovascular diseases. As NSAIDs, beta-blockers are not highly persistent, but they are present in the environmental due to their high volume of use. Due to same mode of action of beta-blockers, it has been found that the

mixture of beta-blockers showed concentration addition indicating a mutual specific nontarget effect on algae (63). These compounds are generally found in aqueous phase because of their low sorption affinity and elevated biodegradability (64). Atenolol, metoprolol and propranolol have been frequently identified in wastewaters, where atenolol was detected in the highest concentrations, in some cases ranging up to 1 μg/L (65-67). As a result of the incomplete removal during conventional wastewater treatment, these compounds were also found in surface waters in ng/L to low mg/L range (68).

Lipid lowering drugs, with statins and fibrates particularly, are used in the treatment and prevention of cardiovascular disease. In the last decade, statins became the drug of choice to lower cholesterol levels and their usage is increasing. According to a report of the National Center for Health Statistics of USA (69), from 1988–1994 and 2003–2006, the use of statin drugs by adults 45 years of age and over, increased almost 10-fold, from 2% to 22%. Among lipid lowering drugs and pharmaceuticals, in general, clofibric acid is one of the most frequently detected in the environment and one of the most persistent drugs with an estimated persistence in the environment of 21 years (49). It has been detected in the ng/L range concentrations in influent, without big difference in the concentrations at the effluent. Many analogues of clofibrate, such as gemfibrozil, bezafibrate and fenofibrate, were detected in samples of sewage plants in concentrations up to low μg/L at the influent (66). Among statins, atorvastatin, mevastatine and prevastatine were detected in various environmental matrices including raw and treated wastewater as well as surface water near the points of discharge (68, 70-71)

Antibiotics are destined to treat diseases and infection caused by bacteria. They are among the most frequently prescribed drugs for humans and animals in modern medicine. Beta-lactams, macrolides, sulfonamides, fluoroquinolones, and tetracyclines are the most important antibiotic groups used in both human and veterinary medicine. High global consumption of up to 200 thousand tons per year (72) and high percentage of antibiotics that may be excreted without undergoing metabolism (up to 90%) result in their wide spread presence in the environment (73). Unmetabolized, thus pharmaceutically active forms of antibiotics concentrated in raw sludge may promote the development of bacterial resistance. Bacteria in raw sludge are more resistant than bacteria elsewhere (74). Many active antibiotic substances were found in raw sewage matrices, including both aqueous and solid phase. Beta-lactams are among the most prescribed antibiotics. Despite their high usage, they readily undergo hydrolysis, and thus have been detected in very low concentrations in treated wastewater, or not at all detected (Le-Minh et al, 2010). Sulfonamides, fluoroquinolones, and macrolide antibiotics show the highest persistence and are frequently detected in wastewater and surface waters (73). Sulfamethoxazole is one of the most detected sulfonamides (75-79) that was reported with various concentration and up to 8μg/L (in raw influent in China) (80). Sulfamethoxazole is often administrated in combination with

trimethoprim, and commonly analyzed together (81). Trimethoprim exhibit high persistence with little removal being effected by WWTPs, thus is ubiquitously detected in ranges from very low ng/L to 1 μg/L in wastewater influent and effluent (75, 82). Fluoroquinolone antibiotics ciprofloxacin and norfloxacin have been frequently detected in various streams in low μg/L (59, 75, 83-84). Even though it is greatly reduced during treatment, ciprofloxacin was found to be present in effluent wastewater at average concentration from 0.1 to 0.6 μg/L (82, 85). The class of tetracyclines, widely used broad-spectrum antibiotics, with chlortetracycline, oxytetracycline, and tetracycline as mostly used, was detected in raw and treated sewage in many studies in the ng/L (86) to μg /L concentrations (87). Tetracyclines and fluoroquinolones form stable complexes with particulates and metal cations, showing the capacity to be more abundant in the sewage sludge (36, 88). Some of the most prescribed antibiotics – macrolides clarythormycin, azythromycin, roxythoromycin and dehydro-erythromycin – were found in various environmental matrices in concentrations ranging from very low ng/L to few μg/L (81, 89-90). High effluent concentrations were reported for dehydro-erythromycin, up to 2.5 μg/L (87, 91). In the final US EPA report CCL-3 from September 2009, erythromycin was one of three pharmaceuticals included as priority drinking water contaminant, based on health effects and occurrence in environmental waters (3).

According to the report of National Center for Health Statistics of USA (69), the usage of *antidiabetic drugs* by adults 45 years and over increased about 50%, from 7% in 1988–1994 to 11% in 2003–2006, what has as a consequence an increase in the detection of antidiabetics in the environment over time. Still, only few data are reported on the occurrence and fate of antidiabetics. Glyburide (also glibenclamide) was found to be ubiquitous in both aqueous and solid phase of sewage treatment (60, 68, 92).

Histamine H2-receptor antagonists are used in treatment of peptic ulcer and gastro esophageal reflux disease. Certain preparations of these drugs are available OTC in various countries. Among H2-receptor antagonists, cimetidine and ranitidine have been frequently detected in wastewater and sludge. As for all the other pharmaceuticals, the reported concentration vary from very low ng/L to a few μg/L (60-61, 93).

While antiepileptic carbamazepine is one of the most studied and detected pharmaceuticals in the environment, there in not many information on the occurrence and fate of other of psycho-active drugs in WWTPs. Carbamazepine is one of the most widely prescribed and very important drug for the treatment of epilepsy, trigeminal neuralgia and some psychiatric diseases *(e.g.* bipolar affective disorders (94). In humans, following oral administration, it is metabolized to pharmacologically active carbamazepine-10,11-epoxide, that is further hydrolyzed to inactive carbamazepine-

10, 11-trans-dihydrodiol and conjugated products which are finally excreted in the urine. Carbamazepine is almost completely transformed by metabolism with less than 5% of a dose excreted unchanged (95). Still, glucuronide conjugates of carbamazepine can presumably be cleaved during wastewater treatment, so its environmental concentrations increase (66). In fact, carbamazepine and its metabolites have been detected in both wastewaters and biosolids (96). Carbamazepine is heavily or no degraded during wastewater treatment and many studies have found it ubiquitous in various environment matrices (groundwater, river, soil) (97)). The concentrations of carbamazepine vary from one plant to another, and they are usually around hundreds ng/L, and incidentally up to few μg/L (66, 98).

1.7. Occurrence of pharmaceuticals in sewage sludge

Sewage sludge is a semi-solid residue generated during the primary (physical and/or chemical), the secondary (biological) and the tertiary (often nutrient removal) treatment. In the last years the quantities of sludge have been increasing in EU because of the implementation of the Directive 91/271/EEC on urban wastewater treatment. There were nearly 9 million tons (dry matter) produced in 2005 in EU Member states. Similar regulation in USA was introduced by USA EPA regulations 40 Code of Federal Regulations Part 503 (40 CFR 503) that established the minimum national standards for the use and disposal of domestic sludge. By this regulation, sludge was classified into two different microbiological types according to the extent of pathogen removal achieved by the sludge treatment process, i.e. Class A (usage without end-use restrictions) and Class B (controlled/limited disposal). The amount of sludge generated in 2006 was estimated to be more than 8 million tons of which 50% were land applied (99).

Due to the physical-chemical processes involved in the treatment, the sludge tends to concentrate heavy metals and poorly biodegradable trace organic compounds as well as potentially pathogenic organisms (viruses, bacteria etc) present in waste waters. Sludge is, however, rich in nutrients such as nitrogen and phosphorous and contains valuable organic matter that is useful when soils are depleted or subject to erosion. It has been used in agriculture over a long time. In EU, since 1986, the utilization of sewage sludge has been ruled by the EU Directive (86/278/EEC), which encouraged the use of sludge regulating its use with respect to the quality of sludge, the soil on which it is to be used, the loading rate, and the crops that may be grown on treated land. None of the regulations cover the question of pharmaceuticals and other emerging pollutants that may be transported to soil after land application of biosolids, having the potential to enter surface water, leach into groundwater or be accumulated by vegetation or other living organisms.

Most of the studies on the fate of pharmaceuticals in WWTPs focused only on the aqueous phase, and concentrations of the compounds in sludge were rarely determined mainly due to the demanding efforts required in the analysis of such difficult matrix. Out of 117 publications studied by Miege *et al* (58), only 15 reported the concentrations of pharmaceuticals in sludge and 1 in suspended solid, and none of these papers reported the removal obtained taking into account both aqueous and solid phases of WWTPs. Still, the screening of sewage sludge showed that these micropollutants are very present in this medium (100-103). High aqueous-phase removal rates for some compounds would suggest very good removal of these compounds during wastewater treatment. But, only a certain percent of the total mass input is really lost (i.e. biodegraded) during the treatment. The rest accumulates in sludge or end up discharged with the effluent. The sorption behaviour of pharmaceuticals can be very complex and difficult to assess. These compounds can absorb onto bacterial lipid structure and fat fraction of the sewage sludge through hydrophobic interactions (e.g., aliphatic and aromatic groups), adsorb onto often negatively charged polysaccharide structures on the outside of bacterial cells through electrostatic interactions (e.g., amino groups), and/or they can bind chemically to bacterial proteins and nucleic acids (104). Also the mechanisms other than hydrophobic partitioning, as hydrogen bonding, ionic interactions and surface complexation, play a significant role in the sorption of pharmaceuticals in sludge. Therefore, also the compounds with low $LogD_{ow}$ and $LogK_d$ values may sorb onto sludge. Despite their negative D_{ow}, fluoroquinolones have a high tendency for sorption because of their zwiterionic character (pK_{aCOOH} = 5.9−6.4, pK_{aNH2} = 7.7−10.2) (83). These antibiotics were reported to be present in the highest concentration in sewage sludge samples from various WWTPs (84, 100, 105). Also tetracycline and sulfonamides exhibit strong sorption onto sludge particles, higher than expected based on their hydrophobicity.

In general, data on the occurrence of pharmaceuticals in sludge are sparse, where the group of antibiotics was mostly analyzed and found to be the most abundant. Kinney *et al* (8) measured erythromycin-H2O, sulfamethoxazole, and trimethoprim in microgram per kilogram concentrations in 9 different biosolids. Out of the 72 pharmaceuticals and personal care products targeted in the study of EPA in its 2001 National Sewage Sludge Survey, 38 (54%) were detected at concentrations ranging from the low ng/g to the µg/g range. All the analyzed antibiotics constituted about 29% of the total mass of pharmaceuticals per sample (102). Only few studies reported data on the occurrence of NSAIDs and some other therapeutic classes (psycho-active drugs, lipid lowering drugs etc) (60, 105-108). Figure 1.4 summarizes literature data (cited throughout the thesis) on the occurrence of frequently analyzed pharmaceuticals in sewage sludge in various WWTPs.

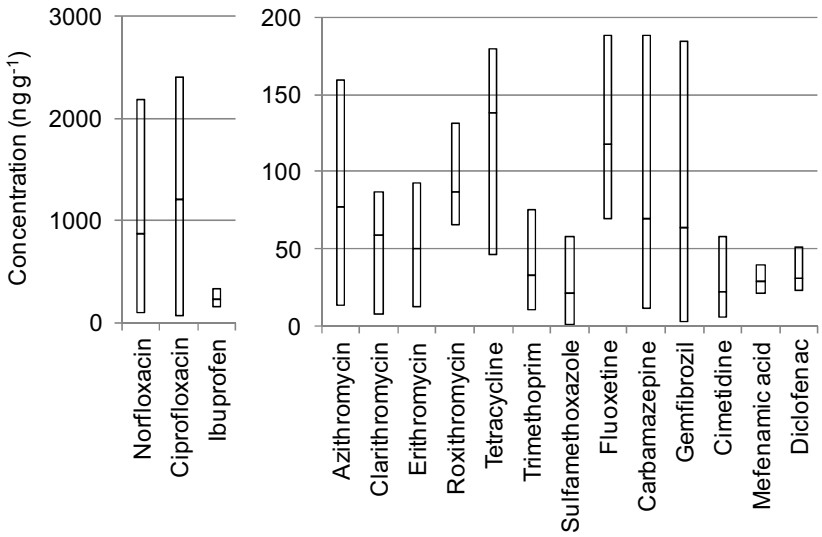


Figure 1.4. Concentrations of pharmaceuticals in sewage sludge (25th, median and 75th percentile, for the values found in the literature)

1.8. Removal of pharmaceuticals during conventional wastewater treatment

Municipal wastewater treatment plants were basically designed to remove pathogens and organic and inorganic suspended and flocculated matter, but not pharmaceuticals. When speaking about pharmaceuticals, the term *removal* refers to the conversion of a pharmaceutical to a compound different than the analyzed one (i.e. the parent compound) and only in few cases will mean as well *elimination*. Thus, it accounts for all the losses of a parent compound produced by different mechanisms of chemical and physical transformation, biodegradation and sorption to solid matter. All these processes (mainly biodegradation, sorption, and photodegradation) are limited in some way for the following reasons: (a) pharmaceuticals are designed to be biologically stable; (b) the sorption depends on the type and properties, and concentration of the suspended solids (sludge); (c) and although they are photoactive, because many of them have aromatic rings, heteroatoms and other functional groups that could be susceptible to photodegradation, they may also give by-products of environmental concern.

In some studies, negative values of removal (i.e. difference in the loads between influent and effluent) have been reported. The explanation for this could be found in sampling protocols (109); not only because they could be inadequate, but because of the nature of disposal of pharmaceuticals. The fact is that the substances arrive in a small number of wastewater packets to the influent of WWTP, in unpredictable amounts and time intervals, thus the influent loads, especially, are easily systematically underestimated. Therefore, well-planned and performed sampling is the

key point in pharmaceutical residue analysis. Even though the analysis of effluent and sludge yields more certain results, because they come from stabilization processes, the sampling in general may result in underestimated and even negative removals. Furthermore, the negative removal can be explained by the formation of unmeasured products of human metabolism and/or transformation products (e.g. glucuronide conjugate, methylates, glycinates etc.) that passing through the plant convert back to the parent compounds. This can be considered as a reasonable assumption since the metabolites and some derivatives of pharmaceuticals are well-known (e.g. hydroxy- and epoxy-derivatives of carbamazepine; 4-trans-hydroxy and 3-cis-hydroxy derivatives of glibenclamide; ortho- and parahydroxylated derivatives of atorvastatin, etc.) (96, 110-111). During complex metabolic processes in human body and bio-chemical in wastewater treatment, various scenarios of transformation from parent compound to metabolite and derivatives and vice versa can occur. Generally speaking, metabolites tend to increase the water solubility of the parent compound. Metabolites can be just as active as their parent compounds. Therefore, the occurrence of metabolites and transformation products and the transformation pathways should be included in the future studies in order to obtain accurate information on removal of pharmaceuticals during treatment and to determine treatment plant capabilities.

1.8.1. Removal mechanisms

The extent to which one compound can be removed during wastewater treatment is influenced by chemical and biological properties of the compound, wastewater characteristics, operational conditions and treatment technology used. For this reason, and as shown in the following examples, high variations in elimination may be expected, and no clear and definitive conclusion can be made on removal of any particular compound, and even less on the fate of a therapeutic group. Considering the fact that the conditions of loading and operation of full-scale activated sludge processes are variable and the systems are inherently dynamic, the variations in removal efficiencies are observed also for a single plant (112).

Removal of pharmaceuticals during biological wastewater treatment is ruled by aerobic/anaerobic biotransformation, sorption (and desorption), and volatilization. Biodegradation and sorption are main removal mechanisms since the polar pharmaceuticals exhibit generally low volatility. Operating parameters such as hydraulic retention time (HRT), solid retention time (SRT), redox conditions and temperature affect sorption and biodegradation mechanisms, and consequently the removal of pharmaceuticals during conventional treatment (113). Of all the operating parameters, SRT is the most critical parameter for activated-sludge design as it affects the treatment process performance, aeration tank volume, sludge production, and oxygen requirements (112, 114-115). It has been proven that longer SRT, especially, influences and improves the elimination of most of the pharmaceuticals during sewage

treatment (77, 116-117). WWTPs with high SRTs allow the enrichment of slowly growing bacteria and consequently the establishment of a more diverse biocoenosis with broader physiological capabilities (e.g., nitrification or the capability for certain elimination pathways) than WWTPs with low SRTs (116).

Biodegradation. Biodegradation is the most important process for the removal of pollutants from the environment, and it can be defined as the biologically catalyzed reduction in complexity of chemicals (118). The biodegradation of pharmaceuticals manly depends on their intrinsic biodegradability. Joss *et al* (119) determined kinetic degradation rate constants for 35 pharmaceuticals, hormones and personal care products in activated sludge from a nutrient-removing municipal WWTP, and divided these compounds into different classes according to their biodegradability in the WWTP: a) no degradable: K_{biol}<0.1 L $kg_{SS}^{-1}d^{-1}$ (macrolide and sulfonamide antibiotics); b) moderately degradable: 0.1<K_{biol}<10 L $kg_{SS}^{-1}d^{-1}$; and c) transformed by more than 90%: K_{biol}>10 L $kg_{SS}^{-1}d^{-1}$ (ibuprofen, paracetamol, estrone and 17β-estradiol). They observed that: a) the dilution of wastewater due to rain or infiltration to the sewer reduces the degradation for a given reactor loading; b) treatment at the source is favorable if dilution can be avoided; and c) dividing of the available reactor volume into reactor cascades can appreciably improve performance, except in case of significant sorption (K_d>0.1 L gSS^{-1}).

During biological wastewater treatment, organic compounds can be biodegraded by micro-organisms by anabolic (i.e. used for growth of biomass), or cometabolic activities (i.e. the compounds are modified, but not used for growth). Biodegradation of pharmaceuticals is likely due to cometabolic activity as they are not present in concentrations high enough to support substantial biomass growth (120-122). This means that the transformation of pharmaceuticals occurs during active degradation of the primary substrates present in wastewater (123). The environmental importance of cometabolism is that hazardous organic compounds may be structurally altered to less harmful products which can be mineralized by other organisms. Nevertheless, cometabolic processes can yield intermediates that can be susceptible to polymerization, sorption, and the formation of bound residues, in which organic compounds are covalently bound to humic material (118). Thus, a very important point for better understanding of pharmaceutical fate during wastewater treatment, and in the environment as well, is the analysis of transformation products of pharmaceuticals and the assessment of ecotoxicity of the formed products. The cometabolic transformation processes have been extensively studied for industrial pollutants (e.g. alkanes, alkenes, aromatics, ethers, thioethers, primary amines and chlorinated aliphatics) (124-125). Pharmaceuticals may have similar structures (e.g. polyaromatic ring) with these compounds and thus may share common cometabolic mechanisms. A variety of mono- and di-oxygenase enzymes from bacterial strains present in nitrifying activated sludge are known to aerobically cometabolise various organic pollutants and

pharmaceuticals (126-128). Ammonia monooxygenase (AMO) from *Nitrosomonas europaea* was one of the most studied. Still, it is not clear if AMO cometabolism is kinetically dominant in full-scale WWTPs among all enzymes that may transform pharmaceuticals, as it was found that AOM provides initial transformation to intermediates that can be further degraded by heterotrophic organisms present in nitrifying mixed culture (127). Further research is needed to clarify the degradation mechanism, and the importance and accumulative/synergistic effect between nitrifying and heterotrophic micro-organisms for pharmaceutical degradation in activated sludge (123).

Sorption of pharmaceuticals in wastewater treatment implies both adsorption and absorption from aqueous phase to activated sludge biomass. The affinity of a compound for the particulate matter is expressed as partitioning (distribution) coefficient (K_d), which is experimentally determined as the ratio of the quantity of the compound sorbed to solid to the amount of the compound remaining in solution at equilibrium. Partitioning coefficients have been determined for primary, secondary and anaerobically digested sludge in a number of studies to investigate the sorption of pharmaceuticals during wastewater treatment (115, 129-130). Ternes et al. (131) determined K_d for selected pharmaceuticals (antiphlogistics, estrogens, lipid regulators, anti-epileptic and cytostatic agents) and polycyclic musk fragrances (HHCB, AHTN) in lab-scale batch reactors with primary and secondary sludge slurries. For pharmaceuticals, K_d values ranged from <1 to 500 L kg^{-1}, and they were different for primary and secondary sludge: acidic pharmaceuticals had higher K_d values for primary sludge, and opposite for neutral pharmaceuticals, and ethinylestradiol. Radjenovic et al. (132) determined K_d for 15 pharmaceuticals in primary and activated sludge from a full scale WWTP, and sludge from two pilot-scale MBRs (hollow-fibre ultra-filtration and micro-filtration flat-sheet membranes). Except for loratidine, the K_d values were < 500 L kg^{-1}. The estimation of K_d is useful for numerical evaluation of the importance of sorption as a removal mechanism. For most of studied pharmaceuticals, removal by sorption is negligible in comparison to the total mass balance, because the K_d values are relatively low (<500 L kg^{-1}) (131). Carballa et al. (133) estimated K_d for several pharmaceuticals (carbamazepine, ibuprofen, naproxen, diclofenac, iopromide, sulfamethoxazole and roxithromycin) in anaerobically digested sludge. The K_d values were lower than 200 L kg^{-1} for all the pharmaceuticals, but since the sorbed amount depends on the solids concentration as well, which is quite high during the sludge treatment, the limit of relevance below which the sorption can be neglected is around 1 L kg^{-1}.

Sorption behavior of pharmaceuticals depends much on the chemical structure of the compounds. In contrast to highly lipophilic environmental contaminants such as PCBs and chlorinated pesticides (e.g. aldrine, dieldrine or DDT), which do not have ionisable nature, pharmaceuticals are complex chemical molecules which may contain

(more than one) acidic and basic groups within the same molecule. Beside hydrophobic, also ionic interactions are possible sorption mechanisms. For that reason, distribution between two phases such as sludge and water (or sorption K_d) depends on pH. The distribution, sorption, solubility, hydrophobicity of pharmaceuticals are pH-dependent. Additionally, some pharmaceuticals contain planar aromatic structures which favor intercalation into the layers of solids. Therefore and as previously explained, the sorption of such compounds depends not only on $LogK_{ow}$ (logP), as lipophilicity parameter of sorbed molecules, but it is governed by pH, redox potential, stereo chemical structure and chemical nature of both the sorbed molecule and the sorbent.

The characteristics of biomass such as particle size and surface properties have important impact on the sorption mechanism between pharmaceuticals and activated sludge biomass. These properties affect mass transfer between the biomass and target compounds, and sorption/desorption of the compounds, as well as the viability of bacteria and their enzymatic activity etc. SRT was found to influence the particle size and surface properties of flocs (134). Flocs surface charge and hydrophobicity are important in terms of flocculation and adhesion and, hence, settleability, dewaterability and rheology of sludge and consequently influence the fate of pharmaceuticals during treatment.

Activated sludge flocs are formed from three major components: microorganisms, extracellular polymers (EPS) and water. Most bacteria carry a net negative charge within a pH range of 5-9 (135). Both sludge solid and extracellular polymers (EPS) carry on overall negative charge (136) that originates mostly from the presence of uronic acids, but also from hydroxyl, methyl, sulfate and phosphate groups present in EPS (137). The amino groups in proteins in sludge flocs carry positive charges and can neutralize some of the negative charges from carboxyl and phosphate groups (134). The extent of negative charge depends on the physiological conditions of microorganisms in sludge, e.g. negative surface charge decrease with longer SRT (due to the high ratio of proteins to carbohydrates in EPS), and increase with nutrient deficiency (due to the higher C/N ratio) (138). A significant portion of EPS (at least 7% in terms of dissolved organic carbon) was found to be hydrophobic (139). Hydrophobic interactions are responsible for the strong interactions between hydrophobic molecules (or hydrophobic parts of molecules) and suspended solids in wastewater (or mixer liquor). The hydrophobic properties of flocs originate from proteins, not from carbohydrates and thus longer SRT and nutrient deficiency make the flocs being more hydrophobic (139-140).

1.8.2. Removal efficiency

NSAIDs have been the most studied group in terms of both occurrence and removal during wastewater treatment. As noted, the pharmaceuticals are grouped

according to the therapeutic applications, thus high variations in removals were observed within the group due to the differences in chemical properties. While ibuprofen and naproxen are generally removed with very high efficiency, diclofenac is only barely removable during conventional treatment. Removal of ibuprofen and naproxen are commonly higher than 75% and 50%, respectively (115, 141-145). Diclofenac shows rather low and very inconsistent removals, between 0 and 90% (115, 142-145). Its persistence is attributed to the presence of chlorine group in the molecule. Some studies on removal during wastewater treatment showed no influence of SRT on the removal of diclofenac (114, 116, 146). The removal of ibuprofen, ketoprofen, indomethacin, acetaminophen and mefenamic acid is reported to be very high (>80%) or even complete for SRT typical for nutrient removal (10<*SRT*<20 days) (114-115,144).

Beta-blockers were reported to be only partially eliminated by conventional biological treatment (65, 147). Data on their removal are very inconsistent and they vary from less than 10% up to 95% depending on the treatment. Maurer *et al* (148) proved that the elimination of beta-blockers in WWTPs depends on the HRT, which could be a good explanation for the variable removals reported in the literature. The highest average removal can be observed for atenolol and sotalol (around 60%). But, for the same compounds low removals were reported as well. Maurer *et al* (148) and Wick *et al* (149) reported removal efficiency lower than 30% for sotalol, and Castiglioni *et al*. (141) reported a removal of 10% for atenolol during winter months. In the same study, for atenolol was achieved better elimination in the summertime (i.e. 55%) due to a higher microbial activity (141). In most cases, metoprolol showed very low removal, ie. 10-30% (65,147,150). The occurrence and microbial cleavage of conjugates is well known to influence the mass balance inWWTPs (151-153). The microbial cleavage of conjugates of metoprolol could be responsible for an underestimation of its removal efficiency. For propranolol, as well, were observed various removals, mostly moderately low (149). This compound is by far the most lipophilic beta-blocker and the only one with a bioaccumulation potential. Sludge-water partition coefficients were found to be less than 100 L/Kg_{ss} for sotalol and atenolol, and 343 L/Kg_{ss} for propanolol (148). For $K_{d,sec}$ values less than 500 L/Kg_{ss} the removal by sorption in a WWTP with a typical sludge production of 0.2 g_{SS}/L is less than 10% (131) and hence does not significantly contribute to the removal of beta-blockers in activated sludge units (149). Therefore, the partial removal of beta-blockers can be assumed to be due to biotransformation.

No significant to medium removal was reported for *lipid regulators*. Zorita *et al* (59) reported a medium removal of 61% during primary and biological treatment, where during the letter one no removal was observed. Lower removal rates were reported by some other authors, <35% (141, 154-155). No or low removal of clofibric acid was observed in different WWTPS of Berlin (156). Winkler *et al*. (157) found no

evidence for biotic degradation of clofibric acid. Radjenovic *et al* (132) showed that clofibric acid may be removed more efficiently with MBR (72–86%) compared to the activated sludge process (of 26–51%). The removal of fibrates bezafibrate, fenofibrate and gemfibrozil vary between 30 and 90% (114-115, 158). Variety of removal efficiencies during biological treatment in 10 WWTPs in Spain were reported in studies of Jelic *et al* (60) and Gros *et al* (68).

Results from various studies showed that *anticonvulsant carbamazepine* is recalcitrant to biological treatment and it is not removed neither during conventional wastewater treatment nor membrane bioreactor treatments (71, 92, 97, 159). Physicochemical processes such as coagulation-flocculation and flotation did not give better results concerning its degradation (113, 160-161). It was constantly found at higher concentrations at the effluent of WWTPs. Knowing that the activated sludge have glucuronidase activity, which allow the cleavage of the glucuronic acid moiety in WWTP (48), rational explanation for the increase in concentration is conversion of CBZ glucorinides and other metabolites to the parent compound by enzymatic processes in a WWTP. No influence of SRT on removal of CBZ during conventional wastewater treatment was noticed (116, 146). Except for carbamazepine, information on the removal and fate of other psycho-active drugs in WWTPs is very scarce. This is probably because of the low therapeutic dose resulting generally in low concentrations in the environment (19, 93, 162). Conventional treatment achieve a removal lower than 10 % in case of diazepam (98). Zorita *et al* (59) reported very high removal efficiency in case of fluoxetine and its active metabolite norfluoxetine. Still, the lowest observed effect concentration of fluoxetine for zooplankton and benthic organisms is close to the maximal measured WWTP effluent concentrations (163). Low effluent concentrations can be due to the fact that fluoxetine rapidly passes to solid phase where it appears to be very persistent (164). Additionally, it was found that it has a high bioaccumulation potential when detected in wild fish (165).

The removal of several other drugs such as the *histamine H2-receptor antagonists* cimetidine, famotidine and ranitidine varied from low to very high. Radjenovic *et al* (132) reported rather poor and varying removal of histamines during conventional treatment (15-60%). Castiglioni *et al* (141) found that removal of ranitidine depends on season, and showed 39% removal in winter and 84% in summer. High removal of ranitidine during activate sludge treatment (89%) was observed in a study of Kasprzyk-Hordern *et al* (61).

Antibiotics cover a broad range of chemical classes, and it is very difficult to characterize their behaviour during activated sludge process due to varying removal efficiencies reported from studies undertaken worldwide. Due to their limited biodegradability and sorption properties, sulfonamides and trimethoprim appear to be only partially removed by conventional wastewater treatment. The removal of these

antibiotics has been reported to vary significantly (75-78, 150, 166). The explanation could be found in different operational parameters as HRT, SRT, temperature; but also in the fact that sulfonamides are easily transform from their parent compounds to their metabolites and vice versa, thus the removal efficiencies may be easily or underestimated or overestimated. In case of trimethoprim, only minor removal was noticed during primary and biological treatment, but the advanced treatment (77) and nitrification organisms appear to be capable of degrading it (167-168). This suggests an important role for aerobic conditions for the biotransformation of trimethoprim. Consistent with this, removal efficiency of trimethoprim appears to be enhanced by long SRT during biological treatment, which is conducive to nitrification (169).

Also macrolide antibiotics are often incompletely removed during biological wastewater treatment. Studies from different conventional WWTPs have revealed that the removal of macrolides varied from high negative values, to around 50% (77). Karthikeyan and Meyer (90) found that 43% to 99% of erythromycin was removed by activated sludge process and aerated lagoons. In the study of Kobayashi *et al* (170), 50% of clarithromycin and azithromycin were removed from three conventional WWTPs. Gobel *et al* (77) proposed gradual release of the macrolides (e.g. clarithromycin) from feces particles during biological treatment as an explanation for the possible negative removal rates for these antibiotics. Sorption of macrolides to wastewater biomass is attributed to hydrophobic interactions (high partitioning coefficient). But, knowing that the surface of activated sludge is predominantly negatively charged, and under typical wastewater conditions, the basic dimethylamino group (pKa > 8.9) is protonated, sorption could occur due to cation-exchange interaction as well (171). Greater adsorption of azithromycin to biomass compared to clarithromycin has been reported (170).

Varying removal was reported for fluoroquinolone antibiotics, as well. Lindberg *et al* (172) found that norfloxacin and ciproloxacin were removed with 78% and 80%, respectively, where around 40% was removed during the biological treatment. Similar removal was reported by Zorita *et al* (59) during secondary and tertiary treatment. The predominant removal mechanism of fluoroquinolones has been suggested to be adsorption to sludge and/or flocs rather than biodegradation (59, 83-84, 169). Ciprofloxacin and norfloxacin sorbed to sludge independently of changes in pH during wastewater treatment, and more than 70% of the total amount of these compounds passing through the plant was ultimately found in the digested sludge (172). These findings indicated sludge as the main reservoir of fluoroquinolones that may potentially release the antibiotics into the environment when applied to agricultural land. Karthikeyan and Meyer (90) reported removal efficiency of 68% for tetracycline, and Yang *et al* (79) have reported removals of 78% and 67% for chlorotetracycline and doxycycline, respectively, during activated sludge processes. Also some tetracyclines have significant potential for absortion onto solids due to a combination of non-

hydrophobic mechanisms, such as ionic interactions, metal complexation, hydrogen bond formation or polarization (173). Their removal is not so affected by HRT, but SRT appear to significantly influence the removal during biological treatment (86).

1.9. Analysis of pharmaceuticals

In order to study the occurrence and fate of pharmaceuticals during wastewater treatment, a number of analytical methodologies for the analysis of pharmaceuticals in wastewater and sludge have been developed and published. Because of the diversity of physico-chemical properties exhibited by different pharmaceuticals, the majority of the analytical methods reported in the literature focus on a specific therapeutic class, with few methods described for the multi-residue analysis of pharmaceuticals. The advantage of multi-residue methods is that they cover a wide range of physicochemical properties at trace levels while minimizing sample collection and preparation time and overall costs. Nevertheless, for reliable and reproducible multi-residue analytical methods, a compromise in the selection of experimental conditions is required and the analytical performance cannot be optimum for every single compound. Therapeutic classes frequently monitored in wastewater treatment plants are NSAIDs, antibiotics, beta-blockers, antiepileptics, blood lipid-lowering agents, antidepressants, hormones, and antihistamines (49).

A typical procedure for the analysis of pharmaceuticals in wastewater and sludge involves five steps: sampling, sample preparation, separation, detection and data analysis. All these steps are important as their quality and reproducibility significantly impact the final result and conclusions.

Table 1.3 and Table 1.4 summarize frequently cited procedures for the analysis of multi-residue pharmaceuticals in sludge and wastewater, respectively.

1.9.1 Sampling of wastewater and sludge

While there are many publications explaining in detail the analysis of pharmaceuticals, very few have focused on proper sampling and sample preservation, that are very important considerations for a proper characterization of wastewater treatment systems (174). Sampling is the first step in any measurement process or experimental study, and it is perhaps the major source of inaccuracy in the measurement process (175). Therefore, to obtain accurate mass balances and fate data for pharmaceuticals in full-scale systems, beside sophisticated analytical methodologies, rigorously controlled sampling methods must be employed. Obtaining representative samples is a difficult task given the fact that diurnal fluctuations occur in concentrations of pharmaceuticals and sewage flow volume, and seasonal

fluctuations in temperature; and the contribution of industrial wastewater may cause wastewater characteristics to change on short or long period.

Wastewater. Two types of samples are commonly used in wastewater analysis: grab samples and composite samples. Obviously, the type of sample will depend on the analysis to be performed.

Grab samples are used for some specific tests such as pH, dissolved oxygen etc., but they are not representative of the entire wastewater flow (just of the water at a specific time and point), and thus they are not appropriate for the studies on occurrence and removal of pharmaceuticals in WWTP.

Given the unique transport characteristics of wastewater influent, highly dynamic flows and variable pollutant loads, composite sampling is proffered over grab sampling for obtaining more accurate mass balances of pharmaceuticals in wastewater. Composites sampling provides more representative samples, except in the case of analytes that degrade during sampling. Composite samples can be taken as: time-proportional, volume-proportional or flow-proportional samples. Time-proportional samples are subsamples of defined (constant) volume taken at constant time intervals. Volume proportional sampling is performed by taking subsamples of constant volume at variable time intervals after a certain amount of wastewater has passed the sampling point. And, in flow-proportional sampling, the subsamples of varying volumes, proportional to the flow at the sampling time, are taken at constant time intervals. Just because a sample, either composite or grab sample, was drawn from a site under consideration, it doesn't mean that is representative for the site. And if the sampling procedure does not offer representative samples, then the statistical analyses of the generated data may lead to inaccurate conclusions and subsequent decisions (175).

Although sampling procedure is very important for the assessment of data quality, it is addressed and explained in only few studies regarding the occurrence and fate of pharmaceuticals during wastewater treatment (109). Ort el al (176) assessed uncertainties associated with different sampling modes when evaluating loads of pharmaceuticals in sewers and influents to WWTPs. The study showed that conventional sampling devices operated in time- or flow-proportional sampling modes and applying sampling intervals of 30 min or longer may result in the collection of non-representative samples. This study put into doubt the validity of conclusions of many studies on removal of pharmaceuticals during wastewater treatment, since time-proportional sampling was applied in most of the cases (109). They suggested the use of flow-proportional sampling as the unique sampling methodology, accompanied with frequent grab sampling to minimize the error associated with sample collection. Besides, (discrete) flow-proportional sampling mode should be applied with high sampling frequency, which will depend on the catchment and the compounds of

interest, and over consecutive days rather than nonconsecutive days. Additionally, sampling of influent and effluent should be offset by the hydraulic retention time of WWTP (plug-flow scenario) (177). Optimized sampling procedures will provide more reliable data that may be used as a basis for improvements of wastewater treatments and further regulatory actions.

Sludge. The residence time of solids in WWTP (e.g. average SRT of anaerobic digestion process is commonly >15 days) and their continuous recycling (e.g. average SRT ranges from 5 to 15 days in activated sludge unit) provide quenching of input extremes. Therefore, a relatively smaller number of composite samples are needed to confidently establish concentrations in sludge (either digested or treated) in contrast to wastewater sampling (177). Nevertheless, and as in the case of wastewater sampling, proper sludge sampling is an integral part of monitoring of sludge quality, and thus a plan for representative sampling must be developed including the sampling points, volumes/sizes to be drawn, days and times of collection (178-179). A single sampling approach is impossible and inappropriate because sludge quantity and quality vary from one plant to another, and even within a given plant. If possible, a composite sample is always preferred over a grab sample of sludge. A composite sludge sample is many grab samples that have been collected, normally over daily operation period at WWTP, and mixed to form a single sample (178). In the case of digesters, a representative sample should be composed of at least four grab samples obtained during daily operations at the facility. Equal sized grab samples of dewatered sludge (from belt press, filter press, drying bed, centrifuge etc.) may be withdrawn at equally spaced intervals over the period of operation of dewatering unit (178).

In order to provide representative samples and decrease the sampling uncertainty, regardless of sample matrix, sampling method and frequency, as well as sampling location have to be defined and optimized depending on a specific purpose of sampling and the conditions of WWTP. Unfortunately, in most cases, the laboratory staff is not involved in planning/performing the sampling, and unclear results of chemical analysis may easily be a consequence of inadequately realized sampling.

1.9.2. Sample preservation

Preservation refers to sample handling processes aimed at preventing or minimizing chemical or biological activity within the sample after it has been collected. Sludge samples are generally preserved by cooling and maintaining samples at 4° C, since high-solids sludge cannot be mixed with other preservatives (180). The most practical and reliable method of preservation of wastewater in the field is chilling to 4ºC, but pH control or chemical addition (e.g. sulfuric acid (181), hydrochloric acid (182) and sodium azide (115, 183)) can be used as well. Nevertheless, very little evidence has been presented to demonstrate the ability of these agents to preserve

the samples for the analysis of pharmaceuticals. Vanderford et al (174) showed that water samples collected in amber bottles, quenched with ascorbic acid and preserved with sodium azide may be held at 4ºC for 28 days without appreciable loss for selected pharmaceuticals. They also found that the samples may be stored at 4 °C with no chemical preservative and extracted within 72 h.

1.9.3. Sample preparation

Sample preparation depends on experimental goals, sample type, and instrumental analysis used. In order to remove potential interferences from the sample matrix and increase the concentration of the target compounds, convert them into a more suitable form (e.g. via derivatization or pH adjustment), and provide a robust and reproducible method independent on the sample matrix, the preparation of samples for the analysis of pharmaceuticals includes: pre-treatment, clean-up and concentration (184).

1.9.3.1. Pretreatment

Wastewater samples. Once collected, wastewater samples are stored in the dark at 4ºC, and filtered and extracted usually within 48h of the collection. Wastewater samples, especially those collected at the WWTP influent, contain much interference such as natural organic matter (NOM), which can reduce extraction efficiencies in the clean-up step. Hence, the samples are generally filtered through 0.2–0.5μm membrane filters to minimize interferences during extraction (185). Nevertheless, together with particulate matter, filtration removes the fraction of target compounds sorbed to the solid particles. To avoid this, the filters should be washed with methanol after filtration. Solution pH adjustment is included in the pre-treatment prior to sample extraction, elution and concentration, as pH determines the chemical form of the analytes, their stability and the interaction with the sorbent in the subsequent extraction step (186). For example, water samples that contain acidic pharmaceuticals that exist in their ionized form at wastewater pH should be acidified to achieve good recoveries during extraction process (185). On the other hand, acidification of wastewater samples can yield undissociated sulfonamides and cationic fluoroquinolones that are not retained by anion-exchange cartridges, while highly negatively charged NOM are (187). Chelating agents, such as disodium ethylenediaminetetraacetate (Na2EDTA), can be added to sequester metal ions that could complex some compounds, particularly tetracyclines (166, 188).

Sludge samples. As in the case of wastewater samples, sludge samples are kept in cool before further processing. Sludge samples that contain low amount of water (e.g. dewatered sludge) are usually freeze-dried, and those that contain higher amount of water, such as thickened and digested sludge, are first centrifuged and then dried.

The main issue related to the centrifuge phase separation is that a considerable amount of water may still remain in the solid phase, leading to a possible, and usually overlooked, systematic error of the applied procedure. After drying, the samples are grind and processed according to specific analytical procedures.

1.9.3.2. Extraction, clean-up and pre-concentration of samples for chemical analysis

Pharmaceutical residues are present in low concentration levels in complex wastewater and sludge matrices. Prior to instrumental analysis, samples must be purified (clean-up) to remove interferences, found not only in the matrix but in the chemicals, materials and solvents used, and to concentrate the target compounds. Adequate clean-up and enrichment is a prerequisite for successful instrumental analysis of pharmaceuticals. For wastewater samples, extraction and clean-up are carried out in one step, while for sludge samples these two steps are separated.

Several methods have been used for preconcentration of **wastewater samples** for the analysis of pharmaceuticals, but solid-phase extraction (SPE) is today most popular and widely used sample preparation method (189). It replaced laborious, time- and organic solvent- consuming liquid-liquid extraction (LLE) in the environmental field. SPE application areas are always growing with improvements in format, automation and the introduction of new sobents that offer improved recoveries for polar analytes and dual-phase media that provide the capturing of a broader range of analytes within a single extraction (3). Nevertheless, SPE is firstly a preconcentration procedure, and SPE sorbents often lack selectivity and may sequester various matrix components which can interfere with analysis. This is a very important point in the extraction from complex matrix such as wastewater.

To overcome SPE limitations, solid-phase microextraction (SPME) (190-191), liquid-phase microextraction (LPME) (including hollow fiber LPME (HF-LPME), single drop microextraction (SDME) (192-193), and molecularly imprinted polymer (MIPs) sorbents (194) have also been developed and applied for the extraction of pharmaceuticals from wastewater matrices. SPME and LPME provide simultaneous extraction, clean-up and concentration of pharmaceuticals from aqueous samples. Although these two techniques provide a reduction in time, labor, costs and matrix effects, the sensitivity and the precision tend to be worse than SPE, especially in the case of LC-MS analysis (195). MIPs are a promising extraction and clean-up technique for pharmaceuticals in wastewater matrices, because of its increased selectivity and specificity of target compounds that lead to lower matrix effect and better sensitivity (196). However, MIPs are to be custom-made, and they are not suitable for multi-residue analysis (195).

In **SPE,** the separation is based on the selective partitioning of target compounds between stationary (solid) phase and solvent (liquid phase), and thus it

depends on the kind of sorbent (i.e. stationary phase), and on the properties of target compounds. A typical SPE sequence involves: conditioning of the sorbent, loading of water sample, rinsing of the sorbent to remove interfering compounds, followed by the elution to desorb the analytes from the sorbent, and finishes with the sample reconstitution for instrumental analysis. All the steps associated with SPE using appropriate sorbent should be optimized in order to provide high reproducible extraction efficiency and sample extracts of adequate purity (197). This is not an easy task especially in multi-residue analysis. Oasis HLB, **h**ydrophilic-**l**ipophilic-**b**alanced reversed-phase (polymeric) sorbent has been the most commonly applied sorbent for simultaneous extraction of acidic, basic and neutral compounds at wide range of pH. Some other commercial sorbents, such as Oasis MAX, Oasis MCX, LiChrolut ENV+ and LiChrolut C18, have also been used for the preparation of aqueous samples. These sorbents offer good recoveries when compounds of a target (multi-residue) group have similar physico-chemical properties. For instance, basic compounds are extracted with high efficiency using a sorbent type that contains a hydrophobic-lipophilic balanced copolymer for reversed-phase interaction and strong cation-exchange capacity for the selective retention of basic analytes, i.e. Oasis MCX (Mixed-mode Cation eXchange), while for acidic compounds using a mixed mode *anion exchange* and *reversed-phase* sorbent, i.e. Oasis MAX (Mixed-mode Anion eXchange). Lichrolut ENV+ is appropriate for the extraction of polar compounds at low pH (around 2) or neutral compounds at neutral pH, while LiChrolut C18 (non-polar alkyl-bonded silica sorbent) is used for moderately polar to non-polar analytes. Thus, the choice of sorbent will mostly depend on the physico-chemical properties of the selected group of compounds to be analyzed by a multi-residue method (Table 1.3 and 1.4.). Also extraction solvent for the elution step is chosen based on the physico-chemical characteristics of target compounds, but depends on the elution strength of the solvent itself. Methanol, acetone and ethyl acetate, either separately or mixed, have been mostly used as extraction solvents for the analysis of pharmaceuticals in wastewater and sludge (198). SPE can be performed off-line, where clean-up is completely separated from subsequent chromatographic separation (199-201), or online, where it is directly integrated into the analytical system (202-204). Most methods still employ preparation based on off-line SPE, mostly because of the availability of equipment. However, online SPE coupled to LC/MS/MS offers high sensitivity and selectivity, minimum sample preparation, more reproducibility, and automation, and is being increasingly used for the analysis of pharmaceuticals (3).

In the case of **sludge samples**, extraction of pharmaceuticals from sludge samples has been usually done by Soxhlet extraction, microwave-assisted extraction (MAE), ultrasonic extraction (USE), or pressurized liquid extraction (PLE) (Table 1.3). Among these, USE and PLE, especially the letter one, have been most employed techniques for the extraction of pharmaceuticals from solid samples. For USE, the necessary equipment is widely available and it is relatively cheap. The extraction can

be carried out within 10 to 60 min with small volumes of solvents (e.g. 5-25 ml of solvent) and with large amount of samples (e.g. 2-30 g). PLE (also known as ASE – Accelerated Solvent Extraction, Dionex name) is automated extraction technique that offers high extraction efficiency within short time and with low consumption of solvent (205).

Ternes et al (206) found comparable results when PLE and USE were used for the extraction of several pharmaceuticals, iodinated contrast media and musk fragrances in sludge. Although it implies expensive equipment and compulsory clean-up step, PLE has become a well-established extraction technique for pharmaceuticals from solid matrices (Table 1.3.).

PLE uses high pressure and temperature for samples amounts between 0.5 and 5 g, depending on the type of solids and extraction cell. The solid samples are mixed with an inert material such as sand, aluminum oxide, diatomaceous earth or Hydromatrix, to increase the exposure surface area of the sample. Extraction solvent, temperature and extraction time (i.e. number of cycles and static time of cycles) govern the efficiency of PLE. The choice of the solvent (or mixture of solvents) depends on the polarity of target compounds. Given the polar nature of pharmaceuticals, methanol-water mixtures are most often used as they offer good extraction recoveries (Table 1.3.).

Temperature is also very important extraction parameter as it affects solubility, diffusion coefficient and viscosity of the medium. Increased temperature decreases the viscosity and surface tension of solvents, allowing for better diffusion through solid matrix. Nevertheless, high temperature can cause degradation/transformation of target compounds (207), and impurities to be extracted from solid matrix with target compounds decreasing the extraction efficiency (208). Usually, temperatures applied for the extraction of pharmaceuticals from sludge samples range from 80 to 100 ºC (Table 1.3.). Pressure maintains the solvents as liquids above their boiling points. Standard operating pressure of 1500 Psi in PLE is well above the threshold needed to keep the solvents in the liquid state; pressure adjustment is not required. As regards the extraction time, it is split into cycles in order to introduce the fresh solvent and maintain favorable solvent/sample equilibrium, which improves partitioning into the liquid phase. The number of static cycles and single cycle time should be studied together in order to extract target compounds in the most efficient way.

PLE and USE extracts normally require additional clean-up step, which is commonly performed by SPE, as in the case of wastewater samples. In most cases, HLB reversed-phase sorbent was found to be well-suited for multi residue methods, with an appropriate selection of the eluent. Methanol has been mostly used as elution solvent for the analysis of pharmaceuticals in wastewater sludge (Table 1.3.).

Table 1.3. Overview of methods for the analysis of pharmaceuticals in sludge samples

Compounds	Sample preparation	Separation and detection	Detection/Quantification Limits	Reference
Fluoroquinolones (ciprofloxacin and norfloxacin)	PLE: 50 mM aqueous phosphoric acid (pH 2)/ACN (1/1, v/v), 100°C, 100 bar,60 and 90 min for sewage sludge and sludge-treated soil, respectively SPE: MPC disk, pH 3, elution: 5% ammonium solution, 15% MeOH	LC-FLD	LOQ: <0.45 mg/kg sludge <0.18 mg/kg sludge-treated soils	Golet et al., 2002
Sulfonamides,macrolides and trimethoprim	PLE water/methanol (50:50, v/v) 100 ºC, 100 bar; 2 cycles of 5 min ; USE: methanol /acetone (4 times 5 min) SPE: Oasis HLB, pH 4, pH 7, elution: MeOH/ethylacetate/ammonia	LC-ESI-MS^2	LOQ: 3 and 41ng/g	Göbel et al., 2005
Triclosan and transformation products	MAE: MeOH/acetone 1:1 (v:v); SPE: Oasis HLB (pH 2.5) /silica, elution: ethyl acetate	GC-MS^2	0.8	Morales et al., 2005
19 neutral and acidic pharmaceuticals	USE; MeOH/acetone; PLE: MeOH, 100 ºC, 100 bar, 2 cycles x 5min SPE: RP-C18e, neutral pH, elution: MeOH; Oasis MCX; pH 2, elution: acetone	HPLC-ESI-MS^2	LOQ 20-50 ng/g	Ternes et al., 2005
9 fluoroquinolones	USE: MeOH/H_2O 30:70 (v:v) 15 mM sodium tetraborate solution pH 4.2; SPE: Chromabond Tetracycline polypropylene columns	LC-ESI-MS^2 LC-FLD	MS(SIM): 0.3–7.5, MS(SRM): 0.6-6.8 FLD: 11-62	Ferdig et al., 2005
6 compounds (lipid regulators, analgesics and anti-inflammatory drugs)	USE: MeOH/acetone; SPE: Oasis MCX, elution: acetone	GC-EI-MS	4–40	Kimura et al., 2007
Macrolides, sulfonamides, ranitidine, omeprazole and trimethoprim	PLE: water (pH 3):MeOH (1:1, v/v), 80 ºC, 1500 psi, 1 cycle x 5min SPE: Oasis HLB, pH 7, elution: MeOH	LC-ESI-MS^2	LOD: 2 to 11 ng/g	Nieto et al., 2007
27 compounds (analgesics and anti-inflammatory drugs, lipid regulator and cholesterol-lowering statin drugs; psychiatric, psychoactive drugs; antibiotics; ß-blockers; ß-antagonists; diuretics; anticoagulants)	PLE: MeOH/H_2O, 1:1 (v:v), 60ºC, 1500 psi, 2 cycles x 5 min SPE: Oasis HLB, pH 5.5, elution: ethylacetate/acetone (1/1, v/v)	LC-ESI-MS^2	2–580	Barron et al., 2008

Compounds	Sample preparation	Separation and detection	Detection/Quantification Limits	Reference
20 compounds (antiepileptic drug, antibiotics, anti-histamines, lipid regulators, analgesics and anti-inflammatory drugs, antihypertensives)	USE: MeOH/acetone; SPE: Oasis HLB (pH 2; pH 3; pH 6)	LC-ESI-MS^2	0.26–250	Spongberg and Witter, 2008
31 compounds (analgesics and anti-inflammatory drugs, anti-ulcer agent, psychiatric drugs, antiepileptic drug, antibiotics, ß-blockers, diuretics, hypoglycemic agents, lipid regulator and cholesterol-lowering statin drugs, anti-histamines)	PLE: MeOH/H_2O, 1:2 (v:v), 100ºC, 1500psi, 3 cycles x 5 min SPE: Oasis HLB, neutral, elution: MeOH	HPLC-ESI-MS^2	0.35–160 (treated); 0.15–114 (activated); 0.17–256 (MBRs)	Radjenović et al., 2009
Fluoroquinolones, tetracyclines and sulfonamides	PLE: 0.35% phosphoric acid/ ACN (1:1, v/v) (pH 2.50), 100–110 ºC, 30min heat-up time, 100–110 atm, 5 cycles x 10 min SPE: Oasis HLB, pH 2, elution: MeOH	LC-ESI-MS2	LOQ 0.1-160 ng/g	Lillenberg et al., 2009
66 PPCPs (pharmaceuticals and personal care products)	PLE: 100 ºC, 2000 psi, 3 cycles x 5min, (1) water at pH2; (2) methanol at pH4; USE: water/methanol (9/1,v/v) pH 11 SPE: Oasis HLB, Na2 EDTA, pH neutral	LC-ESI-MS2		Okuda et al., 2009
Six pharmaceutical antimycotics	USE: MeOH SPE: Evolute ABN, pH 3, Elution: 0,5% FA/MeOH	LC-ESI-MS^2	MQL <50 ng/g	Lindberg et al., 2010
15 pharmaceuticals tetracyclines, sulfonamides, other pharmaceuticals (i.e. acetaminophen, caffeine, carbamazepine, erythromycin, lincomycin and tylosin	PLE: ACN/water (v/v 7:3), 100 ºC, 100 bars, 3 cycles x 15 min SPE: Oasis HLB, pH 3, elution: MeOH/water (v/v = 1:1)with EDTA	LC-ESI-MS2	LOQ: 1.9-488 ng/g	Ding et al., 2011
4 NSAIDs	Pressurized Hot Water Extraction (PHWE): 0.01MNaOH/water, 120 ◦C, 100 bar,5 cycles x 5 min CLEAN-UP: Three-phase hollow fiber liquid phase microextraction (HF-LPME), pH 1.5	LC-ESI-MS	MDL 0.4–3.7 ng/g	Saleh et al., 2011
18 pharmaceuticals (analgesics, antibacterials, anti-epileptics, β-blockers, lipid regulators and non-steroidal anti-inflammatories	MAE: MeOH/water (3/2, v/v) SPE: continuous SPE Oasis HLB, pH 7, elution: ethyl acetate	GC-MS	LOD: 0.8 - 5.1 ng/kg	Azzouz and Ballesteros, 2012

1.10. Instrumental analysis

Upon extraction, concentration and clean-up, pharmaceuticals are typically analyzed using LC-MS(/MS) or GC-MS(/MS) instrumentation. Beside MS, some other detection techniques are also employed, such as diode array (DAD) and fluorescence detection (FLD). LC-DAD analysis is easy, low cost and available in many laboratories, and thus it can be used for routine analysis (209-210). Because of the inherent fluorescence of fluoroquinolone antibiotics, LC-FLD is especially suitable for their analysis in both liquid and solid samples (208).

Good separation of target compounds by HPLC or GC is useful and important even in the analysis with advanced MS detectors, given the potential presence of substances that could interfere with the analytes in samples.

1.10.1. GC-MS analysis

GC-MS has traditionally been the analytical technique of choice for persistent organic compounds (i.e. priority contaminants) because it provides high selectivity and sensitivity for non-polar (or less polar) and volatile organic compounds (e.g. dioxins, organochlorine pesticides etc.). For the analysis of polar pharmaceutical compounds by GC-MS, an efficient derivatization step is required. By derivatization, polar compounds are converted in less polar, with increased volatility and thermal stability, thereby more suitable for GC-MS analysis (211).

It is carried out using derivatization agents such as acid anhydrides, benzyl halides, alkylchloroformates, diazomethane and silylating reagents including N-methyl-N-(trimethylsilyl) trifloroacetamide (MSTFA) or N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) (196). GC-MS is less prone to water matrix interferences (212), but it requires more complicated and time-consuming sample preparation. Additionally, it requires the use of reagents that may be highly toxic, carcinogenic and potentially explosive (185).

An example of the application of GC-MS with derivatization is reported in the method developed by Azzouz *et al* (213). They analyzed 18 pharmaceuticals (analgesics, antibacterials, anti-epileptics, β-blockers, lipid regulators and non-steroidal anti-inflammatories), one personal care product and 3 hormones in soils, sediments and sludge using microwave-assisted extraction and continuous SPE, followed by derivatization with [N,O-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (TMCS)] and analyzed by GC-MS. Analyte recoveries were from 91-101%, and limits of detection from 0.8-5.1 ng/kg. Togola *et al* (214) developed a method for the analysis of 18 pharmaceuticals belonging to 5 therapeutic groups (e.g. NSAIDs, lipid regulators etc) in wastewater, surface water and drinking water. The target compounds were

simultaneously extracted by off-line SPE, derivatized by MSTFA and analyzed by GC-MS. The limits of detection for wastewater samples ranged from 6.2-28.6 ng/l, and recoveries varied from 70 to 110% depending on compound and matrix.

1.10.2. LC-MS analysis

Because of the time consuming, labor-intensive and often irreproducible derivatization, nowadays most analytical methods for the analysis of pharmaceuticals in environmental matrices are based on HPLC-MS(/MS). Inherent selectivity and sensitivity of HPLC-MS/MS, and the general applicability, has made it become the standard technique throughout the laboratories for quantitative/qualitative analysis of pharmaceuticals (Tables 1.3 and 1.4). For HPLC separation, reversed-phase octadecyl C18-bonded or octyl C8-bonded silica stationary phase (5 µm particles, usually) are most commonly employed, with eluent systems generally consisting of combinations of acetonitrile, methanol and water with additives to improve peak shape, retention, and resolution (215). For the analysis of enantiomers or highly polar compounds, special LC column packing (chromatographic stationary phase) is required. Enantiomers are resolved using enantioselective chromatography, and very polar compounds using hydrophilic interaction chromatography (HILIC). Recently, ultraperformance liquid chromatography (ultra-high performance) coupled to mass spectrometer (UPLC-MS), using chromatographic columns with particles of 1.7 µm, is becoming more popular because it is faster, uses less solvent, and provides better resolution, lower matrix effect and sensitivity during MS detection, comparing to HPLC.

Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the most commonly used LC interfaces, but atmospheric pressure photoionization (APPI) and sonic spray ionization (SSI) are sometimes used as well (3). ESI is well suited for the analysis of polar pharmaceuticals, while APCI is effective in the analysis of less polar compounds. Table 1.4 lists selected, frequently cited multi-residue methods for the analysis of pharmaceuticals in wastewater, published in last five years. As shown, HPLC-MS/MS, with ESI used to interface HPLC to MS, is the most applied technique for the analysis of pharmaceuticals in wastewater and sludge samples. The major drawback of this technique is that its quantitative performance is affected by ion suppression or ion enhancement. In ESI, the ionization takes place in the liquid phase, and thus the presence of compounds in the sample matrix that co-elute with target analytes can interfere with the available charges. This can change the efficiency of droplet production or evaporation, thereby affecting the amount of ion in the gas phase that reaches the detector (216). Compounds with high molecular mass, such as the humic acids, are known to affect the ionization of lower mass molecules, with polar pharmaceuticals being very susceptible to these effects (217). Matrix effects can cause unreliable quantitative data, scarce reproducibility, and linearity and accuracy deficiencies in the overall method. They can vary with the nature of samples,

and can induce the unpredictable response even among replicates of the same sample or when using the same method (218). Since the reliable concentrations are required for the purpose of environmental risk assessments, matrix effects must be eliminated or, at least, overcome. In order to minimize matrix effects, analytical methods require the use of an effective (or additional) sample clean up procedure, improved chromatographic separation, diluted samples or reduced influent flow (nano-ESI interface) (219). When matrix effects cannot be removed by these methods, appropriate calibration techniques that compensate for matrix effects should be used: standard addition, external sample calibration (corresponding to matrix-matched calibration), or internal standard calibration using corresponding isotopic-labeled standards (220-222).

Chromatographic separation is generally optimized by testing the mobile phase composition to determine the conditions that provide the best compromise for all target analytes (166). Common mobile phases include water and organic solvents, among which acetonitrile, methanol or their mixtures are mostly employed (Table 1.4). As regards mobile-phase modifiers used for LC-(ESI)-MS/MS, volatile buffers are the norm. This is because nonvolatile species cause salt deposits on the metal surfaces that can block ion transmission, and if an anion and cation pair too strongly with an analyte, the unfavorable competition for charge prevents the analyte of interest from carrying the excess charge on the droplet surface, resulting in reduced sensitivity (218). Therefore, mobile-phase modifiers that can be used are formic acid, ammonium formate, ammonium acetate, and similar (223).

Although single MS can be used for quantification of pharmaceuticals in the environmental samples, tandem MS detection is preferred for increased analytical sensitivity and selectivity in complex matrices. Triple quadrupole (QqQ), quadrupole ion trap (3DIT) and quadrupole time-of-flight (QqTOF) mass spectrometers are the most widely used mass analyzers (224), primarily owing to their performance, ease of use and cost. QqQ mass analyzers are still a mainstay of research and applications in mass spectrometry because they are robust and exhibit a large linear dynamic range for quantitative work. Quantitative analysis by LC-(QqQ)-MS/MS using the selected reaction monitoring mode (SRM) has been demonstrated to be a very selective and powerful approach. In SRM experiments, two mass analyzers are used as static mass filters, to monitor a particular fragment ion of a selected precursor ion. The selectivity resulting from the two filtering stages combined with the high-duty cycle results in quantitative analyses with unmatched sensitivity. The specific pair of m/z values associated with the precursor and fragment ions selected is referred to as a “transition” (225). Usually, two ion transitions in combination with their intensity ratio are used for quantification and confirmation in order to avoid false positive results.

Recently, a hybrid quadrupole linear ion trap mass spectrometer (QqLIT-MS) has become very attractive for the possibility of multiple ion–ion transitions with a larger ion storage capacity and a higher trapping efficiency, comparing to quadrupole ion trap MS (224), and for its high sensitivity (226-227). In hybrid instruments, i.e. QqLITs and QqTOF, MS/MS data are acquired on a partial mass range centered on a fragment ion (225). As regards the qualitative analysis of pharmaceuticals, QqTOF instruments are widely used for metabolite/transformation product screening and identification, as they provide high mass resolution and accurate mass capabilities for identification. Recently, also a novel hybrid linear ion trap (Finnigan™ LTQ™) – Orbitrap MS has received much attention not only because of its high resolution and mass accuracy, but also for its wide dynamic range and detection power, offering both qualitative and quantitative analysis in complex matrices.

Table 1.4. Overview of methodologies for the analysis of pharmaceuticals in wastewater samples

Compounds	Sample preparation	Separation and detection	Limits of detection/quantification	References
36 pharmaceuticals and PPCP	SPE: Oasis MCX, pH 2, elution: MeOH, 5% NH4OH/MeOH	UPLC-(ESI)-MS/MS LC column: UPLC BEH(Ethylene Bridged Hybrid) C18 (100x2.1mm, 1.7 µm); (PI) A: MeOH and CH3COOH/water pH 2.8, B: CH3COOH/water pH 3.2; (NI) A: pH 3.9, MeOH/ water/CH3COOH/NH4OH, B: pH 5.5, MeOH/water/CH3COOH/NH4OH	MQL: 1-538 ng/L	Kasprzyk-Hordern et al., 2008
48 pharmaceuticals and 6 metabolites	SPE: Oasis MCX (Na2EDTA/ascorbic acid), pH7, elution: (acidic and neutral):ACN; (basic):ACN/5%NH4OH	UPLC- (ESI)- MSMS LC column: UPLC BEH C18 (100 x1.0 mm,1.7 µm) (A1) 0.3% FA/water, (A2) 20 mM NH4OH/water; (B) ACN/MeOH, 2/1	IDL: 1-51 ng/L	Batt et al., 2008
14 fluoroquinolones	MISPE, elution: 2% TFA/MeOH	MISPE/HPLC-FLD LC column: C18 (250x4.6 mm, 5µm) A: 25mM o-H3PO4, pH 3 (NaOH), B: ACN, C: MeOH	×	Benito-Peña et al., 2008
6 (neutral) pharmaceuticals	SPE:Oasis HLB, pH 7.5, elution: MeOH	LC-(APCI)-MS/MS LC column: RP C18 (150x3 mm, 4 µm) A: 10 mM NH4Ac/water, B: CAN	×	Zhao and Metcalfe, 2008
18 pharmaceuticals (basic and acidic)	SPE: Oasis MCX, pH 2, Ethyl acetate,ethyl acetate/acetone (50/50),ethyl acetate/acetone/ammonium hydroxide (48/48/2)	GC-MS derivatization: MSTFA (N-methyl-N-(trimethylsylil)trifluoroacetamide GC column: HP5/MS (30m×0.25mm×0.25µm)	MDL: 3.2-28 ng/L (wastewater)	Togola and Budzinski, 2008
15 pharmaceuticals (in total 53 CEC)	SPE: Oasis HLB, pH 7, elution: MeOH	HPLC-(ESI)-MS/MS LC column: (PI) RP C18 (150x2.1mm, 3.5 µm), (NI) Phenyl-Hexyl (150x2 mm, 3 µm) (PI) A: 5 mM NH4Ac/water, B: 5 mM NH4Ac/MeOH; (NI) water/MeOH, 90/10, B: water/MeOH, 5/95, both with 1 mM of TrBA and 1 mM CH3COOH	LOD 0.3 and 30 ng L−1	Rodil et al., 2009

Compounds	Sample preparation	Separation and detection	Limits of detection/quantification	References
15 pharmaceuticals	SPE: Oasis MCX and Oasis MAX in series: MCX: elution - neutrals and acids (MCX): MeOH, Bases: 2% NH4OH/MeOH MAX: elution- neutrals: MeOH, acids: 2% FA/MeOH	UPLC- (ESI)-QTOF-MS LC column: BEH HSS T3 (100x2.1 mm, 1.8 µm) A: 10 mM CH3COOH/water, B: 10 mM CH3COOH/CAN	IDL: 0.4-6 pg; MDL (influent): 2.5-285 ng/L	Lavén et al., 2009
73 pharmaceuticals	SPE: Oasis HLB (+Na2EDTA), elution: MeOH	HPLC-(ESI)-QqLIT-MS/MS column: RP-18 (125mm×2.0mm, 5µm) (PI) A: water/formic acid 0.1%, B: ACN; (NI) A: water, B: ACN/MeOH, 1:1	MQL: 0.1-50 ng/L	Gros et al., 2009
10 beta-blockers	SPE: Bakerbond C18, neutral pH, elution: MeOH; OASIS MCX, pH 3, elution: 5% NH4OH/MeOH	HPLC-(ESI)-MS/MS LC column 1: Polar ether-linked phenyl phase RP (150x3 mm, 4µm), A:20 mmol ammonia/water, pH 5.7 CH3COOH, LC column 2: RP C18 (150×2.1 mm, 4 µm), A: ACN, B: 20 mM NH4Ac/water	LOQ <10 ng/L (influent)	Scheurer et al., 2010
49 pharmaceuticals and 6 metabolites	SPE: Oasis HLB, pH neutral, elution: MeOH	UPLC-(ESI)–MS/MS LC column: UPLC BEH C18 (100 x2.1 mm,1.7 µm) (PI) A: 0.1% FA/ACN, B: 10mM FA/NH4FA (pH 3.5); (NI) A: MeOH/water,90/10, B:water	LOQ: 0.1 to 50 pg	Huerta-Fontela et al., 2010
11 pharmaceuticals	on-line-SPE-LC;elution: NH4Cl/NH4OH at pH 9.2/ACN	on-line (*HXLPP-WAX-EDA) SPE-LC-UV; *(hypercrosslinked polymer resin (HXLPP) modified with 1,2-ethylenediamine (EDA) moieties, SPE: weak anion-exchange (WAX) sorbent) LC column: RP C18 (150x4.6mm , 5 µm)	LOD < 0.1 µg/L	Fontanals et al., 2010
70 pharmaceuticals and PPCP (adopted EPA Method 1694)	SPE: Oasis HLB, pH neutral, elution: MeOH	HPLC-(ESI)-MS/MS LC column 1: RP C18 (100x 2.1 mm,3.5 µm); A:0.1%FA/water, B:0.1%NH4Ac/0.1%CH3COOH LC column 2: Hydrophilic Interaction Chromatography (HILIC) (100x2.1 mm,3.5 µm); A.ACN, B:10 mM NH4Ac/water, pH 6.7	IDL: 0.3-300 pg	Ferrer et al., 2010
23 pharmaceuticals	SPE: Oasis MAX, pH 2,elution: MeOH	HPLC-(ESI)-MS/MS LC column: RP C18 (50x2.1 mm, 2.4µm) A: 10 mM FA/water, B: MeOH	MDL: 1-65 ng/L	Sousa et al., 2011

Compounds	Sample preparation	Separation and detection	Limits of detection/quantification	References
47 pharmaceuticals	SPE: Oasis HLB, pH neutral, elution: MeOH	UPLC-(ESI)-MS/MS LC column: UPLC BEH column (50x2.1 mm, 1.7 µm) A: water (0.1 mM NH4Ac, 0.01% FA), B:MeOH (0.1 mM NH4Ac,0.01% FA)	IDL: 0.2-86 pg	Gracia-Lor et al., 2011
5 NSAIDs	MEPS (microextraction by packed sorbent)	GC-MS/MS derivatization: EDC/TFEA GC column: HP-5MS (30x0.25 mm, 0.25µm)	LOD: 3-111 ng/L; MDL <540 ng/L (wastewater)	Grueiro Noche et al., 2011
9 pharmaceuticals	SPME: derivatization: ethyl chloroformate, SPME fiber coating: polydimethylsiloxane-divinylbenzene (PDMS-DVB)	SPME- GC-MS Rtx-5MS-fused silica column (15m 0.25mm 0.25 mm) from Restek (Bellefonte, USA) GC column: fused silica capillary column (15mx0.25mmx0.25 µm).	LOQ<0.5 µg/L	de Lima Gomes et al., 2011
81 pharmaceuticals	SPE: Oasis HLB, pH neutral, elution: MeOH	UPLC-(ESI)-MS/MS LC column: (PI) BEH High Strenght Silica (HSS T3) colum (50x2.1 mm, 1.7 µm); A: MeOH, B: 10 mM FA/NH4FA (pH 3.2); (NI) BEH C18 (50x2.1 mm, 1.7 µm) A: ACN, B: 5 mM NH4Ac/ammonia (pH 8)	IDL: 0.05 to10 pg; MDL: <20 ng/L	Gros et al., 2012
74 pharmaceuticals	addition of Na_2EDTA	on-line (LC–MS–MS) HySphere Resin GP cartridge column: RP-18 (125mm×2.0mm, 5µm); (PI) A: water/formic acid 0.1%, B: ACN; (NI) A: water, B: ACN/MeOH, 1:1	MDL: 0.01 to 5 ng/L groundwater, surface water MDL:0.01 to 20 ng/L wastewaters	Lopez-Serna et al, 2010
58 pharmaceuticals and 19 TPs	modifiers: PI - 0.1% FA + 0.02% TFA; NI - 10 mM NH_4Ac and 2% MeOH	on-line (TurboFlow™-LC–ESI-MS/MS) TurboFlow™ columns:Cyclone P, C18-P XL and Cyclone MAX. (1.0 mm × 50 mm) + LC:Betasil Phenyl-Hexyl (50×3mm, 3 µm) PI: 0.1% FA + 0.02%TFA; NI:10 mM NH4Ac	MDL<5 ng/L groundwater, MDL<10 ng/L river water	Lopez-Serna et al, 2012

Chapter II

Occurrence, removal and fate of pharmaceuticals in full-scale biological wastewater treatment plants

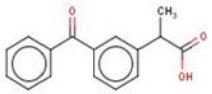
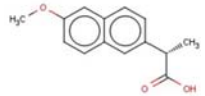
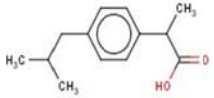
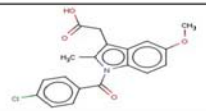
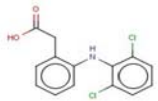
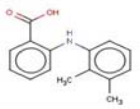
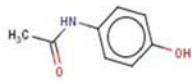
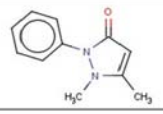
Occurrence, removal and fate of pharmaceuticals in full-scale biological wastewater treatment plants

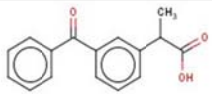
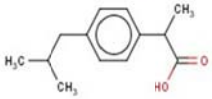
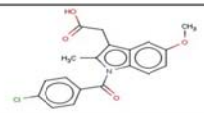
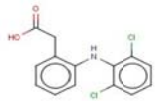
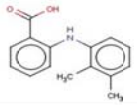
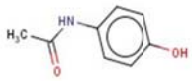
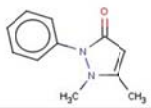
2.1. Analytical methods

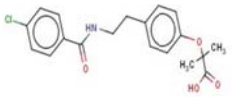
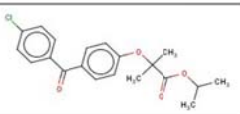
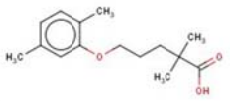
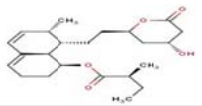
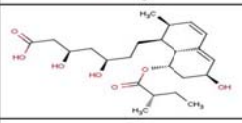
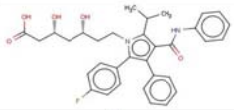
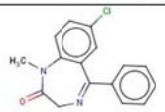
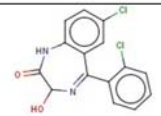
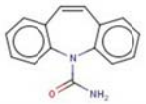
2.1.1. Target compounds

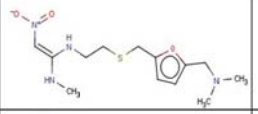
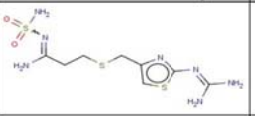
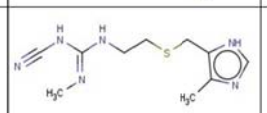
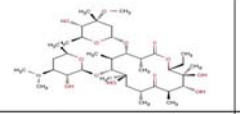
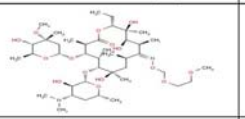
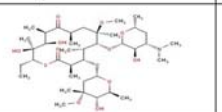
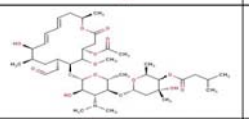
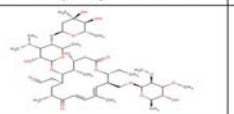
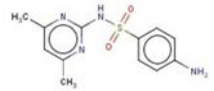
For a better understanding of the potential contribution of pharmaceutical residues to the environmental pollution from sludge reuse and disposal, an analytical procedure to extract and accurately quantify 43 pharmaceuticals in sewage sludge was optimized. The pharmaceuticals were selected on the basis of their high consumption or/and frequently reported detection in wastewaters, and the possibility to be analyzed under the same experimental conditions. A methodology for the analysis of pharmaceuticals in wastewater samples set out by Gros et al (199) was a starting point for developing the method for sludge samples, as it involved numerous compounds of interest and enabled high sample throughput. The target pharmaceuticals belong to different therapeutic classes, i.e. nonsteroidal anti-inflammatory agents and analgesics (treat inflammation, mild to moderate pain, and fever), lipid modifying agents (treat hypercholesterolemia), psycholeptic and antiepileptic drugs (treat epilepsy, bipolar disorder, anxiety etc), beta-blocking agents (manage cardiac arrhythmias, myocardial infarction and hypertension), beta-2-adrenoreceptor agonists (treat asthma and other pulmonary disease states), H2-receptor antagonists (treat peptic ulcer and gastro-oesophageal reflux diseases), antibiotics (treat infections caused by bacteria), angiotensin converting enzyme (ACE) agents (treat hypertension and congestive heart failure), diuretics (treat heart failure, liver cirrhosis, hypertension and kidney diseases) and antidiabetic drugs (blood glucose lowering agents). Table 2.1 summarizes the list of the target compounds and their structures and physicochemical properties, such as acidic and basic pK_a (ionization constant in aqueous solution – measure of acid/base strength), logP (octanol/water partition coefficient for neutral species – measure of molecular hydrophobicity) and logD (octanol/water distribution coefficient for ionic species at a specific pH) at four pH values (i.e. 4, 6, 7, and 9). The properties were predicted using two platforms, Chem Axon's Marvin and JChem cheminformatics platforms (http://www.chemaxon.com), which calculate physicochemical properties from chemical structures.

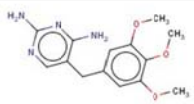
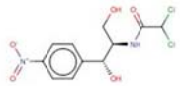
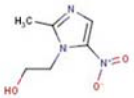
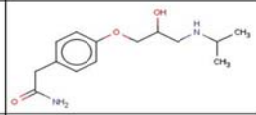
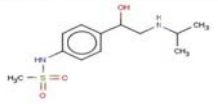
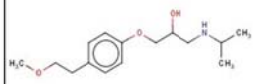
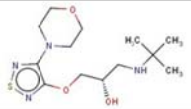
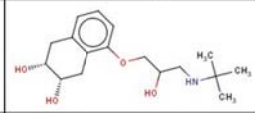
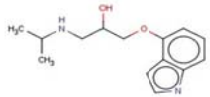
Table 2.1. List of target compounds, their structures and physicochemical properties.

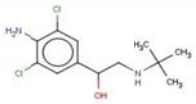
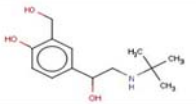
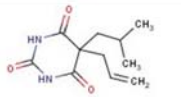
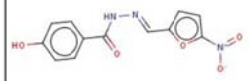
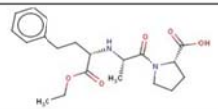
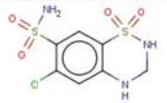
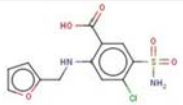
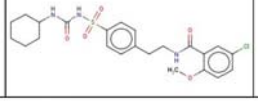
Therapeutic groups	Compounds	CAS Number	Formula	Structure	Mw (g/mol)	Stongest basic pKa	Strongest acidic pKa	Major microspecie at pH 2.8	Major microspecies at pH (6-8)	logP	logD			
											pH4	pH6	pH7	pH9
Nonsteroidal anti-inflammatory agents/analgesics (M01)	**Ketoprofen**	22071-15-4	$C_{16}H_{14}O_3$		230.259	(-)	3.88	neutral	anion	3.61	3.25	1.51	0.64	0.09
	Naproxen	22204-53-1	$C_{14}H_{14}O_3$		254.281	(-)	4.19	neutral	anion	2.99	2.77	1.18	0.25	-0.52
	Ibuprofen	15687-27-1	$C_{13}H_{18}O_2$		206.29	(-)	4.85	neutral	anion	3.84	3.79	2.67	1.71	0.41
	Indomethacine	53-86-1	$C_{19}H_{16}ClNO_4$		357.787	(-)	3.8	neutral	anion	3.53	3.12	1.35	0.50	0.01
	Diclofenac	15307-86-5	$C_{14}H_{11}Cl_2NO_2$		296.148	(-)	4	neutral	anion	4.26	3.96	2.26	1.37	0.74
	Mefenamic acid	61-68-7	$C_{15}H_{15}NO_2$		241.285	(-)	3.89	neutral	anion	5.4 (+1.3)	5.04	3.30	2.42	1.88
	Acetaminophen	103-90-2	$C_8H_9NO_2$		151.17	(-)	9.46	neutral	neutral	0.91	0.91	0.91	0.91	0.78
Phenazone type analgesics	**Phenazone**	60-80-0	$C_{11}H_{12}N_2O$		188.226	(-)	(-)	neutral	neutral	1.22	1.22	1.22	1.22	1.22

Therapeutic groups	Compounds	CAS Number	Formula	Structure	Mw (g/mol)	Stongest basic pKa	Strongest acidic pKa	Major microspecie at pH 2.8	Major microspecies at pH (6-8)	logP	logD			
											pH4	pH6	pH7	pH9
Nonsteroidal anti-inflammatory agents/analgesics (M01)	**Ketoprofen**	22071-15-4	$C_{16}H_{14}O_3$		230.259	(-)	3.88	neutral	anion	3.61	3.25	1.51	0.64	0.09
	Naproxen	22204-53-1	$C_{14}H_{14}O_3$		254.281	(-)	4.19	neutral	anion	2.99	2.77	1.18	0.25	-0.52
	Ibuprofen	15687-27-1	$C_{13}H_{18}O_2$		206.29	(-)	4.85	neutral	anion	3.84	3.79	2.67	1.71	0.41
	Indomethacine	53-86-1	$C_{19}H_{16}ClNO_4$		357.787	(-)	3.8	neutral	anion	3.53	3.12	1.35	0.50	0.01
	Diclofenac	15307-86-5	$C_{14}H_{11}Cl_2NO_2$		296.148	(-)	4	neutral	anion	4.26	3.96	2.26	1.37	0.74
	Mefenamic acid	61-68-7	$C_{15}H_{15}NO_2$		241.285	(-)	3.89	neutral	anion	5.4 (+1.3)	5.04	3.30	2.42	1.88
	Acetaminophen	103-90-2	$C_8H_9NO_2$		151.17	(-)	9.46	neutral	neutral	0.91	0.91	0.91	0.91	0.78
Phenazone type analgesics	**Phenazone**	60-80-0	$C_{11}H_{12}N_2O$		188.226	(-)	(-)	neutral	neutral	1.22	1.22	1.22	1.22	1.22

Therapeutic groups	Compounds	CAS Number	Formula	Structure	Mw (g/mol)	Stonges basic pKa	Strongest acidic pKa	Major microspecie at pH 2.8	Major microspecies at pH (6-8)	logP	log Dow			
											pH4	pH6	pH7	pH9
Lipid modifying agents (C10)	**Bezafibrate**	41859-67-0	$C_{19}H_{20}ClNO_4$		361.819	(-)	3.83	neutral	anion	3.99	3.59	1.83	0.97	0.47
	Fenofibrate	49562-28-9	$C_{20}H_{21}ClO_4$		360.831	(-)	(-)	neutral	neutral	5.28	5.28	5.28	5.28	5.28
	Gemfibrozil	25812-30-0	$C_{15}H_{22}O_3$		250.333	(-)	4.42	neutral	anion	4.39	4.25	2.80	1.85	0.90
	Mevastatin	73573-88-3	$C_{23}H_{34}O_5$		390.513	(-)	14.91	neutral	neutral	3.62	3.62	3.62	3.62	3.62
	Pravastatin	81093-37-0	$C_{23}H_{36}O_7$		424.528	(-)	4.21	neutral	anion	1.65	1.44	-0.14	-1.07	-1.86
	Atorvastatin	134523-00-5	$C_{33}H_{35}FN_2O_5$		558.64	(-)	4.33	neutral	anion	5.39	5.22	3.71	2.77	1.89
Psycholeptics and antiepileptics (N05/N03))	**Diazepam**	439-14-5	$C_{16}H_{13}ClN_2O$		284.7	2.92	(-)	cathion	neutral	3.89	3.04	3.08	3.08	3.08
	Lorazepam	846-49-1	$C_{15}H_{10}Cl_2N_2O_2$		321.2	(-)	12.46	neutral	neutral	3.53	3.53	3.53	3.53	3.53
	Carbamazepine	298-46-4	$C_{15}H_{12}N_2O$		236.269	(-)	0	neutral	neutral	2.77	2.77	2.77	2.77	2.77

Therapeutic groups	Compounds	CAS Number	Formula	Structure	Mw (g/mol)	Stonges basic pKa	Strongest acidic pKa	Major microspecie at pH 2.8	Major microspecies at pH (6-8)	logP	log Dow			
											pH4	pH6	pH7	pH9
Drugs for peptic ulcer and gastro-oesophageal reflux disease (A01)	**Ranitidine**	66357-35-5	$C_{13}H_{22}N_4O_3S$		314.4	8.08	(-)	cathion	cathion	0.98	-2.46	-1.09	-0.13	0.93
	Famotidine	76824-35-6	$C_8H_{15}N_7O_2S_3$		336.449	9.29	8.38	cathion	cathion	-1.88	-3.43	-3.35	-2.95	-1.95
	Cimetidine	51481-61-9	$C_{10}H_{16}N_6S$		252.34	6.53	14.16	cathion	cathion	-0.29	-1.97	-0.83	-0.40	-0.29
Macrolide antibiotics (J01)	**Erythromycin**	114-07-8	$C_{37}H_{67}NO_{13}$		733.93	8.38	12.44	cathion	cathion	2.66	-0.85	0.25	1.20	2.50
	Roxithromycin	80214-83-1	$C_{41}H_{76}N_2O_{15}$		837.047	9.08	12.45	cathion	cathion	3.00	-0.50	0.06	0.93	2.66
	Clarithromycin	81103-11-9	$C_{38}H_{69}NO_{13}$		747.953	8.38	12.46	cathion	cathion	3.24	-0.21	0.89	1.84	3.15
	Josamycin	16846-24-5	$C_{42}H_{69}NO_{15}$		827.995	7.9	13.4	cathion	cathion	3.22	-0.14	1.33	2.27	3.18
	Tylosin A	1401-69-0	$C_{46}H_{77}NO_{17}$		916.1	7.2	13.05	cathion	cathion	2.32	-0.71	1.10	1.91	2.31
Sulfonamide antibiotics (J01)	**Sulfamethazine**	57-68-1	$C_{12}H_{14}N_4O_2S$		279.33	2.04	6.99	neutral	neutral/anion	0.65	0.64	0.61	0.39	-0.26

Therapeutic groups	Compounds	CAS Number	Formula	Structure	Mw (g/mol)	Stonges basic pKa	Strongest acidic pKa	Major microspecie at pH 2.8	Major microspecies at pH (6-8)	logP	log Dow			
											pH4	pH6	pH7	pH9
Other antibiotics (J01)	**Trimethoprim**	738-70-5	$C_{14}H_{18}N_4O_3$		290.32	7.16	(-)	cathion	cathion	1.28	-0.18	0.27	0.92	1.28
	Chloramphenicol	56-75-7	$C_{11}H_{12}Cl_2N_2O_5$		323.132	(-)	13.59	neutral	anion	0.88	0.88	0.87	0.77	0.03
	Metronidazole	443-48-1	$C_6H_9N_3O_3$		171.15	3.09	(-)	cathion	neutral	-0.46	-0.50	-0.46	-0.46	-0.46
Beta-blocking agents (C07)	**Atenolol**	29122-68-7	$C_{14}H_{22}N_2O_3$		266.336	9.67	14.08	cathion	anion	0.43	-2.82	-2.68	-2.14	-0.33
	Sotalol	3930-20-9	$C_{12}H_{20}N_2O_3S$		272.362	9.43	10.07	cathion	cathion	0.05	-3.19	-3.04	-2.47	-0.70
	Metoprolol	37350-58-6	$C_{15}H_{25}NO_3$		267.364	9.67	14.09	cathion	cathion	1.76	-1.48	-1.34	-0.81	1.01
	Timolol	26839-75-8	$C_{13}H_{24}N_4O_3S$		316.42	9.76	14.08	cathion	cathion	1.34	-1.91	-1.79	-1.30	0.51
	Nadolol	42200-33-9	$C_{17}H_{27}NO_4$		309.401	9.76	14.59	cathion	cathion	0.87	-2.38	-2.26	-1.77	0.04
	Pindolol	13523-86-9	$C_{14}H_{20}N_2O_2$		248.32	9.67	14.08	cathion	cathion	1.42	-3.06	-1.93	-1.18	0.67

Therapeutic groups	Compounds	CAS Number	Formula	Structure	Mw (g/mol)	Stonges basic pKa	Strongest acidic pKa	Major microspecie at pH 2.8	Major microspecies at pH (6-8)	logP	log Dow			
											pH4	pH6	pH7	pH9
Beta-2-adrenoreceptor agonists (R03)	**Clenbuterol**	37148-27-9	$C_{12}H_{18}Cl_2N_2O$		277.19	9.63	14.06	cathion	cathion	2.33	-0.91	-0.76	-0.20	1.62
	Salbutamol	18559-94-9	$C_{13}H_{21}NO_3$		239.31	9.40	14.08	cathion	cathion	0.88	-2.36	-2.21	-1.66	0.09
Barbiturates	**Butalbital**	77-26-9	$C_{11}H_{16}N_2O_3$		224.256	12.15	(-)	neutral	neutral	1.59	1.59	1.59	1.58	0.97
Intestinal anti-infective agent (A07)	**Nifuroxazide**	965-52-6	$C_{12}H_9N_3O_5$		275.20	(-)	8.33	neutral	neutral	1.75	1.75	1.75	1.73	1.01
Angiotensin converting enzyme (ACE) inhibitor (C09)	**Enalapril**	75847-73-3	$C_{20}H_{28}N_2O_5$		376.44	5.20	3.67	cathion	anion	2.24	0.59	-0.07	-0.85	-1.28
Diuretics (C03)	**Hydrochlorothiazide**	58-93-5	$C_7H_8ClN_3O_4S_2$		297.74	(-)	9.09	neutral	neutral	-0.58	-0.58	-0.58	-0.58	-0.82
	Furosemide	54-31-9	$C_{12}H_{11}ClN_2O_5S$		330.74	(-)	4.25	neutral	anion	1.75	1.56	0.00	-0.93	-1.81
Antidiabetics (A10)	**Glibenclamide**	10238-21-8	$C_{23}H_{28}ClN_3O_5S$		494.004	(-)	4.3	neutral	anion	3.79	3.64	2.91	2.86	2.85

**We have chosen the strongest acidic pKa and stongest basic pKa values to present as the molecules can have more than one dissociation constant.*

2.1.2. Analytical method for the analysis of selected pharmaceuticals in wastewater samples

Analysis of the selected pharmaceuticals in wastewater samples was carried out according to a methodology developed and validated by Gros et al (199). The method is based on the simultaneous extraction of pharmaceuticals by SPE using Oasis® HLB (Waters, Milford, MA, USA), followed by liquid chromatography tandem mass spectrometry, using a Hybrid Triple Quadrupole/ Linear ion trap mass spectrometer (4000 QTRAP - *Applied Biosystems*/MDS SCIEX, USA).

In outline, right after sampling, wastewater samples were vacuum filtrated through 1 µm glass fiber filters, followed by 0.45 µm nylon membrane filters, and stored on − 20 °C until the analysis. Filtered-aliquots, i.e. 100 mL of influent and 200 ml of effluent wastewater, were processed by SPE Oasis® HLB using a Baker vacuum system (J.T. Baker, Deventer, The Netherlands), and concentrated via elution with pure methanol. The 8 mL eluents were evaporated under a stream of nitrogen and reconstituted in 1 mL of methanol–water mixture (25:75). Prior to instrumental analysis, these samples were fortified by a mixture of internal standards to a final concentration of 20 ng/mL. HPLC analysis was performed using Symbiosis™ Pico (SP104.002, Spark, Holland), equipped with an autosampler and connected in series with a 4000 QTRAP mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA, USA). Chromatographic separation was achieved with a Purospher Star RP-18 endcapped column (125 mm × 2.0 mm, particle size 5 µm) preceded by a C_{18} guard column (4 × 4, 5 µm), both supplied by Merck (Darmstadt, Germany). For the analysis in negative ionization mode, solvent A was HPLC water and solvent B was a mixture of ACN/MeOH (1:1, v/v). The elution started with 20% of eluent B, increasing to 80% in 15 min, raising to 90% in 2 min and then back to initial conditions within 3 min. The column was re-equilibrated for 10 min before another injection. Flow was 0.3 mL/min, and samples injection volume was 20 µL. The analysis in positive ionization mode was performed using HPLC water with 0.1% formic acid as solvent A and ACN as solvent B. The elution started with 5% of eluent B, increasing to 95% in 20 min, raising to 100% in the following 2 min and then back to initial conditions within 5 min. The re-equilibration time was 10 min. The sample injection volume was set at 20 µL and the flow on 0.3 mL/min.

2.1.3. Analytical method for the analysis of selected pharmaceuticals in sewage sludge samples

The developed quantitative analytical procedure for the determination of the selected pharmaceuticals in sludge samples is presented in detail in the **Scientific publication**

Nº1: Multi-residue method for trace level determination of pharmaceuticals in solid samples using pressurized liquid extraction followed by liquid chromatography/quadrupole-linear ion trap mass spectrometry (Talanta (2009) 80 (1) 363-371).

Figure 2.1 shows a flow diagram of the applied procedure.

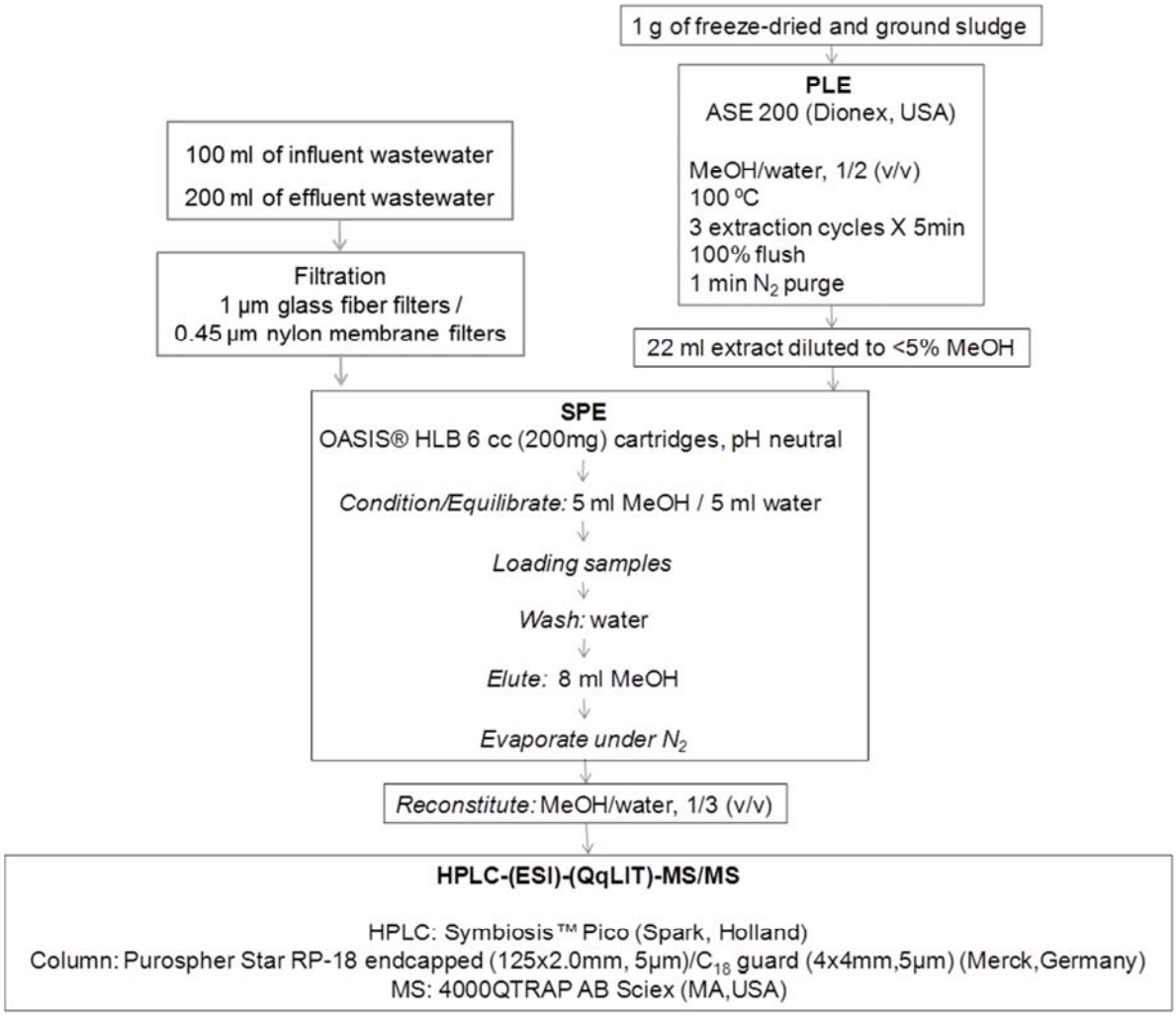


Figure 2.1. Schematic diagram of the analytical procedures for the analysis of pharmaceuticals in wastewater and sludge samples

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Multi-residue method for trace level determination of pharmaceuticals in solid samples using pressurized liquid extraction followed by liquid chromatography/quadrupole-linear ion trap mass spectrometry

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ABSTRACT

A simple and sensitive method for simultaneous analysis of 43 pharmaceutical compounds in sewage sludge and sediment samples was developed and validated. The target compounds were extracted using pressurized liquid extraction (PLE) and then purified and pre-concentrated by solid phase extraction (SPE) using a hydrophilic–lipophilic balanced polymer. PLE extraction was performed on temperature of 100 °C, with methanol/water mixture (1/2, v/v) as extraction solvent. The quantitative analysis was performed by liquid chromatography tandem mass spectrometry using a hybrid triple quadrupole-linear ion trap mass spectrometer (LC–QqLIT-MS). Data acquisition was carried out in selected reaction monitoring (SRM) mode, monitoring two SRM transitions to ensure an accurate identification of target compounds in the samples. Additional identification and confirmation of target compounds were performed using the Information Dependent Acquisition (IDA) function. The method was validated through the estimation of the linearity, sensitivity, repeatability, reproducibility and matrix effects. The internal standard approach was used for quantification because it efficiently corrected matrix effects. Despite the strong matrix interferences, the recoveries were generally higher of 50% in both matrixes and the detection and quantification limits were very low. Beside the very good sensitivity provided by LC–QqLIT-MS, an important characteristic of the method is that all the target compounds can be simultaneously extracted, treated and analysed. Hence, it can be used for routine analysis of pharmaceuticals providing large amount of data. The method was applied for the analysis of pharmaceuticals in river sediment and wastewater sludge from three treatment plants with different treatment properties (i.e. capacity, secondary treatment, quality of influent waters). The analysis showed a widespread occurrence of pharmaceuticals in the sludge matrices.

1. Introduction

The pharmaceutical products have an important role in the treatment and prevention of disease in both humans and animals. They are designed either to be highly active and interact with receptors in humans and animals or to be toxic for many infectious organisms. Because of the nature they can also have unintended effects on animals and micro-organisms in the environment. Although the effects of the pharmaceuticals are investigated through safety and toxicology studies, the potential environmental impacts of their production and use are less understood and have recently become a topic of research interest [1].

The main point of collection and subsequent release of pharmaceuticals into the environment are wastewater treatment plants (WWTP), where they enter via domestic and hospital sewages or through industrial discharges. The studies of effluent waters and river sediment show that wastewater treatment achieves only partial removal of organic pollutants [2–4]. The analysis of effluent and receiving waters itself is not enough to understand comportment of pharmaceuticals during the whole wastewater treatment. Occurrence and distribution of pharmaceuticals in sewage sludge demands detailed investigations, especially because the digested sludge is disposed to landfills or used as agricultural fertilizer. This is another significant route of these micro-pollutants to the environment [5]. It is very difficult to presume the comportment of pharmaceuticals in wastewater treatment because the pharmaceutical products belonging to the same therapeutic groups do not

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show the similar removal. This is caused by the fact that they have different chemical structure and thus they differ in issue. The available data just give very general overview. The existing analytical methods are mostly focused on specific therapeutic classes, paying special attention to the antibiotics due to their potential for antibiotic resistance [6]. But with the quantity and variety of pharmaceuticals and organic pollutants in general, the interactive and synergetic effects in environment are very possible. For this reason, new trends in analytical chemistry are focused on development of methods for simultaneous analysis of many various compounds. The qualitative and quantitative analysis is a good starting point for the further planning of wastewater treatment as well as the establishment of new regulations related to this subject.

Recent development of the advanced instruments and improved analytical methodologies made possible detection of pharmaceuticals in low levels in different environmental matrixes [5,7–11]. Several methodologies have been developed for determination of pharmaceuticals in solid environmental samples. In recent years, the target compounds have usually been extracted by liquid partitioning with ultrasonication (USE) [6,12–15], microwave assisted extraction (MAE) [16] or the more advanced pressurized liquid extraction (PLE). In order to minimize interferences with matrix components and to pre-concentrate target analytes, solid phase extraction (SPE) has been introduced in preparation procedure as a clean-up step. The combination of pressurized liquid extraction (PLE) with solid phase extraction (SPE) as clean-up step becomes mostly employed technique for preparation of solid samples for instrumental analysis. The PLE technique provides good recoveries, saves time and organic solvent, which makes this technique being preferred one for these kind of samples [5,17–21]. Recently, Radjenović et al. [7] used the PLE–SPE combination for the isolation of 31 pharmaceuticals from sludge samples proceeding from the conventional activated sludge treatment and pilot-scale membrane bioreactors. Barron et al. [5] used the same approach for sample preparation for analysis of 27 pharmaceuticals in soil and treated sludge. Also, Nieto et al. [17] and Göbel et al. [22] extracted the target compounds from sludge samples using the PLE–SPE. In all the mentioned methods a hydrophilic–lipophilic balance (HLB) reversed-phase sorbent was used as SPE packaging. This sorbent has been found suitable for multi-residue methods in neutral pH condition, with a proper selection of the eluent (solvent) [23–25].

The majority of current analytical methods for separation and detection of pharmaceuticals uses liquid chromatography–tandem mass spectrometry (LC–MS/MS) because of its versatility, specificity and selectivity [26]. Triple quadrupole (QqQ) [7,12,17,27] and ion trap MS (IT) [5,6,15] have been widely applied in quantitative target analysis. Recently, a hybrid instrument consisting of a quadrupole and a linear ion trap (QqLIT) have been applied for analysis of pharmaceuticals in waters, thus good results related to analysis of pharmaceuticals in sludge could be expected. This instrument allows powerful scan combinations which lead to rapid identification and confirmation of analytes [8,24,28,29].

The objective of the present study was to develop and validate a sensitive multi-residual method for determination of 43 pharmaceuticals in sludge and sediment. The target compounds belong to different therapeutic groups of pharmaceuticals. They are listed in Table 1 classified according to their therapeutic effects. The pharmaceuticals are extracted using PLE and purified by SPE (Oasis® HLB). High performance liquid chromatography (HPLC) coupled to a QqLIT-MS has been applied for separation and determination of the target pharmaceuticals. Quantitation was performed by the internal standard approach. The performance of the method was evaluated through the estimation of the linearity, sensitivity, repeatability, reproducibility and matrix effects.

The developed method offers simple and simultaneous extraction, treatment and analysis of 43 pharmaceutical compounds in sludge and sediment. Comparing to the previously published methods for determination of pharmaceuticals in environmental solid matrices this method exhibits significantly improved sensitivity due to the QqLIT capabilities. Finally, the method was successfully applied for analysis of pharmaceuticals in sludge from three WWTPs which proves that it can be used for routine analysis of pharmaceuticals providing a large amount of data.

2. Experimental part

2.1. Chemicals

All the pharmaceutical standards for target compounds were of high purity grade (>90%). Ibuprofen, neproxen, ketoprofen, diclofenac and gemfibrozil were supplied by Jescuder (Rubí, Spain). Acetaminophen, indometacin, mefenamic acid, phenazone, bezifibrate, mevastatin, fenofibrate, Pravastatin (as sodium salt), carbamazepine, famotidine, ranitidine (as hydrochloride), cimetidine (as hydrochloride), erythromycin (as hydrate), azithromycin (as dehydrate), roxitromycin, clarithromycin, josamycin, tylosin A, sulfamethazine, trimethoprim, chloramphenicol, atenolol, sotalol, metoprolol (as tartrate), timolol, pindolol, nadolol, salbutamol, clenbuterol (as hydrochloride), enalapril (as maleate), glibenclamide, furosemide, hydrochlorothiazide and metronidazole were purchased from Sigma–Aldrich (Steinheim, Germany). Standard atorvastatin (as calcium salt) was provided by LGC Promochem (London, UK), while diazepam, lorazepam and butalbital were from Cerilliant (Texas, USA).

Isotopically labelled compounds, used as internal standards, were sulfathiazole-d_4 from Toronto Research Chemicals, diazepam-d_5 and phenobarbital-d_3 from Cerilliant (Texas, USA), atenolol-d_7, carbamazepine-d_{10}, ibuprofen-d_3, clotrimazole-d_5, enalapril-d_5, hydrochlorothiazide-d_2, glyburide-d_3, albuterol-d_3, cimetidine-d_3, ethyl clofibrate-d_4, antipyrine-d_3, acetaminophen-d_4, diclofenac-d_4 from CDN Isotopes (Quebec, Canada), mecoprop-d_3 from Dr. Ehrenstorfer (Augsburg, Germany) and ^{13}C-erythromycin and ^{13}C-phenacetin from Sigma–Aldrich (Steinheim, Germany).

The solvents, HPLC grade methanol, acetonitrile, water (Lichrosolv) and formic acid 98% were provided by Merck (Darmstadt, Germany). Nitrogen used for drying from Air Liquide (Spain) was of 99.995% purity.

The cartridges used for solid phase extraction were Oasis® HLB (200 mg, 6 ml) from Waters Corporation (Milford, MA, USA). The syringe filters of 0.45 μm pore size were purchased from Pall Corp (USA).

The individual standard solutions as well as isotopically labelled internal standard solutions were prepared on a weight basis in methanol. Furosemide and butalbital were obtained as solutions in acetonitrile, while lorazepam and diazepam were dissolved in methanol, at a concentration of 1 mg/ml. The solutions were stored at −20 °C. Fresh stock solutions of antibiotics were prepared monthly due to their limited stability while stock solutions for the rest of substances was renewed every three months. A mixture of all pharmaceuticals was prepared by appropriate dilution of individual stock solutions in methanol–water (25:75. v/v) and it was renewed before each analytical run. A separate mixture of isotopically labelled internal standards, used for internal standard quantification, was prepared in methanol and further diluted in methanol–water (25:75, v/v) mixture.

2.2. Sample pretreatment

River sediment and sludge samples, provided from three wastewater treatment plants (WWTP), were used for development

Table 1
Target compounds and the corresponding isotopically labelled compounds. SRM transitions and MS/MS parameters for the analysis of the target compounds.

Therapeutic groups	Compounds	CAS number	Internal standards	Precursor ion (m/z)	SRM1	DP-CE-CXP	SRM2	DP-CE-CXP	SRM ratio (SRM1/SRM2)
Analgesics/anti-inflammatories	Ketoprofen	22071-15-4	Mecoprop-d_3	253 $[M-H]^-$	209	40-12-11	197	40-6-9	15.6
	Naproxen	22204-53-1	Ibuprofen-d_3	229 $[M-H]^-$	185	35-10-13	169	35-38-9	2.1
	Ibuprofen	15687-27-1	Ibuprofen-d_3	205 $[M-H]^-$	161	45-10-7	–	–	–
	Indomethacine	53-86-1	Ibuprofen-d_3	356 $[M-H]^-$	312	50-12-3	297	50-24-17	4.7
	Diclofenac	15307-86-5	Diclofenac-d_4	294 $[M-H]^-$	250	40-16-1	214	40-30-15	17.6
	Mefenamic acid	61-68-7	Ibuprofen-d_3	240 $[M-H]^-$	196	45-20-5	180	45-38-35	19.1
	Acetaminophen	103-90-2	Acetaminofen-d_3	150 $[M-H]^-$	107	55-22-7	–	–	–
Phenazone type drugs	Phenazone	60-80-0	Phenazone-d_3	189 $[M+H]^+$	56	76-40-4	147	76-33-4	1.9
Lipid regulators and cholesterol lowering statin drugs	Bezafibrate	41859-67-0	Ethyl clofibrate-d_4	360 $[M-H]^-$	274	70-26-1	154	70-38-5	2.9
	Fenofibrate	49562-28-9	Ethyl clofibrate-d_4	361 $[M+H]^+$	139	76-43-10	–	–	–
	Gemfibrozil	25812-30-0	Ibuprofen-d_3	249 $[M-H]^-$	121	85-20-7	127	85-14-5	21.7
	Mevastatin	73573-88-3	Carbamazepine-d_{10}	391 $[M+H]^+$	185	56-19-16	159	56-39-14	1.6
	Pravastatin	81093-37-0	Carbamazepine-d_{10}	447 $[M+H]^+$	327	81-29-10	–	–	–
	Atorvastatin	134523-00-5	Carbamazepine-d_{10}	559 $[M+H]^+$	440	71-27-20	250	71-63-4	2.0
Psychiatric drugs	Diazepam	439-14-5	Diazepam-d_5	285 $[M+H]^+$	193	91-45-8	154	91-50-15	1.9
	Lorazepam	846-49-1	Diazepam-d_5	323 $[M+H]^+$	174	66-45-18	229	66-45-8	1.2
	Carbamazepine	298-46-4	Carbamazepine-d_{10}	237 $[M+H]^+$	194	76-29-19	–	–	–
Histamine H2 receptor antagonists	Ranitidine	66357-35-5	Cimetidine-d_3	315 $[M+H]^+$	176	56-25-14	130	56-39-6	2.0
	Famotidine	76824-35-6	Cimetidine-d_3	338 $[M+H]^+$	189	56-27-4	259	56-20-8	1.6
	Cimetidine	51481-61-9	Cimetidine-d_3	253 $[M+H]^+$	95	46-30-8	159	46-23-12	1.5
Macrolide antibiotics	Erythromycin	114-07-8	Erythromycin ^{13}C	734 $[M+H]^+$	158	71-41-8	576	71-35-8	3.8
	Roxithromycin	80214-83-1	Erythromycin ^{13}C	838 $[M+H]^+$	158	56-49-14	679	56-31-8	4.2
	Clarithromycin	81103-11-9	Erythromycin ^{13}C	748 $[M+H]^+$	591	61-35-12	158	61-40-12	12.6
	Josamycin	16846-24-5	Erythromycin ^{13}C	828 $[M+H]^+$	174	101-45-14	600	101-37-18	6.6
	Tylosin A	1401-69-0	Erythromycin ^{13}C	916 $[M+H]^+$	174	86-63-14	773	86-41-10	14.2
Sulfonamid antibiotics	Sulfamethazine	57-68-1	Sulfathiazole-d_4	279 $[M+H]^+$	186	71-25-0	124	71-33-10	1.0
Other antibiotics	Trimethoprim	738-70-5	Carbamazepine-d_{10}	291 $[M+H]^+$	230	76-33-0	261	76-31-20	1.2
	Chloramphenicol	56-75-7	Ibuprofen-d_3	323 $[M-H]^-$	152	75-22-13	194	75-18-27	3.5
	Metronidazole	443-48-1	Clotrimazole-d_5	172 $[M+H]^+$	172	61-21-8	82	61-37-6	1.8
ß-blockers	Atenolol	29122-68-7	Atenolol-d_7	267 $[M+H]^+$	145	60-35-8	190	60-35-14	3.6
	Sotalol	3930-20-9	Atenolol-d_7	273 $[M+H]^+$	213	60-25-6	255	60-25-6	1.1
	Metoprolol	37350-58-6	Atenolol-d_7	268 $[M+H]^+$	121	60-35-10	133	60-35-8	1.0
	Timolol	26839-75-8	Atenolol-d_7	317 $[M+H]^+$	261	60-30-20	244	60-30-6	1.2
	Nadolol	42200-33-9	Atenolol-d_7	310 $[M+H]^+$	254	46-30-2	201	46-35-4	1.4
	Pindolol	13523-86-9	Atenolol-d_7	249 $[M+H]^+$	116	60-30-8	98	60-30-14	7.0
ß-agonists	Clenbuterol	37148-27-9	Albuterol-d_3	277 $[M+H]^+$	203	61-23-14	132	61-33-10	2.2
	Salbutamol	18559-94-9	Albuterol-d_3	240 $[M+H]^+$	148	61-25-12	166	61-20-12	1.7
Barbiturates	Butalbital	77-26-9	Phenobarbital-d_5	223 $[M-H]^-$	180	60-16-9	85	60-18-5	3.8
Antihypertensive	Nifuroxazide	965-52-6	Phenacetine ^{13}C	276 $[M+H]^+$	121	81-25-10	65	81-73-4	7.3
	Enalapril	75847-73-3	Enalapril-d_5	377 $[M+H]^+$	234	91-29-12	303	91-35-6	16.8
Diuretic	Hydrochlorothiazide	58-93-5	Hydrochlorthiazide-d_2	296 $[M-H]^-$	78	90-28-17	–	–	–
	Furosemide	54-31-9	Ibuprofen-d_3	329 $[M-H]^-$	205	65-22-19	285	65-32-11	1.2
Antidiabetic	Glibenclamide	10238-21-8	Glibenclamide-d_3	494 $[M+H]^+$	369	81-23-6	169	81-55-12	1.8

and validation of the method. The sludge samples are products of aerobic (WWTP Tudela) and anaerobic digestion (WWTP Arazuri and WWTP Terrassa) of sludge.

The sediment was collected from the middle course of river Ebro (Spain) in 2004. The samples from WWTP Tudela (Sludge I) were collected in October 2007 and July 2008. This plant serves 37,300 inhabitants, with a total capacity of 110,000 equivalent inhabitants. Sludge processing is based on thermophilic aerobic digestion in bacterial beds with approximately 9 days of retention time. The samples from WWTP Arazuri (Pamplona) (Sludge II) were collected in October 2007 and from WWTP Terrassa in April 2007. The WWTP Arazuri serves around 356,000 inhabitants, with a total capacity of 722,000 equivalent inhabitants in 2007. The sludge treatment involves anaerobic digestion with retention time of 19 days. The WWTP Terrassa has a total treatment capacity of 277,000 equivalent inhabitants. The sludge is treated by anaerobic digestion with the solid retention time of approx. 10 days.

All the solid samples were freeze-dried (LioAlfa 6, Telstar) at −40 °C and with 0.044 bar vacuum and stored at −20 °C until the analysis.

2.3. Extraction and clean-up

The samples of sludge and sediment were extracted by PLE using ASE 300 accelerated solvent extractor (Dionex, Sunnyvale, CA) equipped with 11 ml stainless extraction cells. Aliquots of freeze-dried and grinded sludge and sediment (1 g) were mixed in the extraction cells with Hydromatrix. This dispersing agent is used

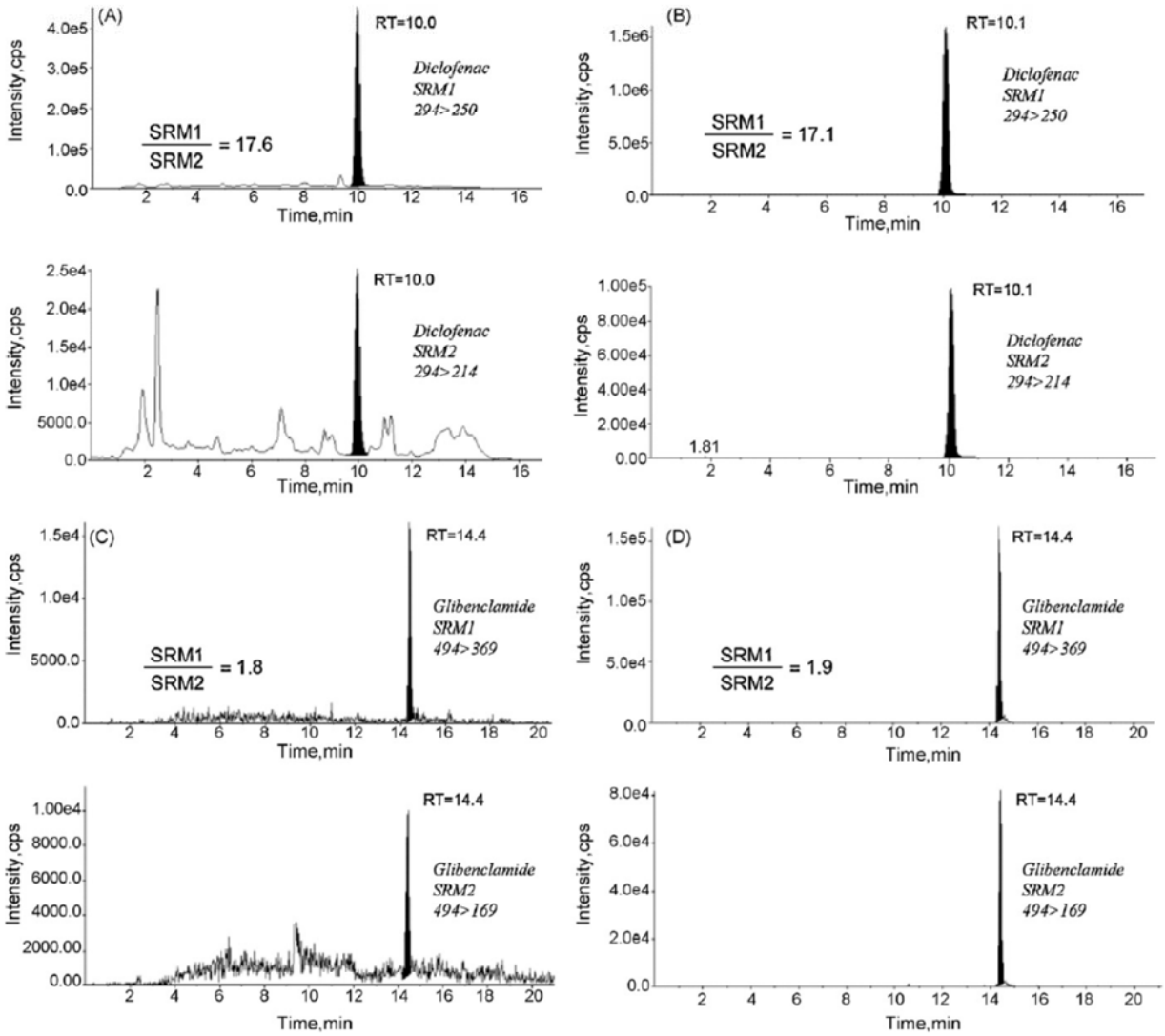

Fig. 1. Chromatograms of the two SRM transitions of diclofenac and glibenclamide in Sludge II (A and C, respectively) and in standard solution (B and D).

to prevent aggregation of sample particles and reduce interstitial volume in the cells [30]. Optimization of extraction parameters included selection of solvent and variation of temperature, time and number of extraction cycles. The extraction method was established with the following parameters: methanol/water, 1/2 (v/v) as extraction solvent, temperature of 100 °C, a preheating period of 5 min, 3 static cycles, each lasting 5 min, total flush volume of 100% of cell with 60 s of nitrogen purge.

The extract obtained in PLE (~22 ml) was diluted in 500 ml of HPLC water (methanol < 5%), and processed by SPE. Oasis HLB cartridges (200 mg, 6 ml) from Waters Corporation (Milford, MA) were used for clean-up. The cartridges were conditioned with 5 ml of methanol followed by 5 ml of HPLC water at neutral pH. Then the dilution of ASE extract was percolated through the cartridges using a Baker vacuum system (J.T. Baker, The Netherlands). Finally, the compounds were eluted with 8 ml of methanol at a 1 ml min^{-1} flow and then the SPE extracts were evaporated under a nitrogen stream and reconstituted with 1 ml of methanol–water mixture (25:75, v/v). Prior to the LC–MS/MS analysis, the samples were passed through 0.45 μm filters and fortified with a standard mixture of the internal standards to the concentration of 20 ng ml^{-1}.

2.4. LC–ESI-(QqLIT)-MS^2 analysis

LC analysis was performed using Symbiosis™ Pico (SP104.002, Spark, Holland), equipped with an autosampler and connected in series with a 4000 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA, USA). Chromatographic separation was achieved with a Purospher Star RP-18 endcapped column (125 mm × 2.0 mm, particle size 5 μm) preceded by a C_{18} guard column (4 × 4, 5 μm), both supplied by Merck (Darmstadt, Germany).

Knowing that an aqueous, purified and pre-concentrated extract of a solid environmental sample is quite similar to a water sample, already established protocol by Gros et al. [8] was used for quantitative analysis of water samples.

The elution gradients were adapted for the LC by Symbiosis™ Pico. For the analysis in negative ionization mode, solvent A was a mixture of acetonitrile–methanol (1:1, v/v) and solvent B was HPLC water. The elution started with 20% of eluent A, increasing to 80% in 15 min, raising to 90% in 2 min and then back to initial conditions within 3 min. The column was re-equilibrated for 10 min before another injection. The analysis in positive ionization mode was performed using acetonitrile as solvent A and HPLC water with 0.1% formic acid as solvent B. The elution started with 5% of eluent A, increasing to 95% in 20 min, raising to 100% in the following 2 min and then back to initial conditions within 5 min. The re-equilibration time was 10 min. The sample injection volume was set at 20 μl and the flow on 0.2 ml/min in both modes.

Data acquisition was performed in selective reaction monitoring (SRM) mode. For each compound two SRM transitions between the precursor ion and two most abundant fragment ions were monitored, as illustrated in Fig. 1. Only one transition was monitored for the isotopically labelled standards since they are normally not present in environmental samples. In total, 70 transitions in positive ionization mode (corresponding to 30 compounds and 12 internal standards) and 29 transitions in negative ionization mode (13 compounds and 7 internal standards) were monitored. In order to obtain additional confirmation, especially for compounds showing poor fragmentation, an Information Dependent Acquisition (IDA) experiment was performed, with SRM as the survey scan and an enhanced product ion scan (EPI), at three different collision energies, as dependent scan. The obtain spectra were compared with library data based on EPI spectra at the three collision energies used. This allows broad accomplishment of the requirements set by the EU regulations (EU Commission Decision 2002/657/EC) [31] related to identification and confirmation of pharmaceuticals in LC–tandem MS analysis.

2.5. Method validation

The performance of the method was evaluated through estimation of the linearity, sensitivity, repeatability, reproducibility and matrix effects of the method.

Quantification, based on peak areas, was performed by internal standard calibration. The internal standards used for quantification of the compounds were following: sulfathiazole-d_4, diazepam-d_5, phenobarbital-d_3, atenolol-d_7, carbamazepine-d_{10}, ibuprofen-d_3, clotrimazole-d_5, enalapril-d_5, hydrochlorothiazide-d_2, glyburide-d_3, albuterol-d_3, cimetidine-d_3, ethyl clofibrate-d_4, antipyrine-d_3, acetaminophen-d_4, diclofenac-d_4, mecoprop-d_3, ^{13}C-erythromycin and ^{13}C-phenacetin. Seven-point calibration curves (0.5–100 ppb) were generated using linear regression analysis. The linearity was qualified by linear correlation coefficient, r^2.

To determine the recoveries, sediment and sludge samples were spiked in triplicate with a standard mixture of analytes in methanol/water, 25/75 (v/v) to 50 ng g^{-1} concentration. The spiked samples were stirred vigorously in order to enable better contact of analytes with the matrix. After 24-h equilibration, these samples together with the correspondent blank samples were extracted and treated by the previously described protocol. The internal standards for correcting matrix effects were added to the final sample extract. The recoveries were determined in triplicate comparing the obtained concentrations, after subtraction of concentrations found in blank samples, with the initial spiking level.

Method detection limits (MDL) and method quantification limits (MQL) were determined as the minimum detectable amount of analyte with a signal-to-noise of 3 and 10, respectively.

Intra-day and inter-day precision were determined from five repeated injections 50 ng g^{-1} standards during the same day (repeatability) and in five successive days (reproducibility). These two parameters were expressed as relative standard deviation of result (RSD, %).

Matrix effect was evaluated. In order to express it as percentage of suppression or enhancement, Eq. (1) was applied. The peak-areas from the analysis of spiked sludge and sediment extracts ($area_{matrix}$) reduced by the peak areas corresponding to the native analytes present in the sample ($area_{blank}$), were compared with the peak areas from spiked solvent at the same concentration ($area_{solvent}$). The spiked concentration was 25 ng g^{-1} for all the solid samples.

$$\text{signal suppression } (\%) = 100 - \left(\frac{(\text{area}_{\text{matrix}} - \text{area}_{\text{blank}}) \times 100}{\text{area}_{\text{solvent}}}\right) \quad (1)$$

The efficiency of internal standard and standard addition calibration were evaluated comparing the relations of calibration curves made in pure solvent with those prepared in matrix extracts.

3. Results and discussion

3.1. PLE conditions

The combination of solvent, temperature, flush volume, number and time of extraction cycles were investigated in order to obtain optimum extraction conditions for analysis of 42 pharmaceutical compounds. This method is one step forward in development of multi-residual analytical methods in our group [7,8]. Thus the starting point for PLE extraction were conditions reported by Radjenović et al. [7], adopted for the extraction of extended list of compounds.

The influence of the parameters was investigated simultaneously. The experiments were organized combining different solvent mixtures with different temperatures in 30 extractions, and then the other parameters were examined (time and number of cycles). Extraction pressure was set to 1500 psi for all PLE experiments, because it is not considered as a critical experimental parameter and it has negligible impact on analytical recovery [30].

Solvent: The solvent must be able to solubilize the target analytes leaving the sample matrix integrate. Since the analysed pharmaceuticals vary in physicochemical properties, the choice of the solvent mixture was limited and it could not match all the compounds in their polarity. The neutral conditions were required. In this study the following extraction solvents tested were: methanol/water (1/1, 1/2, 1/3, 2/1, 3/1, v/v) and acetonitrile/water (1/1, 1/2, 1/3, 2/1, 3/1, v/v). The combination methanol/water, 1/2, (v/v) yielded relatively better recoveries than other ones. For enalapril, metronidazole, bezifibrate and chloramphenicol, higher percent of water (i.e. methanol/water, 1/3, v/v) gave higher recoveries. More methanol (i.e. methanol/water, 1/1, v/v) gave better results for gemfibrozil, sulfamethazine and erythromycin. In the group of experiments with mixtures of acetonitrile and water, the best ratio was 1/2 (v/v) as well, but still it gave lower recoveries than methanol/water combination.

Temperature: This is very important parameter in PLE extraction. Application of higher temperature in PLE decreases the viscosity of solvents, thus allowing its better penetration into the sample matrix. The increase of temperature decreases significantly the dielectric constant of the water so the organic solvents can be used in smaller amount or avoided. But too high temperature can lead to degradation of the compounds or loss in method selectivity due to more efficient extraction of interfering matrix components. In the described experiments, all the combinations of the solvents on three extraction temperatures were tested: 60, 80 and 100 °C. The temperature higher of 100 °C was not tested since thermal

Table 2
Method performance parameters: reproducibility (RSD% for $n=5$), repeatability (RSD% for $n=5$), recoveries (%) and method precision (RSD%), matrix effect (%), method detection (MDL, ng g^{-1}) and quantification limits (MQL, ng g^{-1}) obtained in sludge and sediment samples.

Therapeutic groups	Compounds	Repeat. (RSD%) ($n=5$)	Reprod. (RSD%) ($n=5$)	Sludge				Sediment			
				Recovery,% (RSD,%) ($n=3$)	Matrix effect (%)	MDL (ng g^{-1})	MQL (ng g^{-1})	Recovery, % (RSD, %) ($n=3$)	Matrix effect (%)	MDL (ng g^{-1})	MQL (ng g^{-1})
Analgesics/anti-inflammatories	Ketoprofen	2	2	98.4 (10)	14	0.56	1.86	105 (0.6)	35	0.93	3.11
	Naproxen	5	5	125 (4.6)	41	0.07	0.24	105 (5.2)	48	0.84	2.79
	Ibuprofen	1	2	118 (13)	38	0.10	0.32	129 (1.0)	45	0.12	0.40
	Indomethacine	2	3	107 (7.0)	87	0.22	0.73	81.4 (0.7)	68	0.15	0.49
	Diclofenac	3	2	81.4 (9.1)	79	0.94	3.13	101 (1.2)	54	0.03	0.09
	Mefenamic acid	8	7	69.3 (12)	68	0.07	0.24	95.2 (5.3)	58	0.22	0.74
	Acetaminophen	3	3	40.7 (8.8)	-24	0.07	0.24	60.3 (9.8)	9	0.22	0.74
Phenazone type drugs	Phenazone	6	6	194 (4.1)	76	1.12	3.72	169 (1.8)	73	0.34	1.15
Lipid regulators and cholesterol lowering statin drugs	Bezafibrate	7	13	107 (5.1)	47	0.01	0.05	95.1 (3.8)	56	0.02	0.06
	Fenofibrate	3	11	204 (9.0)	81	1.04	3.46	123 (7.4)	75	0.79	2.62
	Gemfibrozil	4	11	76.1 (13)	82	0.51	1.69	77.7 (4.9)	72	1.24	4.14
	Mevastatin	13	14	94.1 (1.0)	66	8.84	29.4	89.0 (1.5)	85	3.16	10.5
	Pravastatin	12	15	215 (5.4)	78	1.32	4.42	186 (6.9)	57	0.71	2.38
	Atorvastatin	6	5	72.9 (4.5)	97	0.99	3.31	34.6 (3.3)	98	0.72	2.41
Psychiatric drugs	Diazepam	1	4	96.8 (2.5)	93	1.83	6.10	107 (9.3)	77	0.23	0.77
	Lorazepam	2	10	70.2 (13)	79	5.75	19.2	125 (6.3)	71	3.20	10.7
	Carbamazepine	2	8	134 (3.5)	89	0.04	0.13	137 (1.7)	81	0.03	0.09
Histamine H_2 receptor antagonists	Ranitidine	1	9	106 (13)	99	0.03	0.10	126.2 (13)	89	0.02	0.06
	Famotidine	4	14	83.5 (14)	97	0.01	0.05	97.8 (8.1)	73	0.02	0.07
	Cimetidine	7	7	78.3 (14)	80	0.07	0.24	87.4 (7.2)	72	0.01	0.02
Macrolide antibiotics	Erythromycin	6	5	43.2 (1.2)	100	1.17	3.88	68.0 (12)	92	0.01	0.02
	Roxithromycin	5	6	146 (15)	97	6.75	22.5	149 (9.9)	89	0.04	0.13
	Clarithromycin	8	14	38.2 (9.1)	95	3.51	11.7	130 (11)	86	0.10	0.34
	Josamycin	9	8	42.1 (13)	94	0.08	0.27	206 (7.0)	88	0.64	2.12
	Tylosin A	7	5	142 (1.8)	93	0.81	2.69	157 (8.8)	84	0.04	0.14
Sulfonamide antibiotics	Sulfamethazine	6	9	40.3 (14)	74	0.14	0.48	45.7 (8.9)	74	0.32	1.06
Other antibiotics	Trimethoprim	10	11	93.1 (12)	96	0.14	0.47	97.2 (9.6)	83	0.25	0.83
	Chloramphenicol	10	14	76.0 (9.3)	65	0.03	0.09	72.9 (0.3)	45	0.10	0.32
	Metronidazole	9	11	80.5 (15)	62	0.55	1.83	41.7 (3.9)	69	0.38	1.26
ß-blockers	Atenolol	5	4	97.1 (11)	77	0.16	0.54	90.4 (14)	66	0.11	0.36
	Sotalol	3	5	105 (12)	84	0.06	0.20	105 (8.8)	74	0.06	0.20
	Metoprolol	4	8	195 (2.9)	98	0.30	1.00	144 (11)	84	0.21	0.71
	Timolol	9	7	169 (9.0)	98	0.08	0.25	109 (14)	85	0.05	0.17
	Nadolol	6	4	46.1 (8.7)	94	0.06	0.19	66.7 (8.7)	82	0.01	0.03
	Pindolol	4	5	53.0 (7.9)	99	0.42	1.41	77.2 (12)	90	0.03	0.10
ß-agonists	Clenbuterol	5	4	86.4 (14)	88	0.02	0.07	168 (3.3)	97	0.07	0.25
	Salbutamol	0	3	86.0 (4.8)	82	0.08	0.27	84.1 (11)	68	0.02	0.08
Barbiturates	Butalbital	8	7	44.5 (2.1)	69	0.39	1.31	33.2 (1.2)	77	0.05	0.16
Antihypertensives	Nifuroxazide	13	9	65.0 (6.4)	78	0.06	0.20	125 (8.8)	96	0.05	0.17
	Enalapril	6	14	100 (8.8)	96	0.10	0.32	97.7 (5.6)	81	0.01	0.03
Diuretics	Hydrochlorothiazide	9	8	42.3 (12)	73	0.08	0.27	71.0 (13)	65	0.06	0.20
	Furosemide	10	13	106 (3.3)	51	0.19	0.64	77.7 (3.3)	57	0.76	2.52
Antidiabetics	Glibenclamide	6	5	87.8 (11)	89	1.67	5.56	89.1 (9.4)	85	0.29	0.96

degradation can occur at higher temperatures [22]. The recoveries obtained on 60 °C were low for most of analysed compounds. Salbutamol, gemfibrozil, mefenamic acid and diazepam gave better recoveries at temperature of 80 °C. For the compounds that were recovered in small or exaggerated percent is difficult to conclude which temperature suits better (i.e. prevastatine, atorvastatine, macrolides).

Extraction cycles: Optimization of the conditions included the screening of number and duration of static cycles. Each static cycle introduce fresh solvent which is very useful for the samples with complex matrix as sludge, while the longer time of a cycle can allow better diffusion of analytes into the extraction solvent. It is recommended to divide the extraction into more cycles [30]. The extracts of individual cycles of 5 min were collected as well as extracts from 2 and 3 cycles. Through the first and second fraction had almost all the compounds extracted completely, the third cycle was introduced to insure the complete extraction of diclofenac, indometacin, mefenamic acid, gemfibrozil, bezifibrate and lorati-

dine. With the three-cycle extraction, it is presumed that all the spiked and native analytes were removed. Finally, the PLE extraction was performed under the following conditions: 1 g of sample, temperature of 100 °C, as extraction solvent—methanol/water, 1/2 (v/v), a preheating period of 5 min, 3 static cycles, each lasting 5 min, total flush volume was 100% of cell with 60 s of nitrogen purge.

3.2. Method validation

The quantification was based on peak area. The shift of peaks in both transitions was noticed in the matrices comparing to the position in pure solvent. But, the retention times for all the analytes in the matrix varied less than 1%. In Fig. 1 are illustrated the chromatographic peaks of the two SRM transitions of diclofenac and glibenclamide in sludge and standard solution. The parts of the figure marked by A and C stand for the chromatograms of analytes in Sludge II, and B and D for chromatograms of standard solutions of diclofenac and glibenclamide, respectively. As illustrated, the retention times and SRM ratios concord well. Reproducibility and repeatability expressed as relative standard deviation, RSD%, were lower than 13% for intra-day and 15% for inter-day analysis, respectively (Table 2). Seven points calibration curves gave very good fits, $r^2 > 0.99$, over the established concentration range of 0.5–100 ng g^{-1} for all the compounds in all the matrices. The limits of detections in sludge and sediment were lower than 1 ng/g for the most compounds. The method provided lower sensitivity for phenazone, fenofibrate, prevastatine, mevastatine, diazepam, lorazepam, erythromycin, roxitromycin and

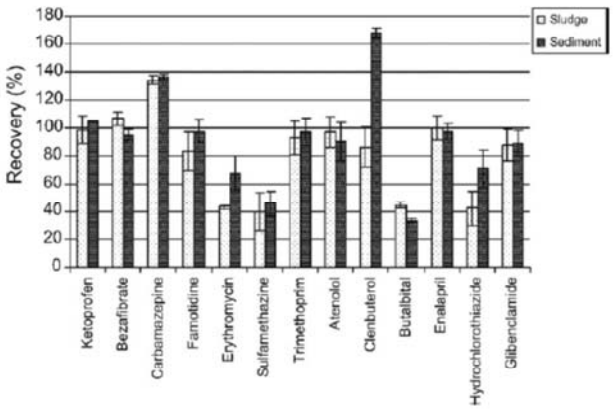

Fig. 2. Recoveries obtained for the representative compounds of each therapeutic group.

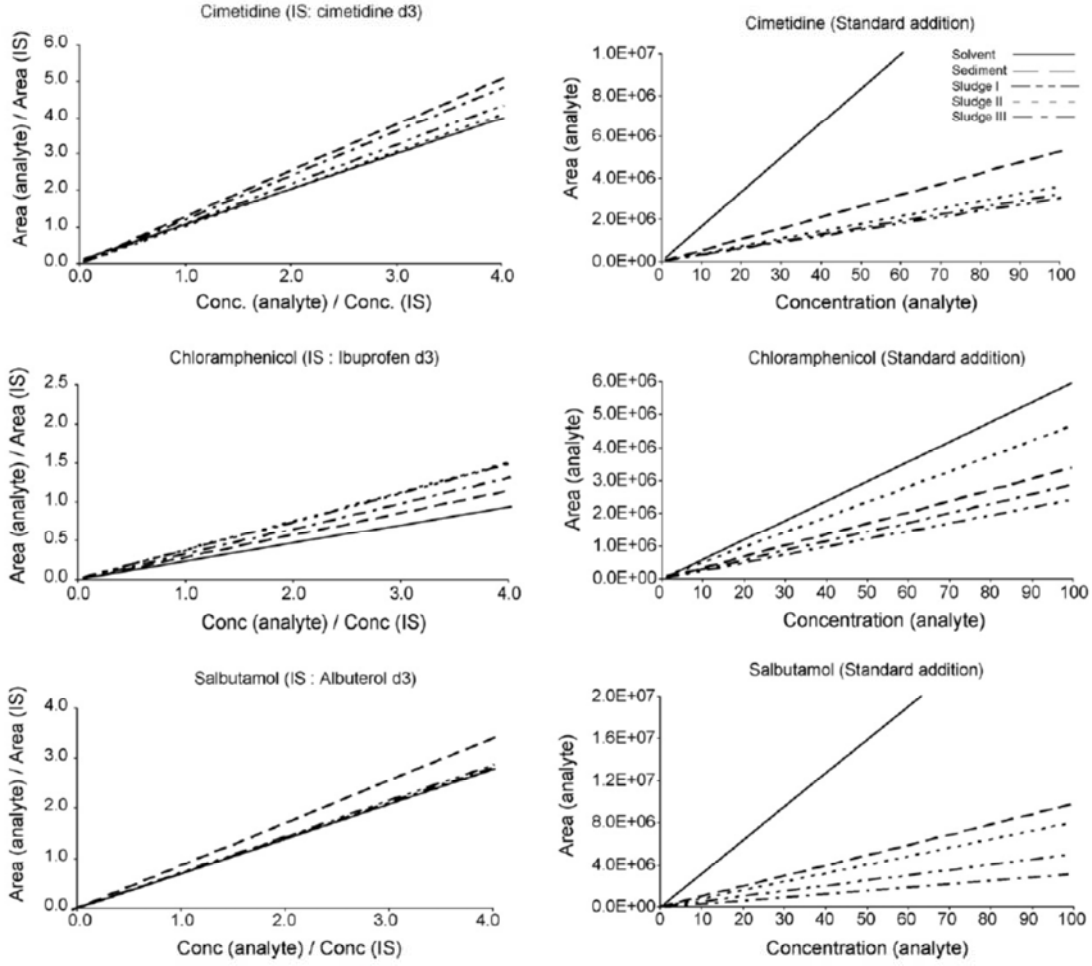

Fig. 3. Internal standard calibration curves (left) and standard addition calibration curves (right) of spiked solvent and extracts of sludge and sediment for cimetidine, salbutamol and chloramphenicol.

clarithromycin in sludge (1.12 < MDL < 6.75) and for mevastatine, gemfibrozil, lorazepam in sediment samples (1.24 < MDL < 3.20). Generally, the sensitivity was lower for sludge than for sediment samples, due to the more complex matrix. The values of MDL and MQL for sludge (Sludge I) and sediment are presented in Table 2. Compared to the method detection limits obtained for pharmaceuticals in solid matrices using some other instruments [5,7,12,13,32], with the LC–QqLIT-MS the improvement in sensitivity is significant.

In Table 2 are listed only the recovery results obtained for Sludge I and sediment, with the corresponding relative standard deviations. The RSD% was less than 15% for all the compounds in both matrices, which is considered as good method precision. The recoveries varied significantly depending on compound and matrix. For the pharmaceuticals in Sludge I only acetaminophen (40.7%), josamycin (42.1%), sulfamethazine (40.3%) and hydrochlorthiazide (42.3%) had recoveries <45%, wherein acetaminophen and hydrochlorthiazide were quantified with their deuterated standards. For the samples of sediment, recoveries were <50% for atorvastatin (34.6%), sulfamethazine (45.7%), metronidazole (41.7%) and butalbital (33.2%). In Fig. 2 are illustrated the results for some representative compounds for each therapeutic group, and the rest of the compounds followed a similar pattern. Some compounds yielded extremely high recoveries (»100%) in both matrices, like prevastatine, phenazone, fenofibrate and metoprolol. Elevated recoveries have already been reported in some studies about pharmaceuticals in sludge [5,7,32]. The properties of matrix itself and the complexity of the interaction with analytes, as well as possible errors in procedure could be the reason for the extreme (high or low) recoveries. Hence, it is difficult to give logical explanation and expect certain values of recoveries. If the accurate results are requested, the recoveries in the investigated matrix must be determined prior to quantification.

Matrix effect: The performance of HPLC–ESI-MS/MS analysis is strongly affected by the ionisable impurities coming from matrix (e.g. natural organic matter, salts, ion-pairing agents, non-target contaminants, etc.) that can interfere with the ionization processes. This may result in a signal suppression or enhancement leading to low sensitivity and inaccurate results. These effects are more extensive when the matrix is more complex as sludge matrix, for example. It is advisable to evaluate matrix effect as a part of validation of the method to ensure the reliability of results obtained. In this study, standard addition and internal standard addition experiments were performed to investigate and minimize matrix effect.

The calibration curves obtained from real sludge and sediment extracts with standard addition were compared with those in pure solvent (methanol/water, 25/75, v/v). All curves were linear over the concentration range of 0.5–100 ng g^{-1} with correlation factors $r^2 > 0.99$. For illustration, Fig. 3 (right) presents calibration curves of cimetidine, chloramphenicol and salbutamol in 4 real matrices (sediment and three samples of sludge) and one in solvent. Significant difference in slopes was observed proving the existence of matrix effects.

Fig. 3 (left) shows the calibration curves of the same compounds acquired by isotopically labeled compounds added into sludge and sediment extracts and in the pure solvent. The curves were linear over the concentration range of 0.5–100 ng g^{-1} with good correlation factors of $r^2 > 0.99$. Despite the fact that the matched isotopically labeled standards were not available for all the target compounds, the calibration curves appear to be overlapped well enough. This means that internal standards compensated the matrix effects considerably; therefore the internal standard addition was used for quantification in this study. Although the selected isotopically labeled standards seem to be appropriate for the target compounds (Fig. 3), cannot be expected that they give very accurate results. More precise results could be achieved using the surrogate standards of the target compounds. It is expected that the surrogate standards compensate for any error that can occur during the sample preparation. But since the surrogate standards were not available for all the analysed compounds and the internal standard quantification gave repeatable results, we decided to use only internal standard approach. The application of only one approach allowed more comparable and reproducible results. The lack of fully compatible surrogate/internal standards is considered as the main limitation of one multi-residual method for pharmaceuticals in environmental samples [33].

Matrix effect was quantified comparing the areas of compounds in spiked matrix samples with the areas obtained in spiked solvent (methanol/water, 25/75, v/v). The effect was expressed by percentage of signal suppression/enhancement and the results are summarized in Table 2. Knowing that the nature of matrix effect is pretty varying, the percentage is just a relative indicator of the degree of suppression and enhancement. The percentage varies from 14 to 100% in sludge and from 9 to 98% for sediment samples. The impact of matrix interferences was different for each compound and except acetoaminophen, all the compounds were subjected to ion suppression. Strong MS signal suppression effects were observed for most of the compounds.

3.3. Application of the method

The developed method was applied for determination of pharmaceuticals in sediment from middle course of river Ebro and in the sewage sludge from WWTPs Tudela, Pamplona and Terrassa. Target pharmaceuticals were not detected in sediment samples. Of

Table 3
Average concentrations of target compounds detected in sludge-samples from WWTP Tudela (Sludge I), Pamplona (Sludge II) and Terrassa (Sludge III).

Compound	Concentration (ng g^{-1}, d.w.)		
	Sludge I	Sludge II	Sludge III
Ketoprofen	BLD[a]	18.9 ± 1.42	21.1 ± 1.3
Naproxen	BLD	5.9 ± 0.7	4.27 ± 0.1
Ibuprofen	43.2 ± 5.5	117 ± 5.9	91.5 ± 3.8
Indomethacine	BLD	2.5 ± 0.3	2.9 ± 0.1
Diclofenac	27.5 ± 0.5	69.1 ± 7.6	74.9 ± 4.1
Mefenamic acid	26.2 ± 3.2	19.3 ± 2.3	14.3 ± 2.2
Acetaminophen	103 ± 9.0	77.8 ± 10.3	42.1 ± 6.4
Phenazone	3.2 ± 0.1	16.0 ± 1.4	BLD
Bezafibrate	2.9 ± 0.2	7.2 ± 0.6	18.7 ± 0.7
Fenofibrate	3.3 ± 0.3	BLD	17.1 ± 3.7
Gemfibrozil	14.3 ± 1.9	33.9 ± 4.61	31.8 ± 3.8
Atorvastatin	42.1 ± 2.8	65.0 ± 3.21	21.4 ± 2.7
Diazepam	3.20 ± 0.1	8.5 ± 0.2	4.6 ± 0.4
Carbamazepine	10.1 ± 0.1	11.0 ± 1.60	12.7 ± 1.4
Ranitidine	0.2 ± 0.02	2.3 ± 0.3	BLD
Famotidine	2.14 ± 0.3	12.4 ± 1.3	14.7 ± 2.3
Cimetidine	0.5 ± 0.06	2.5 ± 0.2	6.1 ± 0.5
Roxithromycin	BLQ	BLQ[b]	BLQ
Clarithromycin	BLD	47.0 ± 2.9	27.0 ± 2.1
Josamycin	BLD	4.8 ± 0.5	47.8 ± 4.8
Sulfamethazine	BLD	BLQ	1.1 ± 0.4
Trimethoprim	BLD	9.2 ± 0.8	11.2 ± 1.2
Chloramphenicol	BLD	1.2 ± 0.3	BLD
Metronidazole	BLD	10.6 ± 0.6	BLD
Atenolol	10.8 ± 1.1	8.8 ± 0.7	3.96 ± 0.4
Sotalol	1.7 ± 0.2	BLD	BLD
Nadolol	0.8 ± 0.1	3.3 ± 0.5	2.70 ± 0.3
Pindolol	13.6 ± 1.1	23.1 ± 1.9	BLD
Clenbuterol	BLD	40.2 ± 2.5	BLD
Salbutamol	BLD	3.8 ± 0.6	BLD
Nifuroxazide	BLD	0.5 ± 0.05	BLD
Hydrochlorothiazide	29.0 ± 3.6	126 ± 8.5	30.5 ± 1.9
Furosemide	10.1 ± 0.3	16.8 ± 1.4	11.7 ± 0.4
Glibenclamide	7.7 ± 0.6	15.8 ± 1.7	42.4 ± 1.5

BLD and BLQ determined for each matrix individually.
[a] BLD—below limits of detection.
[b] BLQ—below limits of quantification.

43 analysed compounds, 34 were detected in sewage sludge samples from WWTPs. The average concentrations ($n = 5$) determined in those samples are summarized in Table 3. The recoveries and limits of detection and quantification were determined for each kind of sludge individually, prior to quantification. The concentrations below the limits of detection and quantification are not presented. In general, the pharmaceuticals were identified in concentrations of 0.1–120 ng/g. Ibuprofen, acetaminophen, diclofenac, atorvastatin and hydrochlorthiazide were detected in the highest concentrations in samples from all 3 WWTP. These compounds were present in average concentrations from 43.2 to 117 ng g^{-1}, 42.1 to 103 ng g^{-1}, 27.5 to 74.9 ng g^{-1}, 21.4 to 65 ng g^{-1} and 29.0 to 126 ng g^{-1}, respectively. Carbamazepine, mefenamic acid, gemfibrozil, furosemide and glibenclamide were frequently detected as well. The presence of these compounds was reported in recent works related to pharmaceuticals in sludge in similar concentrations [7,13,14]. WWTP Pamplona serves more inhabitants and has higher capacity of treatment than the other two, so the samples from this plant are more contaminated by analysed pharmaceutical residues.

4. Conclusion

The study involved development and validation of a method for simultaneous analysis of 43 pharmaceutical compounds in sludge and sediment. Differences in physicochemical properties of the compounds required neutral experimental conditions. The pharmaceuticals were isolated from solid samples using PLE followed by SPE clean-up step, and submitted to the analysis in LC–QqLIT-MS.

The influence of extraction parameters (i.e. solvent composition, temperature, and number and time of extraction cycles) on the extraction and overall recoveries were evaluated. Strong effects of signal suppression and extreme values of recoveries ($\gg$100%) were noticed as result of matrix interferences. The chosen isotopically labelled compounds compensated for the matrix effects well enough; therefore the internal standard approach was used for quantification. Linearity of the method was satisfactory ($r^2 < 0.99$) in the defined concentration range (0.5–100 ng g^{-1}). The instrument exhibited very good sensitivity with the MDLs and MQLs lower than 1 ng g^{-1} for most of the compounds. The instrumental precision was acceptable with RSDs < 15% for intra- and inter-day analysis and <1% for peak position shifts.

The applicability of the method for routine multi-residue analysis was demonstrated through the analysis of sludge samples from three WWTPs. The recoveries were higher of 50% for most of the compounds and the variance of values was observed depending on the origin of the sludge samples. The presence of 35 pharmaceuticals was confirmed, wherein ibuprofen, acetaminophen, diclofenac, atorvastatin and hydrochlorothiazide were detected in the highest concentrations.

Acknowledgements

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2.1.4. Data analysis – Quantification/confirmation criteria

Acquired selected reaction monitoring (SRM) data were analyzed by Analyst 4.2 (Applied Biosystems – MDS SCIEX, USA). Automatic peak integrations were reviewed manually. Although MRM detection offers good selectivity, false positive findings can occur (249). In order to avoid false positives, we applied following criteria as introduced by Commission Decision 2002/657/EC, implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results:

1) The retention time of a target analyte in a sample matrix should be within 2.5 % of retention times of the corresponding analytical standard and isotope-labeled compound (if available) in the sample matrix (Figure 2.3).
2) Two SRM transitions of a target analyte were used for quantification and confirmation purposes, i.e. the most abundant transition was used for quantitative analysis ("quantifier" ion), and the second one was used for confirmation purposes (qualifier ion). To positively confirm the presence of the analyte in samples, the ratio between the abundances (e.g. area counts) of these two transitions of the analyte in sample matrix should be equal to the ratio of the two transitions of the corresponding analytical standard in calibration standard solutions or spiked samples (Figure 2.2).

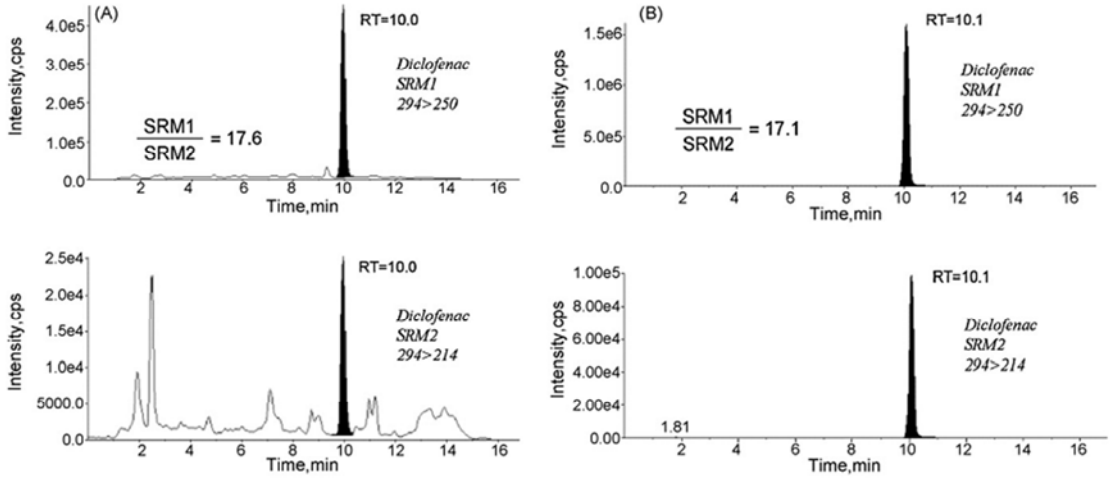

Figure 2.2. Chromatograms of two SRM transitions of diclofenac in sludge matrix (A) and in standard solution (B) following the identification and confirmation criteria for the analysis of contaminants defined by Commission Decision 2002/657/EC

Depending of the intensity ratio of two transitions, maximum permitted tolerance is indicated in the EC Decision (Table 2.2):

Table 2.2. Maximum permitted tolerance for relative ion intensities using LC-MS^n

Relative intensity (% of base peak)	LC-MS^n (relative)
> 50%	± 20 %
> 20 % to 50 %	± 25 %
> 10 % to 20 %	± 30 %
≤ 10 %	± 50 %

By analyzing two MS/MS transitions, 4 Identification points (IPs, 2002/657/EC) were earned for the employed HPLC-MS/MS analysis. Besides, an Information Dependent Acquisition (IDA) experiment was used as an additional confirmation tool. Here, recorded spectra were compared with library data, and it was applied for the compounds that showed poor fragmentation. With all the information, we ensured that the requirements set by the Decision 2002/657/EC were fulfilled (i.e. minimum 3 IPs).

2.1.5. Matrix effect and recovery analysis

With the principal aim being the development of multi-residue method for the extraction of numerous pharmaceuticals, characterized by different physico-chemical properties, we had to accept that, under the chosen extraction conditions, the performance of the method cannot be optimal for each single compound but rather acceptable for all compounds. Out of thirty combinations of solvents and temperatures, we decided to proceed with the extraction using MeOH/water, 1/2 (v/v) at 100 ºC as it allowed for better recoveries of most of the analyzed compounds. Nevertheless, the applied temperature was high enough to extract also matrix organic components, which was perceptible to the eye as a darker ASE extract, and by MS as slightly lower method selectivity. The same effect was observed for 3-cycles extraction. Because of this, SPE was a compulsory purification step after the ASE extraction. Applying the developed procedure for the extraction of the selected pharmaceuticals from sludge, and using the MS system, i.e. QqLIT-MS, that offers excellent sensitivity, very low LODs and LOQs were reached, i.e. <1 ng/g, for most of the compounds, in the sludge and sediment matrixes.

The developed analytical procedure was applied for the analysis of various sludge samples from different WWTPs or lab-scale treatments over the PhD period. All sludge samples had always different appearance, consistency, and quality, which influenced the performance parameters of the applied extraction/quantification method, such as recovery of the analytes, matrix effect and quantification limits. It is now well known that sample matrix affects the performance of MS detector, causing suppression or enhancement of the target analyte response. We calculated the matrix effect as the ratio of the peak area of the chemical standard added to the calibration

solution and the peak area of the standard added to each different sample matrix. Figure 2.3 shows an example of ion suppression (matrix effect) for carbamazepine (CBZ) in three types of sludge, i.e. thickened, digested, and treated sludge from one activated sludge WWTP.

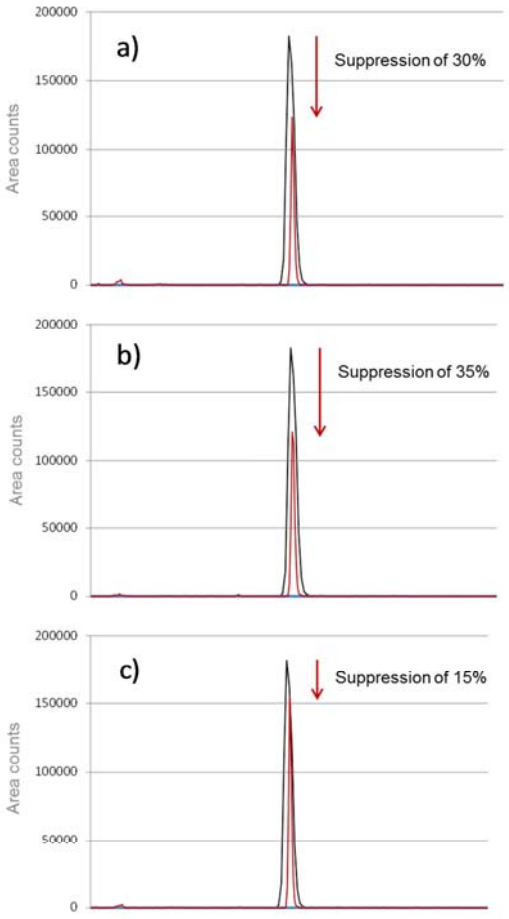

Figure 2.3. Signal response comparisons for CBZ added to (a) thickened, (b) digested and (c) treated sludge. The samples were spiked with CBZ at 50 ng/g. Black line represents the signal of the analyst in MeOH/water, 1/3 (v/v), while red line shows the signal in different matrices

To compensate for matrix effects, we used the internal standard calibration approach for quantification supposing that the ion suppression is identical (or almost identical) for both the analyte and isotope-labeled internal standard. Nevertheless, if matrix effect exists and reduces the signal of the analyte or internal standard to the point where S/N is compromised to the point where accuracy and precision are negatively affected method, the ion suppression should be evaluated (250).

The relative change in peak areas of the added pharmaceutical standards reflected only ion suppression or enhancement due to sample matrix since the isotope-labeled compounds were added just before the MS/MS analysis. Therefore, the losses due to sample preparation were calculated separately from recovery analysis with spiked samples (n=3 as minimum) carried out for each different sludge matrix before the corresponding samples were analyzed. Quantitative analysis was performed by internal standard approach, and two isotope-labeled compounds, i.e. mecoprop-d_3 for NI mode, and sulfathiazole-d_3 for PI mode, were added to all the samples before the extraction as control standards. Table 2.3 shows recovery values, LOQs and concentrations of carbamazepine analyzed in the same samples (251).

Table 2.3. Analysis of carbamazepine in different types of sludge

Type of sludge	Recovery (RSD%, n=3)	LOQ (RSD%, n=3)	Concentration (average) (ng/g)	Concentration range (Min-Max) (ng/g)
Thickened sludge	60 (9%)	0.5 (3%)	10.0	8.0-12.5
Digested sludge	52 (13%)	0.8 (13 %)	10.0	8.0-11.0
Treated sludge	83 (11%)	0.2 (9 %)	9.0	7.5-11.0

2.2. Occurrence, removal and fate of pharmaceuticals in wastewater and sludge from activated sludge WWTPs

The results obtained in three studies aimed at assessment of the occurrence, removal and fate of the selected pharmaceuticals during full-scale biological wastewater treatment are presented and discussed in detailed in the scientific publications titled:

Nº2: Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment (Water Research (45) 3: 1165–1176)

Nº3: Tracing pharmaceuticals in a municipal plant for integrated wastewater and organic solid waste treatment (Science of The Total Environment (433) 352–361)

Nº4: New indexes for compound prioritization and complexity quantification on environmental monitoring inventories (Environmental Science and Pollution Research (2012) 19 (4): 958-970)

2.2.1. Sampling sites

The first study (i.e. Nº2) was carried out in the scope of the project SOSTAQUA - Technological Developments through the Auto-sustainable Urban Water Cycle (Aguas de Barcelona/ CENIT). Samples were obtained from three full-scale WWTPs in Catalonia (Spain). Composite influent and effluent wastewaters samples, as well as grab dewatered sludge samples, were collected in eight sampling campaigns between July 2007 and March 2009, in intervals of 2 to 3 months. Even though the selected WWTPs do not cover all the available types of sewage treatment technologies, which is practically impossible, they still can be considered as representative for some typical WWTP configurations existing in the region, employed in order to fulfill the requirements of Council Directive 91/271/EC concerning urban wastewater treatment. The Directive defines the amount of macro-contaminants that can be discharged from urban waste water treatment plants (BOD, COD, total suspended solids, total phosphorus and total nitrogen). They differ in the relative contribution of industrial vs. municipal sewage water: while WWTP1 and WWTP2 receive predominantly municipal wastewater, WWTP3 have a contribution of about 75 % of industrial wastewater. The WWTPs essentially consist of a primary sedimentation followed by activated sludge (secondary) treatment step. WWTP1 additionally treats the secondary effluent in a two-layer sand microfiltration/disinfection process (tertiary treatment). In WWTP1, gravity thickened primary sludge and flotation thickened waste activated sludge are mixed and dewatered via centrifuge, and sent for composting. Sludge generated from

primary and secondary clarifiers is thickened and treated by mesophilic anaerobic digestion, and dewatered via centrifuge (WWTP 2) or on belt filter press (WWTP 3).

In the subsequent study (Nº3), carried out in collaboration with the University of Verona, we further examined the occurrence and behavior of the selected pharmaceuticals in a small WWTP in Veneto region (Italy) that treats municipal wastewater. Wastewater is treated in an intermittent aeration activated sludge process for biological nutrient removal (BNR) process. Infiltration of groundwater in the sewer system lead to a dilution of influent wastewater (low-loaded wastewater) and for that reason primary sedimentation was omitted in order to preserve COD and improve BNR process performances. Wasted activated sludge (WAS) is mixed with the Organic Fraction of Municipal Solid Waste (OFMSW), thickened and treated in a single-stage mesophilic anaerobic digestion (AcoD), followed by mechanical dewatering. Supernatant from the AcoD and from belt press is treated by a short-cut nitrification-denitrification process in a demonstration sequencing batch reactor (SBR) and then sent back to wastewater headwork. Samples were collected over three consecutive days during two campaigns as: volume proportional 24h-composite influent and effluent wastewater samples, and combined (five) grab samples daily of each sludge type, i.e. thickened, digested and dewatered sludge samples. All the samples were prepared and analyzed according to the previously explained methodologies brought by (199, 226).

2.2.2. Results and discussion

Scientific publications:

Nº2: Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment, Water Research (2011) 45 (3): 1165–1176

Nº3: Tracing pharmaceuticals in a municipal plant for integrated wastewater and organic solid waste treatment, Science of the Total Environment (2012) 433: 352–361

Nº4: New indexes for compound prioritization and complexity quantification on environmental monitoring inventories, Environmental Science and Pollution Research (2012) 19 (4): 958-970

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Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment

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ABSTRACT

During 8 sampling campaigns carried out over a period of two years, 72 samples, including influent and effluent wastewater, and sludge samples from three conventional wastewater treatment plants (WWTPs), were analyzed to assess the occurrence and fate of 43 pharmaceutical compounds. The selected pharmaceuticals belong to different therapeutic classes, i.e. non-steroidal anti-inflammatory drugs, lipid modifying agents (fibrates and statins), psychiatric drugs (benzodiazepine derivative drugs and antiepileptics), histamine H2-receptor antagonists, antibacterials for systemic use, beta blocking agents, beta-agonists, diuretics, angiotensin converting enzyme (ACE) inhibitors and anti-diabetics. The obtained results showed the presence of 32 target compounds in wastewater influent and 29 in effluent, in concentrations ranging from low ng/L to a few μg/L (e.g. NSAIDs). The analysis of sludge samples showed that 21 pharmaceuticals accumulated in sewage sludge from all three WWTPs in concentrations up to 100 ng/g. This indicates that even good removal rates obtained in aqueous phase (i.e. comparison of influent and effluent wastewater concentrations) do not imply degradation to the same extent. For this reason, the overall removal was estimated as a sum of all the losses of a parent compound produces by different mechanisms of chemical and physical transformation, biodegradation and sorption to solid matter. The target compounds showed very different removal rates and no logical pattern in behaviour even if they belong to the same therapeutic groups. What is clear is that the elimination of most of the substances is incomplete and improvements of the wastewater treatment and subsequent treatments of the produced sludge are required to prevent the introduction of these micro-pollutants in the environment.

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1. Introduction

Pharmaceuticals are a large and diverse group of compounds designed to prevent, cure and treat disease and improve health. They have long been used in significant quantities throughout the world. Their usage and consumption are increasing consistently due to the discoveries of new drugs, the expanding population and the inverting age structure in the general population, as well as due to expiration of patents with resulting availability of less expensive generics (Daughton, 2003). After intake, these pharmaceutically active compounds undergo metabolic processes in organism. Significant fractions of the parent compound are excreted in unmetabolized form or as metabolites (active or inactive) into raw sewage and wastewater treatment systems. Sewage treatment plant effluents are discharged to water bodies or reused for irrigation, and biosolids produced are reused in agriculture as soil amendment or disposed to landfill. Thus body metabolization and excretion followed by wastewater treatment is considered to be the primary pathway of pharmaceuticals to the environment. Disposal of drug leftovers to sewage and trash is another source of entry, but its relative significance is unknown with respect to the overall levels of pharmaceuticals in the environment (Ruhoya and Daughton, 2008).

Continual improvements in analytical equipment and methodologies enable the determination of pharmaceuticals at lower and lower concentration levels in different environmental matrices. Pharmaceuticals and their metabolites in surface water and aquatic sediment were subject of numerous studies concerning pharmaceuticals in the environment (Bartelt-Hunt et al., 2009; Nilsen, 2007); Vazquez-Roig et al., 2010. Several studies investigated the occurrence and distribution of pharmaceuticals in soil irrigated with reclaimed water (Gielen et al., 2009; Kinney, 2006); Ternes et al., 2007 and soil that received biosolids from urban sewage treatment plants (Carbonell et al., 2009; Lapen et al., 2008). These studies indicated that the applied wastewater treatments are not efficient enough to remove these micro-pollutants from wastewater and sludge, and as a result they find their way into the environment. Once entered the environment, pharmaceutically active compounds can produce subtle effects on aquatic and terrestrial organisms, especially on the former since they are exposed to long-term continuous influx of wastewater effluents. Several studies investigated and reported on it (Cleuvers, 2004; Nentwig et al., 2004; Schnell et al., 2009).

Therefore, the occurrence of pharmaceutical compounds and the extent to which they can be eliminated during wastewater treatment have become active subject matter of actual research. Conventional systems that use an activated sludge process are still widely employed for wastewater treatment, mostly because they produce effluents that meet required quality standards (suitable for disposal or recycling purposes), at reasonable operating and maintenance costs. However, this type of treatment has limited capability of removing pharmaceuticals from wastewater (Kasprzyk-Hordern et al., 2009); Wick et al., 2009. Most of the studies on the fate of pharmaceuticals in WWTPs focused only on the aqueous phase, and concentrations of the compounds in sludge were rarely determined mainly due to the demanding efforts required in the analysis in this difficult matrix. Out of 117 publications studied by Miege et al (Miège et al., 2009), only 15 reported the concentrations of pharmaceuticals in sludge and 1 in suspended solid, and none of these papers reported the removal obtained taking into account both aqueous and solid phases of WWTPs. Still, the screening of sewage sludge showed that these micro-pollutants are very present in this medium (Lillenberg et al., 2009; Lindberg et al., 2010; McClellan and Halden, 2010; Radjenovic et al., 2009a).

In this study we aimed to determine the contamination of wastewater and sludge with 43 pharmaceutical compounds in order to obtain more information on their fate during conventional wastewater treatment. The selected pharmaceuticals belong to different therapeutic groups (i.e. non-steroidal anti-inflammatory drugs (NSAIDs), lipid modifying agents (fibrates and statins), psychiatric drugs (benzodiazepine derivative drugs and antiepileptics), histamine H2-receptor antagonists, antibacterials for systemic use, beta blocking agents, beta-agonists, diuretics, angiotensin converting enzyme (ACE) inhibitors and anti-diabetics). The samples were provided from three conventional full-scale activated sludge sewage treatment plants with anaerobic digestion of sludge, from the region of Catalonia (Spain). The preparation and analysis of the samples were performed using high performance liquid chromatography coupled to a hybrid triple quadrupole – linear ion trap mass spectrometer (HPLC-QLIT- MS/MS) according to the previously developed multi-residual methodologies for analysis of pharmaceuticals in wastewater and sludge samples (Gros et al., 2009; Jelic et al., 2009).

2. Experimental part

2.1. Chemicals

All the pharmaceutical standards for target compounds were of high purity grade (>90%). ibuprofen, naproxen, ketoprofen, diclofenac and gemfibrozil were supplied by Jescuder (Rubí, Spain). acetaminophen, indomethacin, mefenamic acid, phenazone, bezifibrate, mevastatin, fenofibrate, pravastatin (as sodium salt), carbamazepine, famotidine, ranitidine (as hydrochloride), cimetidine (as hydrochloride), erythromycin (as hydrate), azithromycin (as dehydrate), roxitromycin, clarithromycin, josamycin, tylosin a, sulfamethazine, trimethoprim, chloramphenicol, atenolol, sotalol, metoprolol (as tartrate), timolol, pindolol, nadolol, salbutamol, clenbuterol (as hydrochloride), enalapril (as maleate), glibenclamide, furosemide, hydrochlorothiazide and metronidazole were purchased from Sigma–Aldrich (Steinheim, Germany). Standard atorvastatin (as calcium salt) was provided by LGC Promochem (London, UK), while diazepam, lorazepam and butalbital were from Cerilliant (Texas, USA).

The isotopically labelled compounds, used as internal standards, were sulfamethazine-d_4, famotidine-$^{13}C_3$, rac-timolol-d_5 maleate, clarithromycin-n-methyl-d_3, atorvastatin-d_5 sodium salt, fenofibrate-d_6, metoprolol-d_7, metronidazole hydroxyl-d_2, pravastatin-d_3, ketoprofen-^{13}C,d_3, indomethazine-d_4, rac-naproxen-d_3, mefenamic acid-d_3, gemfibrozil-d_6, bezafibrate-d_4 and furosemide-d_5 from Toronto Research Chemicals;

diazepam-d_5 and phenobarbital-d_3 from Cerilliant (Texas, USA); atenolol-d_7, carbamazepine-d_{10}, ibuprofen-d_3, clotrimazole-d_5, enalapril–d_5, hydrochlorothiazide-d_2, glyburide-d_3, albuterol-d_3, cimetidine-d_3, antipyrine-d_3, aceta-minophen-d_4, diclofenac-d_4, clofibric-d_4 acid, hydrochlorothiazide-3,3-d_2 from CDN Isotopes (Quebec, Canada); sotalol hydrochloride d_6 from Dr. Ehrenstorfer (Augsburg, Germany) and erythromycin-^{13}C,d_3 (N-methyl-^{13}C,d_3) from Isotec (Ohio, USA).

The solvents, HPLC grade methanol, acetonitrile, water (Lichrosolv) and formic acid 98% were provided by Merck (Darmstadt, Germany). Nitrogen used for drying from Air Liquide (Spain) was of 99.995% purity.

The cartridges used for solid phase extraction were Oasis® HLB (200 mg, 6 mL) from Waters Corporation (Milford, MA, USA). The syringe filters of 0.45 μm pore size were purchased from Pall Corp (USA).

The individual standard solutions as well as isotopically labelled internal standard solutions were prepared on a weight basis in methanol. Furosemide and butalbital were obtained as solutions in acetonitrile, while lorazepam and diazepam were dissolved in methanol, at a concentration of 1 mg/mL. The solutions were stored at −20 °C. Fresh stock solutions of antibiotics were prepared monthly due to their limited stability while stock solutions for the rest of substances was renewed every three months. A mixture of all pharmaceuticals was prepared by appropriate dilution of individual stock solutions in methanol-water (25:75, v/v) and it was renewed before each analytical run. A separate mixture of isotopically labelled internal standards, used for internal standard quantification, was prepared in methanol and further diluted in methanol-water (25:75, v/v) mixture.

2.2. Sample collection

Samples (i.e. influent and effluent wastewater, and sewage sludge) were obtained from three full-scale wastewater treatment plants (WWTPs) in the region of Catalonia (Spain). Table 1 (A and B) summarizes some characteristics of the three investigated WWTPs (source: Annual report of Catalan Water Agency for 2008). WWTP1 and WWTP2 treat predominantly municipal wastewater, while the WWTP3 influent has an important industrial contribution. The wastewater treatment process in WWTP1 consists in pre-treatment, primary settling, and biological treatment (anoxic/aerobic) followed by secondary settling. The secondary effluent then passes through coagulation/flocculation and lamella settling, and after microfiltration and chlorination is discharged as tertiary effluent. The gravity thickened (primary sludge) and flotation thickened waste activated sludge are mixed and dewatered via centrifuge, and sent for composting. The WWTP1 is designed for 210000 equivalent inhabitants (eq.inh.) and to treat up to 47500 m^3/day of wastewater. It is situated in the tourist coastal area where the amount and the quality of water entering the plant are significantly affected by the seasonal population growth. The wastewater flow in WWTP1 changes from 15000 m^3/day, during the winter months, to 32000 m^3/day during the summer months. During 2008, it treated (on average) 25000 m^3/day for 74000 eq.inh. The WWTP2, which usually works with 80% of designed treatment capacity, treated around 26000 m^3/day in 2008, serving a population equivalent of around 170000. The treatment includes pre-treatment and primary clarifier, followed by activated sludge treatment and secondary clarifier. Sludge generated from primary and secondary clarifiers is thickened and blended and fed to anaerobic digester system, and dewatered via centrifuge. WWTP3 employs primary sedimentation, followed by a secondary biological treatment for nitrogen and phosphorus removal. The sludge mixture proceeding from the primary and secondary settlers is thickened by gravity, treated by anaerobic digestion and dewatered on belt filter press. The WWTP3 treated an average of 21000 m^3/day of urban and industrial wastewater in 2008, which is about 80% of the total treatment capacity of the plant. Schematic diagrams of the treatment processes are shown in Fig. 1.

Wastewater and sludge samples were collected in eight sampling campaigns between July 2007 and March 2009, in campaign intervals of 3 months covering all the seasons of the year. The sampling was carried out at dry weather flow, according to the established sampling protocols and locations defined by Aguas de Barcelona, and following the occupational health and safety regulations. Composite influent and effluent wastewater samples (24-h) were collected using an ISCO automated sampler (GLS Compact Comosite Samplers) with an integrated 5 L amber glass bottle and cooling system that was providing temperatures below 4 °C. The sampling program was set to collect 50 mL of wastewater every 30 min during 24 h. The effluent samples were taken according to the retention times estimated with the current data for each WWTPs and sampling campaign. The influent wastewater samples were collected in the pre-treatment building of WWTP1, in the influent homogenization ponds in WWTP2, while in WWTP3, three different intakes in the pumping well of the plant were taken proportionally to the flow and volume and mixed. The effluent wastewater samples were taken after the secondary treatment at WWTP2 and WWTP3, and after the tertiary one in WWTP1. The samples were kept at 4 °C until extraction (within 48 h). Prior to extraction, the water was vacuum filtered through 1 μm glass fiber filters, followed by 0.45 μm nylon membrane filters (Teknokroma, Barcelona, Spain).

The analyzed samples of sludge were collected at the final phase of the process, i.e. treated sewage sludge. Ten grab samples of equal volume were taken from the exit belt to make up the composite sample of sludge. Sludge samples were mixed and chilled to 4 °C for transportation and freeze-dried (LioAlfa 6, Telstar) at −50 °C and under 0.044 bar vacuum and stored at −20 °C until the analysis.

2.3. Sample preparation

Procedures for preparation of water and sludge samples for instrumental analysis were described in detail previously (Gros et al., 2009; Jelic et al., 2009).

In brief, in the filtered-aliquots of wastewater (100 mL for influent and 200 mL for effluent) Na_2EDTA was added to a concentration of 0.1vol%. Then the target compounds were separated by solid phase extraction (Oasis HLB cartridges, 6 cc, 200 mg; Waters Corp., Milford, MA) using a Baker vacuum system (J.T. Baker, Deventer, The Netherlands), and concentrated via elution with pure methanol. The 8 mL eluents were evaporated under a stream of nitrogen and reconstituted in 1 mL of methanol-water mixture (25:75). Prior to instrumental

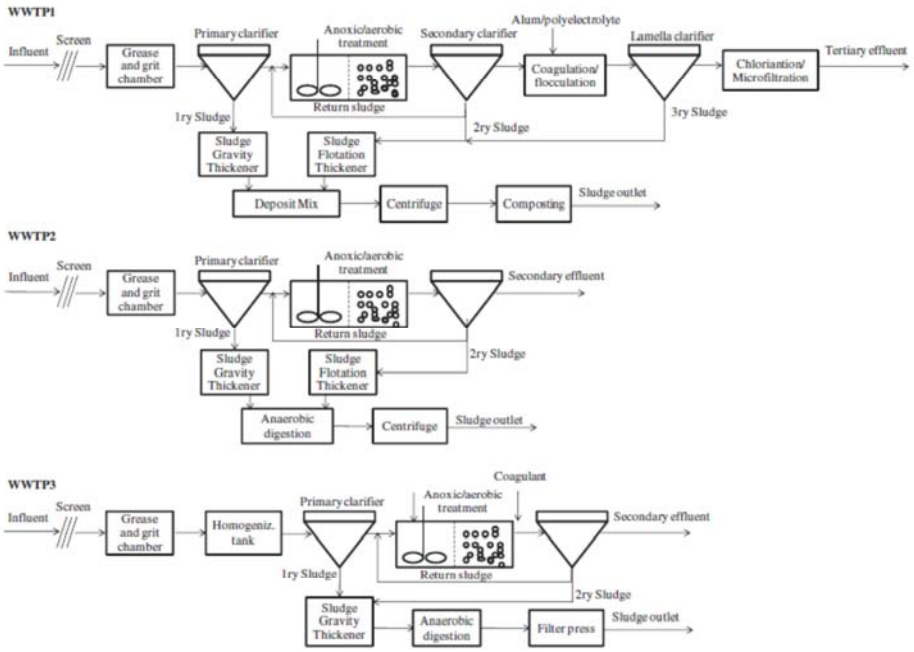

Fig. 1 – Schematic diagram of the studied wastewater treatment plants.

analysis, these samples were fortified by a mixture of internal standard to a final concentration of 20 ng/mL.

Sludge samples were extracted using an accelerated solvent extraction (ASE) (Dionex ASE 200, Dionex; Sunnyvale, CA). The extractions were carried out using a methanol-water mixture (1:2) as extraction solvent, at 1500 psi and 100 °C in 3 static cycles, each lasting 5 min. Finally, the cell was flushed with 100% cell volume of fresh solvent. Concentrated extracts were dissolved in water in order to reduce the content of methanol (<5 Vol%) and processed further as water samples. Instrumental analysis of all samples was done by HPLC-QLIT-MS/MS.

2.4. *Instrumental analysis*

The analytical method used in this study was already developed by M. Gros et al (Gros et al., 2009). Samples were analyzed using high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS/MS). LC analysis was performed using Symbiosis™ Pico (SP104.002, Spark, Holland), equipped with an autosampler and connected in series with a 4000 QTRAP Hybrid Triple Quadrupole - Linear Ion Trap mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA, USA). Chromatographic separation was achieved with a Purospher Star RP-18 endcapped column (125 mm × 2.0 mm, particle size 5 μm) preceded by a C_{18} guard column (4 mm × 4 mm, particle size 5 μm), both supplied by Merck (Darmstadt, Germany).

The mobile phases for the analysis in negative ionization (NI) mode were a mixture of acetonitrile-methanol (1:1, v/v) (i.e. eluent A), and HPLC grade water (i.e. eluent B). The analysis in positive ionization (PI) mode was performed using acetonitrile as eluent A and HPLC grade water with 0.1% formic acid as eluent B. The target compounds were scanned in MRM, monitoring two transitions between the precursor ion and the most abundant fragment ions for each compound. Further information on the methodology and its performances can be found elsewhere (Gros et al., 2009; Jelic et al., 2009).

2.5. *Removal rate calculation*

In this study we employed a mass balance approach in order to asses quantitatively the removal of the selected pharmaceuticals during wastewater treatment. Even when dealing with such a complex system, we can assume that the WWTP behaves as a black-box with only one entrance (i.e. influent water) and two outlets (i.e. effluent water and treated sludge) and operates at steady state over the studied period of two years. Then, from the measured concentrations and the operation parameters (i.e. flow rates of influent, $\dot{V}_{influent}$, and $\dot{V}_{effluent}$, and sludge production, $\dot{P}_{sludge}$) could be written as follows:

$$\dot{R}_{Overall} = \dot{m}_{in} - \dot{m}_{out} \quad (1)$$

$$\dot{m}_{in} = \dot{m}_{influent} \quad (2)$$

$$\dot{m}_{out} = \dot{m}_{effluent} + \dot{m}_{sludge} \quad (3)$$

$$\dot{V}_{influent} = \dot{V}_{effluent} = \dot{V}_l \quad (4)$$

$$\dot{R}_{Overall} = c_{influent} \times \dot{V}_l - \left(c_{effluent} \times \dot{V}_l + c_{sludge} \times \dot{P}_{sludge}\right) \quad (5)$$

where $\dot{m}_{in}$, $\dot{m}_{out}$, $\dot{m}_{influent}$, $\dot{m}_{effluent}$ and $\dot{m}_{sludge}$ are the mass flow rate (in g/day) of inlet, outlet, influent liquid, effluent liquid and sludge, respectively. $\dot{R}_{Overall}$ (g/day) is the mass load lost

per unit of time due to the sum of all processes that can occur during wastewater treatment. Mass flow rates of pharmaceutical compounds in influent and effluent streams were calculated by multiplying the measured concentrations in a given stream by the appropriate flow rate of that stream. Thus, the concentration of each pharmaceutical in the daily influent and effluent samples ($c_{influent}$ or $c_{effluent}$, [g/m^3]) was multiplied by the flow rate for that day (i.e.$\dot{V}_l$, [m^3/day]) to give the mass of the pharmaceutical entering or leaving the plant that day (g/day) (i.e. daily mass load). Similarly, the concentration of pharmaceuticals in the treated sludge ($\dot{c}_{sludge}$, [ng/g d.w.]) was multiplied by the production rate of sludge (tons/day) to determine the mass of pharmaceuticals removed with the sludge (g/day). From these data, both removal from aqueous-phase, $R_{Aqueous\ phase}$(%), and overall removal (i.e. mass loss), $R_{Overall}$(%), of the target compounds were calculated according to the eqs. (3) and (4), respectively:

$$R_{Aqueous\ phase}(\%) = 100 \times c_{influent} \times \frac{\dot{V}_l - c_{effluent} \times \dot{V}_l}{c_{influent} \times \dot{V}_l} \quad (6)$$

$$R_{Overall}(\%) = 100 \times \frac{\dot{R}_{Overall}}{c_{influent} \times \dot{V}_l} \quad (7)$$

Taking into account that the sampling at the influent and the effluent was performed in accordance with retention time and flow rate of the plants, the removal rates were calculated from pair-wise data and then averaged. Fig. 4 shows average percentages of the detected pharmaceuticals that were discharged with effluent, sorbed to sludge, and removed during treatment (i.e. overall removal rate).

3. Results and discussion

3.1. *Occurrence of pharmaceuticals in wastewater and sludge*

Table 2 shows the frequencies of detection and the limits of quantification of the pharmaceutical compounds detected in wastewater and sludge from the studied WWTPs. Out of 43 analyzed pharmaceutical compounds, 32 were detected in influent, 29 in effluent and 21 in sludge samples. The analysis of wastewater and sludge showed huge variation in concentration levels from campaign to campaign of a given plant. This can be due to changes of the composition of influent waters in different seasons, weather conditions and operational conditions of the plant, as well as due to the amount of the drug that is used. But, the sampling protocol itself has great influence on concentration values obtained (Ort et al., 2010). The fact is that the substances arrive in a small number of wastewater packets to the influent of WWTP, in unpredictable amounts and time intervals, thus the influent loads, especially, are easily systematically underestimated. In order to minimize the effect of sampling, triplicate of composite samples from 8 sampling campaigns were analyzed. The results are shown in Fig. 2 as box plots displaying 25th, median and 75th percentiles as boxes, and minimum and maximum concentration values, as line and triangle, respectively, so the overall uncertainty (including variability and reducible uncertainty) could be understood better. The uncertainty due to analysis is not shown in the Fig. 2, and it was less than 5%, 6% and 11% for wastewater influent, effluent and sludge, respectively (Gros et al., 2009; Jelic et al., 2009).

According to the daily loads and population served by each plant, the amount of the selected pharmaceuticals disposed in these plants is estimated to be 5.6, 2.0 and 0.4 g/day/1000 equivalent inhabitants for WWTP1, WWTP2 and WWTP3, respectively (Fig. 3). The highest levels at the influent of all three WWTPs were observed for NSAIDs that were expected due to their high consumption. In addition, topical application of the NSAIDs results in greater discharge of these compounds in unmodified forms. This result is in fairly good agreement with previously reported studies (Gracia-Lor et al., 2010; Miège et al., 2009). At the influent of the plants, this group accounts for ca. 65% of all the therapeutic groups analyzed, as can be seen in the Fig. 3. Naproxen, ketoprofen and diclofenac were detected in all the samples in average concentration ranges 4.2–7.2 μg/L, 1.1–2.3 μg/L and 0.4–1.5 μg/L, respectively. Ibuprofen and acetaminophen were not included in the discussion because they yielded to high concentrations which can be due to the strong matrix effect and/or to interactions that may produce false identification and thus incorrect concentration values. Lower but still significant levels of lipid modifying agents (including fibrates and statins) (7–12%), diuretics (8–10%), and beta-blockers (5–9%) were detected entering these WWTPs. Furosemide, bezafibrate, atenolol and carbamazepine were quantified in all influent samples from the three WWTPs in average concentrations ranging from 0.4 to 1.4 μg/L.

The amount found in effluent or sludge depended on the removal efficiency of plant and/or the physicochemical properties of the compounds. As the influent concentrations can give us information about the consumption of pharmaceuticals, the effluent and the sludge concentrations are important from the environmental point of view, since the pharmaceuticals find their way to the environment through discharges of treated waters to rivers, or disposal of sludge to agricultural and forest land. In the effluent waters, NSAIDs were present in the highest percentage (35–44%), followed by the lipid modifying agents (8–29%) and psychiatric drugs (both antiepileptic and benzodiazepine derivative drugs) (17–30%) (Fig. 3). The highest concentrations in the effluents of all the WWTPs were found for naproxen, diclofenac and carbamazepine, and they ranged from 0.4 to 1 μg/L depending on the compound and the removal efficiency of the plant. In the treated effluent of WWTP2, ketoprofen, bezafibrate, atenolol and furosemide were detected in much higher average concentrations (0.7, 0.4, 0.4 and 0.9 μg/L, respectively) than in the other two plants. Analysis of sludge samples showed the presence of 21 out of 43 analyzed pharmaceuticals, covering a wide range of physicochemical properties, as in the case of wastewater effluent. Diuretics accounted for ca. 19%, antibacterials for 16–21% and lipid modifying agents 15–20% of all the pharmaceuticals analyzed (depending on the plant). Hydrochlorthiazide, furosemide, atorvastatine, clarithormycin, carbamazepine and diclofenac were ubiquitous in samples from all three WWTPs, in average concentrations from 30 to 60 ng/g. On the other hand, beta-blockers, beta-agonist and histamine H_2-receptor antagonists were found in very low concentrations in sludge.

Table 2 – Frequency of detection (%) and limits of quantification (LOQ) of pharmaceuticals detected in wastewater influent (WWI), effluent (WWE) and sewage sludge from the studied WWTPs during 8 sampling campaigns.

Compounds	Frequency of detection, %			LOQ (ng/L)		LOQ (ng/g)
	WWI	WWE	Sludge	WWI	WWE	Sludge
Ketoprofen (KTP)	100	54	0	13	7.0	1.3
Naproxen (NPR)	100	88	0	21	3.0	0.9
Diclofenac (DCL)	92	100	100	4.0	4.0	2.0
Indomethacine (INM)	65	58	0	3.0	2.0	1.0
Mefenamic acid (MFA)	54	77	83	16	5.0	0.4
Bezafibrate (BZF)	100	100	100	4.0	0.4	0.4
Fenofibrate (FNB)	42	0	79	0.5	0.5	2.5
Gemfibrozil (GMB)	38	58	50	3.0	1.0	1.7
Atorvastatin (ATR)	100	77	96	4.0	2.0	2.5
Pravastatin (PRV)	73	65	0	25	9.0	2.4
Mevastatin (MVS)	12	8	0	2.0	2.0	4.5
Diazepam (DZP)	54	54	88	3.0	1.2	4.1
Lorazepam (LRZ)	81	85	79	7.0	4.0	5.1
Carbamazepine (CBZ)	100	100	100	2.0	2.0	0.2
Clarithromycin (CLR)	73	85	83	5.0	4.0	7.1
Cimetidine (CMTD)	100	69	88	0.6	0.4	0.2
Ranitidine (RNTD)	92	88	92	3.0	2.0	0.3
Famotidine (FMTD)	19	8	96	1.0	0.7	0.1
Sulfamethazine (SLFM)	58	65	33	2.0	1.0	0.8
Trimethoprim (TRM)	100	96	88	1.0	0.4	0.6
Metronidazole (MTR)	62	62	0	6.0	0.7	5.6
Chloramphenicol (CHLR)	12	46	0	2.0	0.6	0.2
Atenolol (ATN)	100	100	88	9.0	9.0	0.7
Sotalol (STL)	65	58	54	5.0	2.0	0.4
Metoprolol (MTP)	35	62	0	2.0	2.0	1.2
Timolol (TML)	42	65	0	0.3	0.3	0.9
Nadolol (NDL)	100	69	54	0.8	0.2	0.3
Salbutamol (SLB)	69	58	0	0.3	0.2	0.3
Enalapril (ENL)	96	46	0	5.0	0.7	0.4
Glibenclamide (GLB)	85	65	92	5.0	4.0	3.5
Furosemide (FRS)	100	96	83	4.0	2.0	1.0
Hydrochlorthiazide (HCRT)	0	0	100	13	6.0	0.5

The total loads of analyzed pharmaceuticals that leave the plants unmodified (including sludge and effluent water) were calculated to equal 1.1, 0.9 and 0.1 g/day/1000 equivalent inhabitants for WWTP1, WWTP2 and WWTP3, respectively, of which only 3–9% (depending on the plant) was retained by sludge. The amount of pharmaceutical compounds detected in this study exiting the plants is not of great concern if we compare it with the results from some other studies done in this field (Castiglioni et al., 2006; Zorita et al., 2009).

3.2. *Overall removal of pharmaceuticals during wastewater treatment*

The daily mass loads of target compounds in wastewater influent and effluent, and in sludge, in g/day, were calculated as explained previously, and these values were used for the estimation and the comparison of the aqueous phase removal and the overall removal rates. The mass loads of pharmaceuticals that were discharged with effluent, sorbed to sludge and removed during treatment were normalized on influent of a given plant and presented in Fig. 4. Considering the fact that pharmaceuticals are grouped by the therapeutical applications for which they are used and not on the basis of their physicochemical similarity, their removal during treatment is expected to be diverse. Here the term removal refers to the conversion of a pharmaceutical to a compound different than the analyzed one (i.e. the parent compound). Thus, the overall removal refers to all the losses of a parent compound produced by different mechanisms of chemical and physical transformation, biodegradation and sorption to solid matter.

High aqueous-phase removal rates for some compounds (i.e. lipid regulator fenofibrate and histamine H2-receptor antagonists famotidine) would suggest very good removal of these compounds during the wastewater treatment. But, as shown in Fig. 4, only a certain percent of the total mass input is really lost during the treatment (overall removal). The rest was accumulated in sludge or discharged with the effluent. Sorption of fenofibrate, atorvastatine, diazepam and clarithromycin contributed to the elimination from the aqueous phase with more than 20% related to the amount of these compounds at the influent. This finding clearly indicates the importance of the analysis of sludge when studying wastewater treatment performances. Since many of the analyzed compounds were found in the sludge samples, the overall removal rate was the parameter used to compare the removal performances of the studied treatment plants.

In general, the removal rates varied strongly without evident correlation to the compound structure, as can be seen in the Fig. 4. The antihypertensive enalapril and NSAIDs ketoprofen and naproxen were removed in all the three cases with very

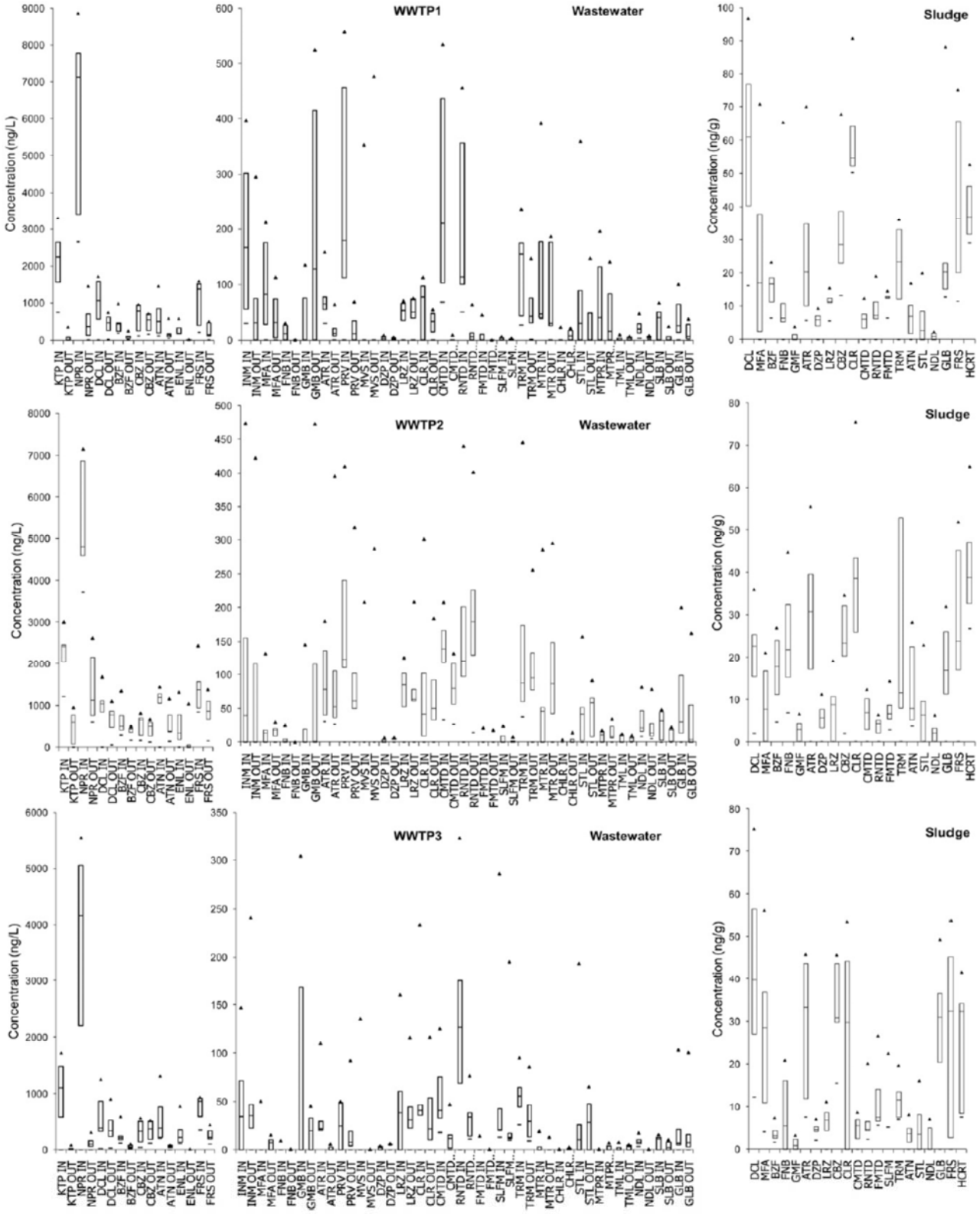

Fig. 2 – Box plots of concentration ranges (Min (–), P 0.25, Median, P 0.75 and Max (▲) of the pharmaceuticals detected in wastewater influent (IN), effluent (OUT) and sewage sludge from the studied wastewater treatment plants (WWTP1, WWTP2 and WWTP3) during 8 sampling campaigns (compound abbreviations are indicated in Table 2).

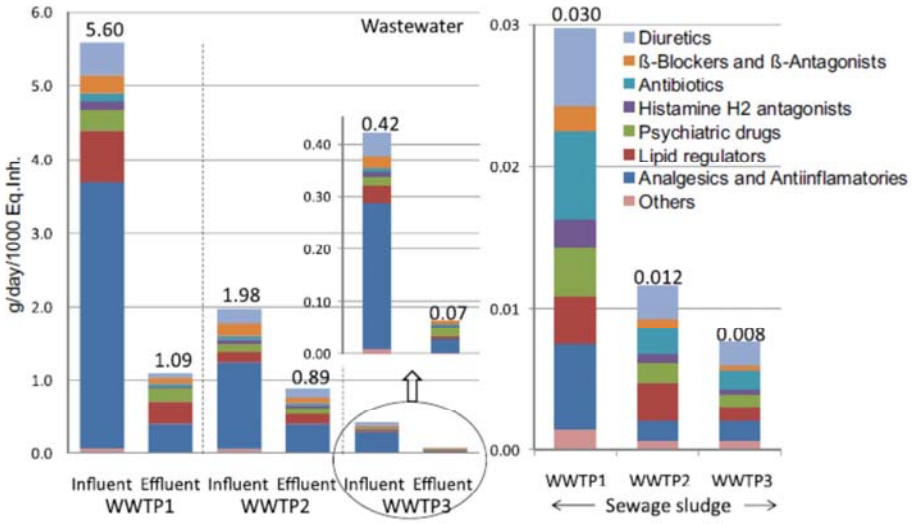

Fig. 3 – Daily mass loads (g/day per 1000 eq.inh.) of different therapeutic groups at the influent and effluent, and in the sludge from the studied WWTPs.

good removal efficiency (>80%) and they did not accumulate in sludge. Similar removal of these compounds from aqueous phase, under conventional treatment conditions, was observed in various studies on this topic (Lishman et al., 2006; Sim et al., 2010; Zorita et al., 2009). But then, the most analyzed anticonvulsant carbamazepine showed very low removal (<25%) regardless of the treatment applied. The results concerning its persistence and ubiquitous occurrence match with those from previous studies (Joss et al., 2005; Pérez and Barceló, 2007; Radjenovic et al., 2009b). No significant overall removal during the studied treatments (<30%) was observed for antibiotics trimethoprim and metronidazole, and benzodiazepine lorazepam. The incomplete removal of these compounds during conventional treatment has been reported by several studies (Bendz et al., 2005; Göbel et al., 2007; Kasprzyk-Hordern et al., 2009). A benzodiazepine diazepam and antimicrobial chloramphenicol were detected in concentrations close to their corresponding LOQs thus no reliable conclusion could be made on their behaviour.

Cholesterol lowering statin drugs pravastatin and mevastatin, antibiotic sulfamethazine, beta-blockers metaprolol and timolol, beta-agonist salbutamol were not accumulated in sludge and they showed a variety of removal rates between 30 and 80%. Inconsistent overall removal was also observed for NSAIDs mefenamic acid, indometacine and diclofenac, histamine H2-receptor antagonists cimetidine, famotidine and ranitidine, and diuretic furosemide. Some previous studies have also reported quite variable removal efficiencies of these compounds. For example, Castiglioni et al. (Castiglioni et al., 2006) observed low or no removal for salbutamol, furosemide and bezafibrate, whereas Kasprzyk-Hordern et al. (Kasprzyk-Hordern et al., 2009) noted higher removal for these compounds (>70%). Regarding the removal of histamine H2-receptor antagonists, the reported removals varied from rather low (Radjenovic et al., 2009b) to high rates (i.e. 86% in (Kasprzyk-Hordern et al., 2009). It is difficult to give a final conclusion on removal of majority of the studied pharmaceuticals, but it seems that the removal was mainly influenced by wastewater characteristics, operational conditions and treatment technology used. Comparing to the other two plants, WWTP1 offers better removal for the majority of the analyzed compounds (Fig. 4). This activated sludge plant featured by a tertiary treatment in WWTP1 improves the removal of diclofenac to 60%, while in the other two plants removal is much lower (<24%). Low removals of diclofenac were already reported in some publications on this topic (Cirja et al., 2008; Kimura et al., 2007; Quintana et al., 2005) imputed its persistence to the presence of chlorine group in the molecule. Some studies on removal during wastewater treatment showed no influence of solid retention time on the removal of diclofenac (Clara et al., 2005; Kreuzinger et al., 2004; Lishman et al., 2006). Furosemide, pravastatin, and ranitidine that were eliminated with removal ca. 80% and 60% in WWTP1 and WWTP3, respectively, marked very low (ca. 30%) removal rates in WWTP2. Better performances of WWTP1 and WWTP3 may be due to longer both hydraulic and solid retention times. As a compound spends more time in reactors wherein bacteria growth is promoted, the biological transformation may occur to a greater extent (Reif et al., 2008).It has been proven that longer SRT, especially, improves the elimination of most of the pharmaceuticals during sewage treatment (Clara et al., 2005; Göbel et al., 2007); Suárez et al., 2005.

The negative values of removal rates (omitted in the Fig. 4) refer to an increase in the concentration of an analyzed parent compound during treatment. This phenomenon of "negative removal" for some compounds was already reported in the literature (Gros et al., 2009; Joss et al., 2005); Wick et al., 2009. Hydrochlorothiazide was not detected in influent neither effluent water samples, but it was detected in sludge. This was not at all expected according to its low logP and the fact that >95% of the dose of this pharmaceutical is excreted unchanged (EMA, 2009). Lipid-regulating agent gemfibrozil was detected in higher concentration in the effluent than in the influent water samples. Similar was observed for macrolide clarythromycin,

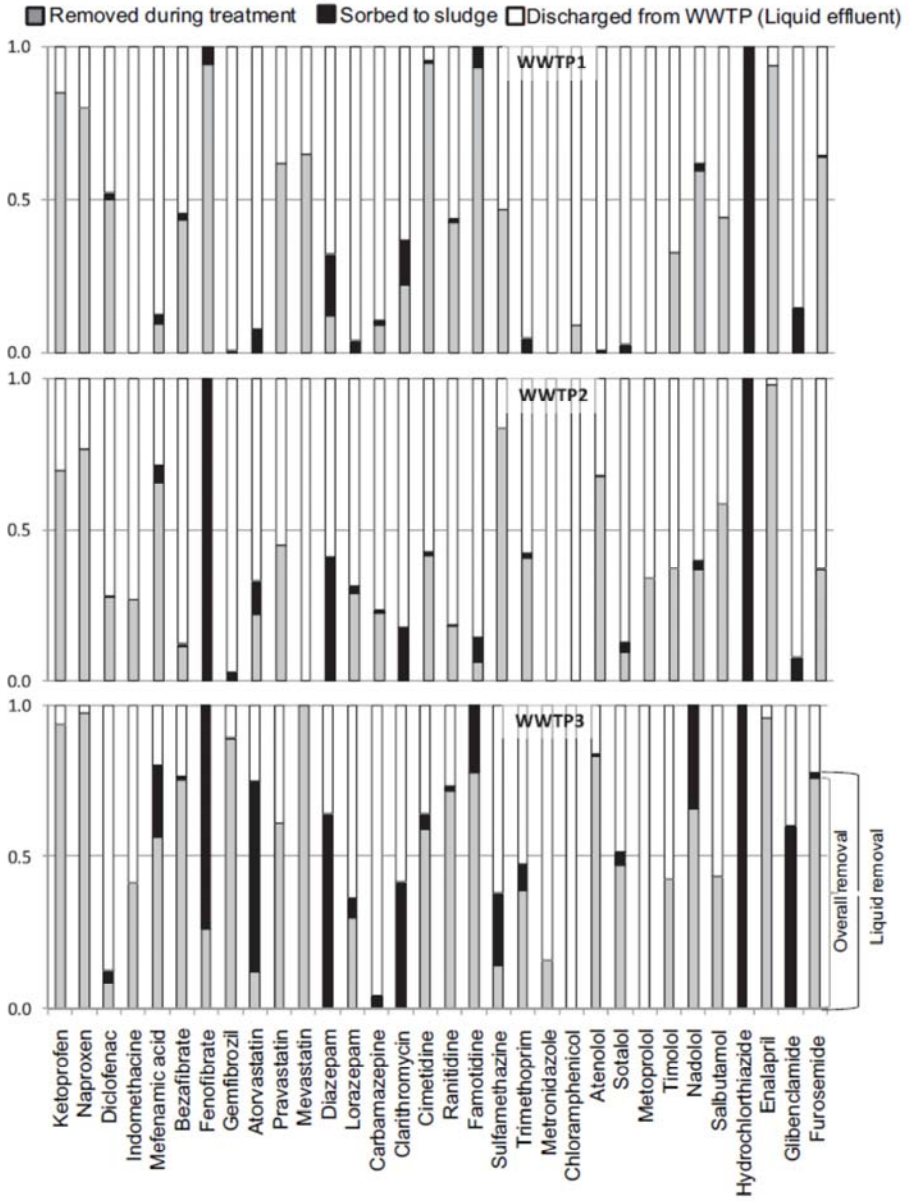

Fig. 4 – Normalized mass loads of the selected pharmaceuticals entering the studied WWTPs (i.e. fraction discharged with effluent, sorbed to sludge, and removed during treatment (overall removal rate))

anti-diabetic glibenclamide, lipid regulators fenofibrate and atrorvastatin, as well as for carbamazepine in one of the plants, which yielded higher concentration levels at the exit of a plant (i.e. including effluent and sludge) than at its entrance.

The explanation for this could be found in sampling protocols, as noted before; not only because they could be inadequate, but because of the nature of disposal of pharmaceuticals. Even though the analysis of effluent and sludge yields more certain results, because they come from stabilization processes, the sampling in general may result in underestimated and even negative removals. Furthermore, the negative removal can be explained by the formation of unmeasured products of human metabolism and/or transformation products (e.g. glucuronide conjugate, methylates, glycinates etc.) that passing through the plant convert back to the parent compounds. This can be considered as a reasonable assumption since the metabolites and some derivates of the mentioned compounds are well-known (e.g. hydroxy and epoxy-derivatives of carbamazepine; 4-trans-hydroxy and 3-cis-hydroxy derivatives of glibenclamide; ortho- and parahydroxylated derivatives of atorvastatine; gemfibrozil acyl glucuronide etc.) (Aviram et al., 1998; Miao et al., 2005); Shipkova and Wieland, 2005. Gobel et al. (Göbel et al., 2007) proposed gradual release of the macrolides (e.g. clarithormycin) from feces particles during biological treatment as an

explanation for the possible negative removal rates for these antibiotics. During complex metabolic processes in human body and bio-chemical in wastewater treatment, various scenarios of transformation from parent compound to metabolite and derivatives and vice versa can occur. These metabolites can be just as active as their parent compounds. Therefore, the occurrence of metabolites and transformation products and pathways should be included in the future studies in order to obtain accurate information on removal of pharmaceuticals during treatment and to determine treatment plant capabilities.

4. Conclusions

The study showed that, even though the WWTPs meet the regulatory requirements for wastewater treatment (Directive 91/271/EEC), they are only moderately effective in removing pharmaceutical compounds. Two plants that operated with longer SRT (i.e. WWTP1, with a tertiary treatment, and WWTP3) offered better removal of the majority of the analyzed pharmaceuticals. The calculated removal rates may have been underestimated due to at least three factors: removal efficiency was calculated from the mean concentration values; the metabolites and transformation products of pharmaceuticals and their amounts were not defined; and the time-proportional sampling may not be perfectly suitable for pharmaceutical analysis, especially on influent (Ort et al., 2010). Still, the fact is that 29 pharmaceuticals were detected in effluent wastewater and 21 in sludge samples. These pharmaceuticals cover a wide range of physical-chemical properties and biological activities. Although the chronic toxicity effects of such a mixture are unknown and thus the risk that it could pose to the environment could not be fully assessed, their presence must not be ignored. The results of this and similar studies are very useful for the estimations of (a) the magnitude of pharmaceuticals that reach the environment via either effluent or sludge and (b) the efficiency of the currently applied wastewater treatments, regarding the elimination of pharmaceuticals. More information on quality, quantity and toxicity of pharmaceuticals and their metabolites are definitely needed especially when attempting to reuse wastewater and dispose sludge to agricultural areas and landfills.

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Tracing pharmaceuticals in a municipal plant for integrated wastewater and organic solid waste treatment

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ABSTRACT

The occurrence and removal of 42 pharmaceuticals, belonging to different therapeutic groups (analgesics and anti-inflammatory drugs, anti-ulcer agent, psychiatric drugs, antiepileptic drug, antibiotics, ß-blockers, diuretics, lipid regulator and cholesterol lowering statin drugs and anti-histamines), were studied in the wastewater and sewage sludge trains of a full scale integrated treatment plant. The plant employs a biological nutrient removal (BNR) process for the treatment of municipal wastewater, and a single-stage mesophilic anaerobic co-digestion for the treatment of wasted activated sludge mixed with the organic fraction of municipal solid waste (OFMSW), followed by a short-cut nitrification–denitrification of the anaerobic supernatant in a sequential batch reactor. Influent and effluent wastewater, as well as thickened, digested and treated sludge were sampled and analyzed for the selected pharmaceuticals in order to study their presence and fate during the treatment. Twenty three compounds were detected in influent and effluent wastewater and eleven in sludge. Infiltration of groundwater in the sewer system led to a dilution of raw sewage, resulting in lower concentrations in wastewater (up to 0.7μg/L in influent) and sludge (70 ng/g d.w.). Due to the dilution, overall risk quotient for the mixture of pharmaceuticals detected in effluent wastewater was less than one, indicating no direct risk for the aquatic environment. A wide range of removal efficiencies during the treatment was observed, i.e. <20% to 90%. The influent concentrations of the target pharmaceuticals, as polar compounds, were undoubtedly mostly affected by BNR process in the wastewater train, and less by anaerobic-co-digestion. Mass balance calculations showed that less than 2% of the total mass load of the studied pharmaceuticals was removed by sorption. Experimentally estimated distribution coefficients (<500 L/kg) also indicated that the selected pharmaceuticals preferably remain in the aqueous phase, and that biodegradation/transformation is the primary removal mechanism for these compounds during wastewater treatment.

1. Introduction

Occurrence, fate and eco-toxic effects of pharmaceuticals in the environment are a topic that has received increasing scientific attention over the past decade. Metabolism and excretion of pharmaceuticals followed by wastewater treatment, as well as disposal of expired and unused drugs to sewage and trash, and the release of waste from pharmaceutical manufacturing plants have been recognized as the most important sources of human pharmaceuticals to the environment (Larsson et al., 2007; Li et al., 2008a, 2008b; Ruhoya and Daughton, 2008). Wastewater treatment plants (WWTPs) have been identified as the main point of collection and subsequent release of human pharmaceuticals into the environment, where they enter via the discharge of raw and treated sewage from residential users and/or medical facilities. The presence and fate of pharmaceuticals have been principally studied in the wastewater treatment train of conventional activated sludge plants, and in a limited number of pilot-scale membrane bioreactors. Less is known about the occurrence and fate of pharmaceuticals during sewage sludge treatment processes, due to the complex nature and varying quality of sludge, and also due to the need for sophisticated analytical equipment (Jones-Lepp and Stevens, 2007).

Most commonly applied sludge treatment is anaerobic digestion (AD) because it offers sludge volume reduction, nutrient recycling and renewable energy production while stabilizing the sludge. Although important for energy recovery, the AD process may involve expensive equipment and is generally economic only for large-scale systems. But, when sewage sludge is co-digested with biowaste (e.g. organic fraction of municipal solid waste (OFMSW), food-processing

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wastes etc.), the energy yields and the stability of AD system increase substantially, and it may be applied also for smaller WWTPs (Bolzonella et al., 2006; Singh and Harvey, 2010). The anaerobic co-digestion (AcoD) has particularly advanced in Europe, where EU Landfill directive (Council Directive 1999/31/EC) imposed the requirement for the reduction of biodegradable waste to landfill, encouraging the separate collection, material and energy recovery and recycling of waste. For both economic and environmental reasons, the number of AcoD facilities treating different kinds of biodegradable wastes is increasing in Europe and worldwide, with very successful examples from Denmark, Sweden, Germany and Switzerland (Braun and Wellinger, 2003). AcoD offers a higher degradation of organics than the AD of separate substrates (Fountoulakis et al., 2008; Mata-Alvarez et al., 2011), which may be an important point for the degradation of pharmaceuticals as well. Although AD is the most studied sludge stabilization method, the data on the occurrence and behavior of pharmaceuticals during this treatment are very scarce. They mainly come from single studies performed in laboratory- and pilot-scale AD plants (Carballa et al., 2007a, 2007b), or from monitoring studies conducted in full-scale WWTPs (Golet et al., 2003; Lindberg et al., 2006; Radjenović et al., 2009).

The present paper shows results of a study aimed at assessing the occurrence and behavior of selected pharmaceuticals during the full scale integrated treatment of municipal wastewater and OFMSW. The pharmaceuticals of interest belong to different therapeutic groups, i.e. analgesics and anti-inflammatory drugs, anti-ulcer agent, psychiatric drugs, antiepileptic drug, antibiotics, ß-blockers, diuretics, lipid regulator and cholesterol lowering statin drugs and anti-histamines (Table S1, Supplementary data). These compounds were selected on the basis of their high consumption or/and frequently reported detection in wastewaters, and the possibility to be analyzed under the same experimental conditions. Samples of influent (i.e. aqueous phase and suspended solids), and effluent; as well as thickened, digested (from AcoD) and treated sludge were collected to determine the concentrations and mass flows of the target compounds throughout the WWTP and to obtain more information on their fate during the treatment. The analysis was performed according to previously optimized methodologies for the analysis of the selected pharmaceuticals in wastewater and sludge samples (Gros et al., 2009; Jelic et al., 2009).

2. Materials and methods

2.1. Chemicals

Naproxen, ketoprofen, diclofenac and gemfibrozil were supplied by Jescuder (Rubí, Spain). Indomethacin, mefenamic acid, phenazone, bezifibrate, mevastatin, fenofibrate, pravastatin (as sodium salt), carbamazepine, 10,11-epoxycarbamazepine, famotidine, ranitidine (as hydrochloride), cimetidine (as hydrochloride), erythromycin (as hydrate), azithromycin (as dehydrate), roxitromycin, clarithromycin, josamycin, tylosin a, sulfamethazine, trimethoprim, chloramphenicol, atenolol, sotalol, metoprolol (as tartrate), timolol, pindolol, nadolol, salbutamol, clenbuterol (as hydrochloride), enalapril (as maleate), glibenclamide, furosemide, hydrochlorothiazide and metronidazole were purchased from Sigma-Aldrich (Steinheim, Germany). Standard atorvastatin (as calcium salt) was provided by LGC Promochem (London, UK), while diazepam, lorazepam and butalbital were from Cerilliant (Texas, USA).

The isotopically labeled compounds, used as internal standards, were sulfamethazine-d_4, rac-timolol-d_5 maleate, clarithromycin-n-methyl-d_3, atorvastatin-d_5 sodium salt, fenofibrate-d_6, metoprolol-d_7, metronidazole hydroxyl-d_2, pravastatin-d_3, rac-naproxen-d_3, mefenamic acid-d_3, gemfibrozil-d_6, bezafibrate-d_4 and furosemide-d_5 from Toronto Research Chemicals; diazepam-d_5 and phenobarbital-d_3 from Cerilliant (Texas, USA); atenolol-d_7, carbamazepine-d_{10}, ibuprofen-d_3, enalapril-d_5, hydrochlorothiazide-d_2, glyburide-d_3, albuterol-d_3, cimetidine-d_3, antipyrine-d_3, acetaminophen-d_4, diclofenac-d_4, clofibric-d_4 acid, hydrochlorothiazide-3,3-d_2 from CDN Isotopes (Quebec, Canada); sotalol hydrochloride d_6 from Dr. Ehrenstorfer (Augsburg, Germany) Isotec (Ohio, USA). All the pharmaceutical and the corresponding isotopically labeled internal standards were of high purity grade, >97%, except atorvastatin (90.2%) and diclofenac-d_4 (92.8%).

The individual standard solutions as well as isotopically labeled internal standard solutions were prepared on a weight basis in methanol. Fresh stock solutions of antibiotics were prepared monthly due to their limited stability, while stock solutions of the rest of substances was renewed every 3 months. The solutions were stored at −20 °C. A mixture of all pharmaceuticals was prepared by appropriate dilution of individual stock solutions in methanol–water (25:75, v/v) and it was renewed before each analytical run. A separate mixture of isotopically labeled internal standards, used for internal standard quantification, was prepared in methanol and further diluted in methanol–water (25:75, v/v) mixture.

The solvents, HPLC grade methanol, acetonitrile, water (Lichrosolv) and formic acid (98%) were provided by Merck (Darmstadt, Germany). Nitrogen used for drying from Air Liquide (Spain) was of 99.995% purity.

2.2. Plant description

The studied municipal integrated treatment plant – WWTP A – has a design treatment capacity of 50,000 PE. Low-loaded sewage water (about 18,000 m^3/day) is treated in a multi-zone nitrogen and

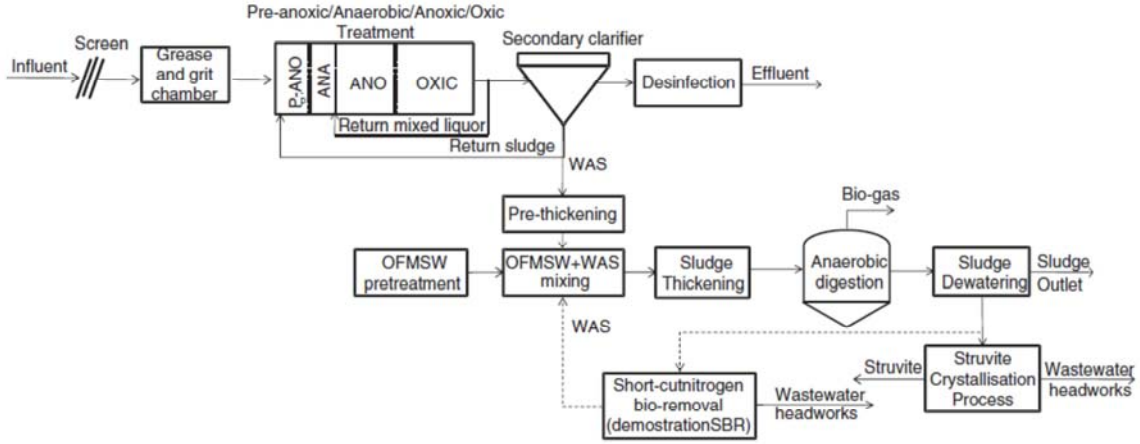

Fig. 1. Schematic diagram of the studied WWTP.

phosphorous biological removal process (BNR) with A2O *plus* Johannesburg configuration. The total hydraulic retention time (HRT) and solid retention time (SRT) were 7 h and 15 days, respectively. The sewage sludge treatment train consists of a single-stage mesophilic AcoD of wasted activated sludge (WAS) and OFMSW, followed by mechanical dewatering. The supernatant from the co-digester and from a belt press is treated by a short-cut nitrification–denitrification process in a demonstration sequencing batch reactor (SBR) (2.7 m^3 reaction volume) before being recycled to the wastewater treatment headwork (Fatone et al., 2011a). Fig. 1 shows the scheme of the process, and Table 1 shows the characteristics of the treated wastewater and sludge.

As regards the OFMSW (Table 1), it comes from separate collection and enters the plant with 25–30% dry matter. The preparation for AcoD starts with waste shredding, followed by removal of iron and ferrous materials, and screening of the residual material in a trommel screen. A final shredding is performed to reduce the size of the biowaste. The waste is then sent to a mixer/separator where the dry matter content is lowered to 7–8% and the floating and inert materials are withdrawn. From this point on, the blend is mixed with WAS and fed to the AcoD. Over the studied period the organic loading rate to the AcoD was about 1.7 kg TVS/m^3 day, made of OFMSW of 5.1 (±95%) tons OFMSW/day and 0.84 (±30%) tons WAS/day.

2.3. Sample collection and analysis

Samples of wastewater and sludge were collected over three consecutive days in 1 week in December 2010 and another week in February 2011. The weather was stable and without precipitation; the environmental temperatures ranged from −2 to 7 °C during the sampling periods, and the temperature of wastewater ranged from 10 to 15 °C. Flow proportional 24 h-composite samples of influent and effluent wastewater were collected using portable automatic samplers (Sigma 900 Max, Hach Lange Srl. Italy) that were programmed to collect 50 mL for every 100 m^3, resulting in about 10–12 L of water over 24 h. The effluent sampling was adjusted for the 7-hour HRT. The influent flow was around 800 m^3/h over the period from 11 am till 11 pm, when it decreased for about 30% during the night, and increased rapidly by 50% over the mean flow between 7 and 9 am.

The water samples were vacuum filtered through 1 μm glass fiber filters, followed by 0.45 μm nylon membrane filters right after the sampling, and stored on −20 °C until the analysis. The filtered-aliquots, i.e. 100 mL of influent wastewater, and the supernatants from the thickening and AcoD; and 200 mL of effluent wastewater, and the inlet and outlet of the SBR, were processed by solid phase extraction (Oasis® HLB, Waters, Milford, MA, USA) using a Baker vacuum system (J.T. Baker, Deventer, The Netherlands), and concentrated via elution with pure methanol. The 8 mL eluents were evaporated under a stream of nitrogen and reconstituted in 1 mL of methanol–water mixture (25:75). Prior to instrumental analysis, these samples were fortified by a mixture of internal standards to a final concentration of 20 ng/mL. Instrumental analysis was performed by high performance liquid chromatography coupled to a hybrid triple quadrupole–linear ion trap mass spectrometer (HPLC-QLIT-MS/MS) according to the previously developed multi-residual methodology for analysis of pharmaceuticals in wastewater (Gros et al., 2009).

The samples of thickened, digested and dewatered (treated) sludge were collected as five grab samples daily, and mixed to give a single sample (around 2 L) of each sludge type per day. The suspended particulate matter from the influent was taken using a membrane equipped automatic sampler designed and engineered for this purpose (Fatone et al., 2011b). The samples of the concentrated suspended particulate matter (i.e. liquid and suspended solids at around 2 g SSL^{-1}) proceeded from a 24 h-long ultrafiltration of 500 to 700 L of wastewater, depending on the wastewater influent flow. Thickened and digested sludge as well as the concentrated samples of influent suspended solids were centrifuged at 4500 rpm for

Table 1
Characteristics of the wastewater and sludge from the studied WWTP, and OFSMW used for AcoD (in brackets: RSD% values).

Wastewater						Sludge							
Influent		Aeration tank		Effluent		OFSMW		Thickened		Digested		Treated	
TSS (mg/L)	89 (13)	HRT (h)	6.5 (15)	TSS (mg/L)	3.7 (13)	TCOD (mgO_2/g)	2127 (13)	T (°C)	17.2 (2)	T (°C)	31.4 (6)	Production (t/month)	278 (18)
TCOD (mgO_2/L)	108 (12)	T (°C)	16.6 (6)	TCOD (mgO_2/L)	8.0 (24)			pH	6.7 (2)	pH	7.5 (1)		
BOD (mgO_2/L)	59.5 (13)	DO (mg/L)	1.4 (10)	BOD (mgO_2/L)	4.4 (25)	TKN (mg/g)	30.9 (16)	Alkalinity (mg/L)	1316 (3)	Alkalinity (mg/L)	2077 (2)	Dryness (%)	24.2 (1)
N–NH3 (mg/L)	7.1 (5)	MLSS (g/L)	4.5 (8)	N–NH3 (mg/L)	1.3 (13)	TP (mg/g)	7.1 (27)	TS (g/L)	36.4 (2)	TS (g/L)	24.7 (5)		
TKN (mg/L)	11.0 (3)	MLVSS (g/L)	3.2 (11)	TKN (mg/L)	2.3 (9)			TVS (%)	71 (4)	TVS (%)	61 (4)	TVS (g/L)	8.6 (5)
$NO3^-$ (mg/L)	1.6 (7)	MLVSS (%)	71 (4)	$NO3^-$ (mg/L)	0.6 (20)	TS (mg/g)	296 (31)	$NO3^-$ (mg/L)	1.5 (20)	$NO3^-$ (mg/L)	1.4 (18)	TVS (%)	61 (5)
NO_2^- (mg/L)	0.3 (11)	V_{Sludge} (mL/L)	452 (9)	NO_2^- (mg/L)	0.03 (24)			TKN (mg/g)	20.1 (8)	TKN (mg/g)	20.7 (4)	TKN (mg/g)	22.1 (4)
TP (mg/L)	1.5 (9)	SVI (mL/g)	101 (4)	TP (mg/L)	0.6 (18)	TVS (%)	76 (13)	TP (mg/g)	2.9 (15)	TP (mg/g)	2.6 (12)	TP (mg/gTS)	2.9 (4)

30 min to concentrate the particulate matter. The solid samples were freeze dried and ground, and extracted using an accelerated solvent extraction system as described in detail elsewhere (Jelic et al., 2009). Concentrated extracts were processed as water samples and analyzed by HPLC-QLIT-MS/MS (Gros et al., 2009).

The internal standard calibration approach was used for quantification. To determine the recoveries, three samples of each matrix were spiked with a standard mixture of target analytes. For wastewater, the recoveries ranged from 45 to 146% (RSD<13%) for influent, and from 40 to 141% (RSD<12%) for effluent wastewater samples. For solid samples, the recoveries ranged from 41 to 136% for influent suspended solids, and 31–136%, 35–126% and 35–133% (RSD<20%) for thickened, digested and treated sludge, respectively. The instrumental intra-day precision ranged from 2 to 13%, for five injections of a 50 ng/mL standard mixture. The method quantification limits (MQL), calculated as ten times signal-to-noise, were 0.7–55 ng/L, 0.6–39 ng/L for influent and effluent wastewater, respectively, and 1.3–92 ng/L for the thickened and digested sludge supernatant. For sludge samples, the MQL were 0.2–16 ng/g, 0.2–14 ng/g, 0.3–18 ng/g and 0.2–9.3 for thickened, digested and treated sludge, and influent suspended solids, respectively.

2.4. Calculations

2.4.1. Overall removal

The removal (R_i, %) of a detected pharmaceutical i during the treatment was calculated using the following equations (Eq.) (Joss et al., 2005):

$$\dot{m}_{ij(l)} = c_{ij(l)} \times \dot{V}_j \tag{1}$$

$$\dot{m}_{ij(SS)} = c_{ij(SS)} \times \mathrm{TSS}_j \times \dot{V}_j \tag{2}$$

$$\dot{m}_{ij(Sludge)} = c_{ij(Sludge)} \times \dot{P}_j \tag{3}$$

$$R_i = 1 \frac{\sum_{j=1}^{6}\left(\dot{m}_{ij(l)} + \dot{m}_{ij(SS)}\right) Effluent + \sum_{j=1}^{6} \dot{m}_{ij(Sludge)}}{\sum_{j=1}^{6}\left(\dot{m}_{ij(l)} + \dot{m}_{ij(SS)}\right) Influent} \tag{4}$$

where $\dot{m}_{ij(l)}$ and $\dot{m}_{ij(SS)}$ are daily mass loads (g/day) of a compound i in the aqueous phase (l) and in the suspended solids (SS) of the wastewater influent and effluent; $\dot{m}_{ij(Sludge)}$ are daily mass loads (g/day) of a compound i in sludge. TSS (g/L) is the concentration of suspended solids in the influent. In the equations, $c_{ij(l)}$ (ng/L), $c_{ij(SS)}$ (ng/g), and $c_{ij(Sludge)}$ (ng/g) stand for measured concentrations in the two phases of the influent, effluent and sludge, respectively; and $\dot{V}_j$ (L/day) and $\dot{P}_j$ (g/day) are flow rate of wastewater and the production rate of sludge, respectively, on sampling day j. Removal efficiencies (R_i) are shown as white bars in Fig. 3.

Assuming that the variables in the mass load calculations are independent, the overall uncertainty associated with the mass load values (for each compound) was estimated from the individual uncertainties in sampling ($U_{Sampling}$), flow measurements (U_{Flow}) and chemical analysis ($U_{Analysis}$) as follows (Lai et al., 2011):

$$U_{\dot{m}ij} = \sqrt{U_{Sampling}^2 + U_{Flow}^2 + U_{Analysis}^2} \tag{5}$$

The uncertainty of chemical analysis was estimated from standard deviation of the mean of triplicate (spiked) samples and intra-day precision ($U_{Analysis}$<25% depending on compound). For the applied sampling procedure, the uncertainty associated with sampling was assessed to be 10% (Ort et al., 2010a) for both influent and effluent, although the sample volume is more accurate for less turbid effluent samples. The ideal specified wastewater flow meter error was 2% under laboratory conditions. The actual uncertainty of flow measurements is expected to be higher when used for field wastewater measurements. We used 10% of the flow measurement uncertainty for the estimation of the overall uncertainty for influent and effluent wastewater. Since the quantity of pharmaceuticals in sludge was negligible compared to wastewater, the uncertainties of the mass loads of pharmaceuticals in sludge were omitted from the analysis. The uncertainties associated with influent and effluent mass loadings are shown as error bars in Fig. 3.

2.4.2. Removal of pharmaceuticals during AcoD

In order to account for the complete mass of each pharmaceutical that enters and leaves the digester, daily mass loads were calculated from the concentrations in the centrifuged sludge and the corresponding supernatant according to the equations:

$$\dot{m}_{ij(s)} = \mathrm{TSS}_{ad,j} \times \dot{V}_j \times c_{ij(s)} \tag{6}$$

$$\dot{m}_{ij(l)} = \dot{V}_j \times c_{ij(l)} \tag{7}$$

where: $\dot{m}_{ij(s)}$ and $\dot{m}_{ij(l)}$ are the mass loads (g/L) of a compound i in the solid phase (s) and the corresponding supernatant (l) of thickened or digested sludge on a sampling day j; $c_{ij(s)}$ (ng/g, d.w.) and $c_{ij(l)}$ (ng/L) are concentrations of the compound i measured in dried solids and the corresponding supernatants from the thickened/digested sludge. $\mathrm{TSS}_{a,d,j}$ (g/L) is a concentration of total suspended solids in the thickened/digested sludge and $\dot{V}_j$ (L/day) is the daily flow rate of the sludge entering the AcoD. Hence relative removal of pharmaceuticals during AcoD over the sampling period was calculated as follows:

$$R_{AcoD,i} = 1 - \frac{\sum_{j=1}^{6}\left(\dot{m}_{ij(l)} + \dot{m}_{ij(S)}\right) Digested}{\sum_{j=1}^{6}\left(\dot{m}_{ij(l)} + \dot{m}_{ij(S)}\right) Thickened}. \tag{8}$$

The white bars in Fig. 6 show the removal efficiencies of the selected pharmaceuticals during AcoD ($R_{AcoD,i}$).

Uncertainty associated with the removal efficiency of AcoD was calculated via propagation of uncertainties of flow measurement (Endress Hauser Promag W flow meter, error 5%) of 20%, and chemical analysis (depending on compound, in general<25%) (Fig. 6). Although sampling uncertainty may contribute most to the overall uncertainty, it could not be quantified and was omitted from the analysis.

2.4.3. Solid–water distribution coefficient (K_d)

Solid–water distribution coefficient, K_{d_k} (L/kg), for sample k, was calculated as the ratio of sorbed concentration of a compound i, $C_{ik,s}$ (mg/kg), to the dissolved concentration, $C_{ik,l}$ (ng/L), at equilibrium (Ternes et al., 2004):

$$K_{d_k} = \frac{C_{ik,s}}{C_{ik,l}} \tag{9}$$

where k stands for influent wastewater, thickened or digested sludge sample. Table 2 summarizes K_d values, given as mean ± standard deviation for six measurements. The mean K_d values were derived from pairwise data and then averaged:

$$\overline{K_{d_k}} = \frac{\sum_{j=1}^{6} K_{d_k}}{6} \tag{10}$$

2.4.4. Risk assessment — Risk quotients

To evaluate the environment risk posed by the studied pharmaceuticals on the aquatic system, a risk quotient (RQ) for each compound i and sample j was estimated as follows:

$$RQ_{ij} = \frac{c_{ij}}{PNEC_i} \tag{11}$$

Table 2
Estimated K_d values $\overline{K_{d_s}}$ for pharmaceuticals in influent suspended solids (SS), thickened and digested sludge, given as mean ± standard deviation of six measurements, and comparison with previously reported K_d values for primary and digested sludge.

Compound	Kd, L/kg (measured)			Literature data (measured)	
	Influent SS	Thickened sludge	Digested sludge	Primary sludge	Digested sludge
Ketoprofen	<20	–	–	226±180[a]	
Diclofenac	87±17	134±33	106±17	194±134[a], 459±32[b],<30[c]	18–151[d]
Atorvastation	99±17	–	–	216±82[c]	
Carbamazepine	162±54	–	–	314±205[a],<20[b], 65±5[c], 40–220[e]	20–68[d]
Lorazepam	217±71	–	–		
Trimethoprim	156±48	–	–	427±238[a], 251±99[c]	
Azithromycin	405±109	444±133	367±89		
Clarithromycin	132±20	461±36	715±261		
Furosemide	43±22	127±41	110±39		
Hydrochlorothiazide	50±11	81±19	92±15	25.8±14.4[a]	
Glibenclamide	274±115	–	–	282±307[a], <20[b]	

[a] Radjenovic et al., 2009.
[b] Ternes et al., 2004.
[c] Stevens-Garmon et al., 2011.
[d] Carballa et al., 2008.
[e] Martín et al., in press.

where C_{ik} (ng/L) is maximum concentration of compound i measured during the sampling period in j sample (i.e. influent or effluent wastewater); and $PNEC_i$ (ng/L) is the Predicted No Effect Concentration. The PNEC values were estimated from literature data on acute toxicity, EC50 (i.e. concentration of a drug that produces 50% of the drug's maximal effect) for fish, algae and *Daphnia magna*, corrected by a safety factor of 1000 as it was recommended by the Water Framework Directive (Directive 2000/60/EC). The EC50 values used for the calculation are provided in Supplementary data (Table S3).

The joint effect of n detected compounds in influent and effluent wastewater for three trophic levels was expressed by an overall $RQ_{ij(sum)}$ (Fig. 4, Table S3) as follows (Von der Ohe et al., 2009; Backhaus and Faust, 2012):

$$RQ_{ij(sum)} = \sum_{i=1}^{n} RQ_{ij} \quad (12)$$

when the RQ is below 1, the risk is considered low (Ginebreda et al., 2010).

3. Results and discussion

3.1. Occurrence and overall removal during the treatment

Out of 42 analyzed, 23 pharmaceuticals were detected in wastewater and 11 in sludge. Fig. 2 shows the range of concentrations of pharmaceuticals measured at the influent and the effluent of WWTP A. In general, the detected concentrations were much lower than those previously reported (Gros et al., 2010; Kasprzyk-Hordern et al., 2009). This is mainly due to the fact that the sewer system was combined and subject to infiltration of groundwater that lead to a dilution of the contaminant input. However, no information on the dilution factor due to the infiltration of groundwater into the sewer system was available. Calculated based on BOD values (Council Directive 91/271/EEC), population equivalent of the plant was 19,000 (Table 1). Average total daily loads of the detected pharmaceuticals leaving the plant unmodified (including both effluent wastewater and sludge) was 1.2 g/(day·1000 PE) (Table S2). This value falls within the ranges previously reported for the same group of pharmaceuticals in activated sludge WWTPs (Jelic et al., 2011;

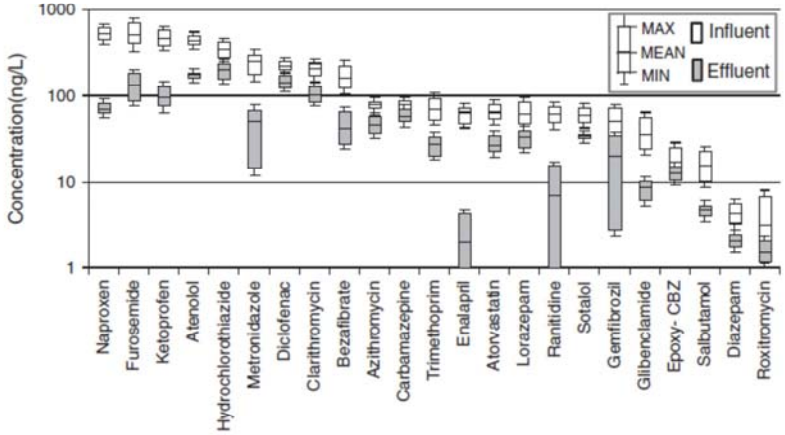

Fig. 2. Concentrations of the pharmaceuticals detected in samples of influent (white bars) and effluent (gray bars) wastewater from WWTP A. Boxes represent the range of measured concentrations for the sampling period, i.e. minimum, (arithmetic) mean and maximum; while the error bars indicate the uncertainty associated with the concentrations.

Zorita et al., 2009). Only 2% of the total mass load of the detected pharmaceuticals, determined from mass balance calculations, was retained by sludge.

Along with the parameters shown in Table 1 (i.e. suspended solids, BOD, COD etc), removal efficiencies of pharmaceuticals were calculated as additional data to evaluate the treatment capability of WWTP A. The removal was estimated from the difference between the total mass loads of an analyzed (parent) compound in the input (wastewater influent) and output streams (wastewater effluent and sludge) of WWTP A for the sampling period (Eq. (4)) (Joss et al., 2005). The mass loads of pharmaceuticals discharged from the plant with effluent and treated sludge normalized on the influent mass loads are shown in Fig. 3. The white part of the bars represents the fraction of pharmaceutical load that was removed by different mechanisms of chemical and physical transformation and biodegradation. Gray bars in Fig. 3 correspond to the part of the load that was discharged from WWTP A with effluent, while the black fields in the figure represent the portion of pharmaceuticals that was sorbed to sludge. The amount of the compounds found in the particulate matter from the influent wastewater has also been included in the mass balance, although it accounted for less than 1% of the total influent mass load.

The observed removal efficiencies ranged from very low to very high, i.e. from 20 to 95% (Fig. 3). Antimicrobial roxithromycin and benzodiazepine diazepam were detected only in wastewater at concentrations close to the MQLs (3.9 and 11 ng/L, respectively) so no reliable conclusion could be made on their removal. The antihypertensive enalapril and histamine H2-receptor antagonist ranitidine were not detected in any of sludge samples and they were removed with a very good efficiency (≥90%). Anti-inflammatory drug naproxen was detected at negligible concentrations in sludge (~MQL 3.2 ng/g d.w. in treated sludge), and it was removed with high efficiency during the treatment (>80%).

On the other hand, very low removal efficiency (<25%) was observed for anticonvulsant carbamazepine. Additionally, the calculated average removal was as high as the uncertainty associated with the determined mass loading of carbamazepine. The results concerning its persistence are in accordance with other published results (Joss et al., 2005, 2006). In the present study, an active metabolite of carbamazepine, 10,11-dihydro-10,11-epoxycarbamazepine (epoxy-CBZ), was analyzed as well. In all the samples of influent wastewater, the ratio of the concentrations of carbamazepine and epoxy-CBZ in influent wastewater was calculated to be around 1/5, and it was held constant in effluent wastewater as well. As for its parent compound, a poor removal was observed for epoxy-CBZ (<25%). The metabolite was not detected in sludge samples. Similar findings were reported in two studies by Miao and his colleagues (Miao and Metcalfe, 2003; Miao et al., 2005). Diclofenac was also barely removed during the treatment. The average diclofenac removal efficiency was calculated to be around 35%, with a significant range of uncertainty. Similar results were reported previously (Cirja et al., 2008; Kimura et al., 2007; Quintana et al., 2005). It was found that removal of diclofenac and carbamazepine is not affected by the operational SRT (Clara et al., 2005; Kreuzinger et al., 2004).

For the other compounds, a range of removal efficiencies were calculated, from 35 to 80% (Fig. 3). As regards the removal of macrolide antibiotics azithromycin and clarithromycin, they were eliminated with average efficiencies of about 35 and 50%, respectively. Kobayashi et al (Kobayashi et al., 2006) reported removals of 50% for these two macrolides in three conventional WWTPs. The calculated removal efficiencies for beta-blockers sotalol and atenolol were 42 and 60%, respectively. Radjenovic et al. (2009) reported similar removal of these compounds in a conventional WWTP that treated a mixture of municipal and industrial full strength wastewaters (SRT 10 d). In general, the data on the removal of beta-blockers found in the literature are very inconsistent and the removal efficiencies vary from less than 10% up to 95% depending on WWTP (Lee et al., 2007; Ternes, 1998; Vieno et al., 2007; Wick et al., 2009). There is no clear correlation between the removal of beta-blockers and SRT, as all the cited WWTP applied SRT>15 days. Moderate removals, i.e. 60–70%, were observed for fibrates bezafibrate and gemfibrozil. In the literature, the reported removal efficiencies ranged from 30 to 90%, and the reduction did not appear to be strongly influenced by SRT (Lishman et al., 2006). The data are in close agreement with those reported by Joss et al. (2006) that observed partial biotransformation of the fibrates in batch experiments. The variation in the removal efficiencies of pharmaceuticals during biological treatments observed in different studies may be a consequence of various factors such as different wastewater characteristics, operational conditions and treatment technologies used (Clara et al., 2005; Göbel et al., 2007), as well as sampling procedures and conditions (Ort et al., 2010a; Ort et al., 2010b).

Even though many of the analyzed pharmaceuticals were removed with significant efficiency, there were a number of compounds having different physico-chemical properties and biological activities that were detected in effluent wastewater and dewatered sludge. Therefore, a risk assessment has been carried out, where the risk quotients (RQ) were calculated by dividing maximum measured concentrations of pharmaceuticals (i.e. exposure) by PNEC values

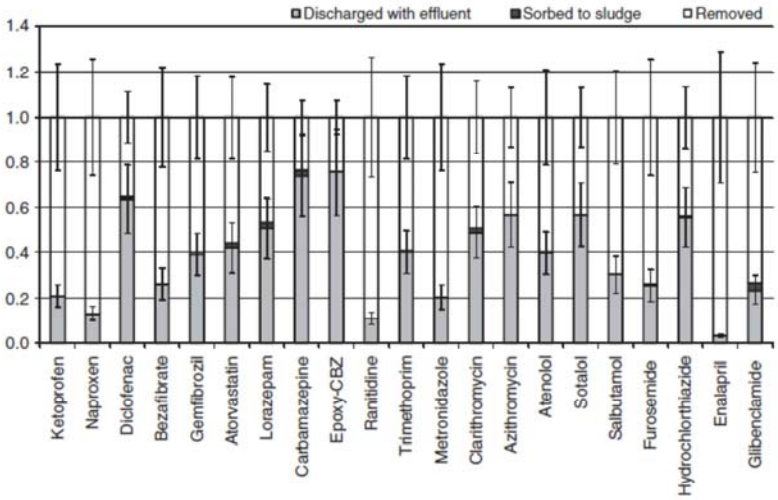

Fig. 3. Mass loads of pharmaceuticals normalized to the influent mass load: fraction discharged with effluent, sorbed to sludge, and removed during treatment. The error bars correspond to the total uncertainty calculated for each mass load.

calculated from the literature acute ecotoxicity data for fish, algae and *D. magna* (i.e. effect) (Eq. (11)). Whenever possible, the PNEC values were determined from experimental data, otherwise the EC50 were estimated using USEPA's ECOSAR (Ecological Structure Activity Relationships) model (ECOSAR v1.00) (López-Serna et al., 2012). The EC50 values used for the calculation are summarized in Table S3. In order to estimate a joint effect of the mixture of the detected compounds, we used a concentration addition (CA) concept (Eq. (12)) (Backhaus and Faust, 2012). The CA is based on a simple addition of individual RQs to obtain an overall risk quotient ($RQ_{ij(sum)}$). The CA mixture toxicity concept was found to serve as a justifiable approximation, irrespectively of the modes/mechanisms of action of the mixture components, for the first estimation of potential risks for an exposed ecosystem when only base-set data are available (Backhaus and Faust, 2012). If we compare the obtained $RQ_{ij(sum)}$ values for the detected compounds and three trophic levels (Table S3), it appears that the three organisms show similar overall susceptibility to the selected group of pharmaceuticals. Obviously, each organism showed different susceptibility for different pharmaceuticals. In most of the individual cases, algae and *D. magna* were much more sensitive to the individual pharmaceuticals than fish. For the WWTP effluent, RQs of the individual compounds, as well as the overall $RQ_{ij(sum)}$ for the three trophic levels were below 1 for all the compounds indicating no risk of adverse effects (Table S3). Atorvastatin contributed the most to the overall $RQ_{ij(sum)}$ for the three organisms. As an example, Fig. 4 shows the RQ for *D. magna*, obtained for each detected compound in the influent and effluent of WWTP A. It is to be noted that the relatively low values of the measured concentrations and RQs were mainly due to the dilution effect of the sewer infiltration.

3.2. Occurrence and removal of pharmaceuticals during AcoD of WAS and OFMSW

Fig. 5 shows the range of measured concentrations of the pharmaceuticals detected in the thickened, digested and dewatered (treated) sludge from the studied integrated municipal WWTP A. The arithmetic mean and the corresponding minimum (min) and maximum (max) values were based on concentration data for the six sampling days. The error bars correspond to the uncertainty due to chemical analysis. Eleven pharmaceuticals were detected in concentrations higher than the calculated MQLs for the sludge samples. As already mentioned, the study deals with a low-loaded wastewater, and then WAS is additionally mixed with OFMSW, thus the concentrations of pharmaceuticals detected in sludge samples were lower than previously reported by other similar studies (Kinney et al., 2006; McClellan and Halden, 2010; Radjenovic et al., 2009). Only azithromycin, clarithrimycin, furosemide, diclofenac and hydrochlorothiaze were detected at concentrations greater than 20 ng/g d.w in the sludge entering AcoD (Fig. 5). Gemfibrozil, naproxen, and sotalol were detected in all the sludge matrices in concentrations close to the corresponding MQLs. The low concentrations of the detected pharmaceuticals are significantly affected by the errors due to sampling and analysis, easily leading to under or over-estimation of the removal efficiencies of the process. In order to avoid ambiguous conclusions, the removal efficiencies were determined only for the compounds detected at concentrations > 20 ng/g. Total mass load of each pharmaceutical leaving AcoD is plotted normalized on the corresponding total inlet mass load (i.e. thickened sludge) (Fig. 6), and the difference represents the fraction removed during AcoD (Eqs. (6), (7) and (8)). In Fig. 6, two different sets of removals are shown as pair of bars associated with each compound. The left bar in a pair represents the removal calculated from the total mass load of a compound entering and leaving the digester, including both dry solids and supernatant (Eq. (8)), while the right bar in a pair shows the removal calculated on a dry weight (d.w.) basis. The difference between these two sets is almost negligible, indicating that the concentrated solid phase of both thickened and digested sludge contained almost all the mass of the pharmaceuticals. Also the measured K_d values for sludge samples (greater than 100 L/kg) suggested that sorption of pharmaceuticals to digested sludge cannot be ignored (Ternes et al., 2004). The removal efficiencies of pharmaceuticals during AcoD were low to moderate, ranging from around 30% (for diclofenac and furosemide) to around 60% (for clarithromycin and atorvastatin) (Fig. 6). Sludge conditioning and dewatering on belt-press had little or no effect on the concentration levels of the pharmaceuticals (Fig. 5).

Given that implementation of anaerobic co-digestion at full operating scale is relatively new, there are no literature data regarding the efficiency of this process for pharmaceutical removal. Actually, there are very few data regarding their removal also during other sludge treatment processes. The information on the removal of pharmaceuticals during AD found in the literature is usually based on lab-scale experiments. Carballa et al. (2007b) studied the behavior of pharmaceuticals during AD in two lab-scale continuously stirred reactors operated under mesophilic and thermophilic conditions. The efficiencies of the removal ranged from very high (e.g. naproxen, sulfamethoxazole, and roxithromycin) to very low (e.g. carbamazepine). In a follow up study, they investigated the effect of pre-treatments (chemical or thermal, and ozonation) on removal efficiency of AD at lab-scale (Carballa et al., 2007a). Except for cabamazepine that was removed up to 60% using ozonation prior to thermophilic AD, the results were very similar to those obtained in the first study. The treatment

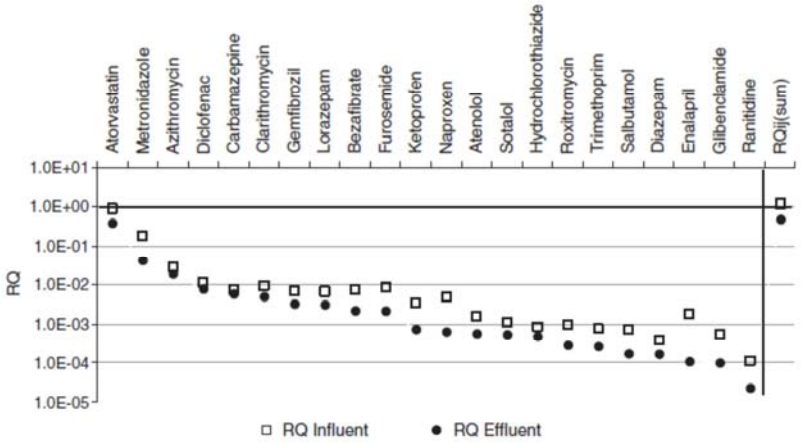

Fig. 4. Influent and effluent RQ values for the pharmaceuticals detected in WWTP A estimated from the maximum measured concentrations and literature data on acute toxicity for *Daphnia magna*.

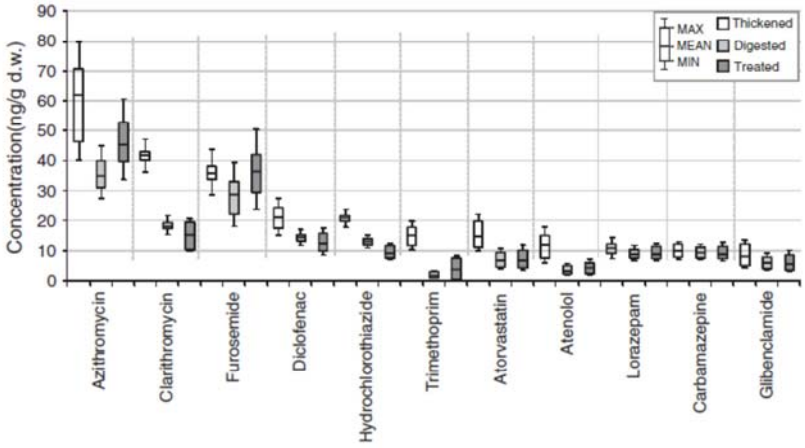

Fig. 5. Concentrations of the pharmaceuticals detected in thickened, digested and treated sludge from WWTP A. The boxes represent the range of measured concentrations for the sampling period, i.e. minimum, (arithmetic) mean and maximum. The error bars correspond to the uncertainty associated with the concentrations.

efficiency of AcoD observed for diclofenac in our study is comparable to their results for AD of WAS (around 30%), where both the organic loading rate and the biodegradability degree of the feed are lower. Radjenović et al. (2009) reported similar results regarding the removal of pharmaceuticals during wastewater treatment that applied mesophilic AD of WAS. In a report prepared for the Biosolids Task Group (BTG) of the Canadian Council of Ministers of the Environment (CCME), the documented removal efficiencies of pharmaceuticals during mesophilic AD in two Canadian WWTPs were highly variable (CCME Report, 2009). Trimethoprim, sulfamethoxazole and naproxen were calculated to have the highest removal efficiencies. A number of compounds either remained unaffected by AD or increased in concentration through the process, e.g. carbamazepine, gemfibrozil etc. For azithromycin and hydrochlorothizide, the estimated removals (0–49%) during mesophilic AD were lower than those estimated in our study.

3.3. Treatment of the anaerobic supernatant

Eleven compounds were detected in the supernatant of AcoD, in concentrations of some dozens (e.g. atorvastatine, carbamazepine) to a hundred of ng/L (e.g. diclofenac, azythromycin). The sludge liquors arising from AcoD of WAS and biodegradable waste are characterized by high concentrations of ammonia resulting from the degradation of proteins and amino-acids present in the substrate. In order to remove nitrogen and to try to remove the pharmaceuticals, this stream was adequately treated in a short-cut nitrification–denitrification process in SBR, before its recirculation to the liquid treatment train of the studied plant (Fatone et al., 2011a). Beside the higher nitrogen removal (Gujer, 2010), sludge enriched with nitrifying bacteria was found to enhance the removal of some pharmaceuticals, e.g. diclofenac, naproxen, roxithromycin and erythromycin, and 17α-ethinylestradiol (Suarez et al., 2010). In our study, very low removal of diclofenac (<15%) was observed during the SBR treatment of anaerobic supernatant. Slightly better, but still poor removals (i.e. <50%) were obtained for the other detected pharmaceuticals as well (Fig. S1).

3.4. Quantitative estimation of sorption

Although biodegradation/transformation were proved to be the most important mechanisms for the removal of pharmaceuticals during wastewater treatment, the fact that these compounds were detected in sludge indicates that the sorption might influence it to a certain extent, as well. In order to quantify the sorption of pharmaceuticals, distribution coefficients (K_d) were experimentally estimated for the detected compounds. K_d was calculated as the ratio of the quantity of the compound sorbed per mass of solid to the amount of the compound in solution, assuming that the system was at equilibrium (Section 2.4.4). Nevertheless, the K_d values must be taken as rough estimates due to, at least, two factors mostly influenced by sampling and sample-handling: a) wastewater and sludge streams are non-homogeneous as the result of short-term and long-term variation in

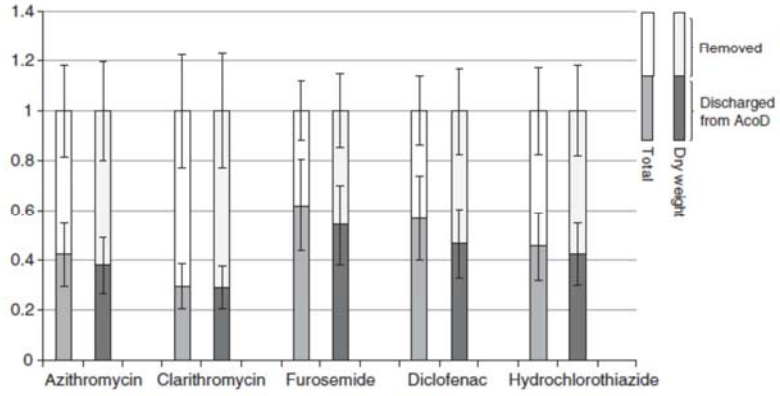

Fig. 6. Removal of selected pharmaceuticals during AcoD estimated based on dry weight concentrations (light gray) and on the total mass flow (white). Mass load of each compound is plotted normalized to the AcoD inlet (thickened sludge) mass load. The error bars correspond to the uncertainty associated with each mass load.

sewers and WWTPs, and b) the solid–liquid phase equilibrium can be easily disturbed (Ort et al., 2010b; Radjenovic et al., 2009).

To compare the sorption characteristics of the particulate matter of the WWTP inlet and outlet streams, K_d were estimated for the influent wastewater and the sludge coming out of the AcoD. It was also measured for the thickened sludge, to discern possible differences between thickened and digested sludge regarding their sorption capacity. As a site-specific parameter, K_d was determined for each compound and matrix, and sampling day. For influent suspended solids (SS), the highest K_d was determined for azithromycin (i.e. 405 ± 109), followed by the K_d of glibenclamide and lorazepam (Table 2). The K_d for carbamazepine, trimethoprim and clarithromycin ranged from 100 to 200 L/kg, and for diclofenac, hydrochlorothiazide and furosemide from 30 to 100 L/kg. Radjenovic et al. (2009) reported similar K_d ranges for glibenclamide and hydrochlorothiazide, but somewhat higher for diclofenac, trimethoprim and carbamazepine (Table 2). As all the determined K_d values lower than 500 L/kg SS for influent suspended solids, the removal of the pharmaceuticals by sorption in the WWTP is considered negligible (Ternes et al., 2004).

Table 2 shows the mean K_d determined for the five compounds that were detected at concentrations > 20 ng/g. d.w. in thickened and digested sludge. The K_d values ranged from 400 to 900 L/kg for the macrolides azithromycin and claithromycin, and around 100 L/kg for furosemide, diclofenac and hydrochlorothiazide. Since the sludge biomass (i.e. both microorganisms and their extracellular polymers) carries an overall negative charge within a pH range typical for wastewater treatment (pH 5–9) (Busch and Stumm, 1968), electrostatic interaction with positively charged dimethylamino group in the molecules of azithromycin and clarithromycin (pKa 8.7 and 8.9, respectively) may be responsible for higher K_d values of these compounds. In fact, sorption of pharmaceuticals was found to be, principally, a result of the electrostatic and hydrophobic nature of sludge (Project POSEIDON, 2001–2004). In the case of clarithromycin, besides electrostatic, also hydrophobic interactions ($\log D_{ow}$ ~1.5–2.5 for pH 6.6–7.6) resulted in higher K_d values than for azithromycin, i.e. 400–500 L/kg for thickened, and 500–900 L/kg for digested sludge. On the other hand, significantly lower K_d values were determined for negatively charged diclofenac and furosemide (pKa 4.1 and 3.9, respectively). Their octanol–water distribution coefficients (D_{ow}) are strongly influenced by changes of pH: the D_{ow} values increase 6–10 times with a decrease in one pH unit. This can be a reason that the K_d values for the digested sludge (pH 7.6) were lower (although slightly) than for the thickened one (pH 6.6). Similar range of K_d values was measured for diclofenac in digested sludge by Carballa et al. (2008). In general, the estimated K_d values for the selected compounds were higher for thickened and digested sludge than for the influent suspended solids. The enhanced sorption ability of digested sludge might come from the higher concentration and the composition of the organic matter due to the addition of the OFMSW to wastewater sludge, that resulted in increased interactions compared to those in the influent suspended solids. Carballa et al. (2008) compared the K_d values of selected pharmaceuticals obtained for digested sludge with the corresponding literature values for primary sludge, and found that the digested sludge had lower sorption capacity. Nevertheless, the variation in the composition of sludge and pH, as well as the concentration of compounds in the aqueous phase, can result in large differences in K_d values calculated for different matrices of a single WWTP.

4. Conclusions

As a result of incomplete removal of the selected pharmaceuticals during applied wastewater treatment, twenty three compounds were detected in wastewater effluent and eleven in treated sludge. The predominant compounds in effluent wastewater were atenolol, hydrochlorothiazide and diclofenac with concentrations ranging up to 200 ng/L. Out of eleven pharmaceuticals detected in treated sludge solids, azithromycin and furosemide were present in the highest concentrations, i.e. up to 50 ng/g d.w. The observed concentrations are generally lower than other previously reported ones, due to groundwater infiltration in the sewer system. The diluted concentrations resulted in no risk for the aquatic environment, with the overall risk quotients below 1 for fish, algae and *D. magna*. The observed overall removal during treatment varied widely from compound to compound, from a low of 20% (e.g. carbamazepine) to 95% (e.g. enalapril). The sorption to influent suspended solids was found to be low, with K_d below 500 L/kg for the studied pharmaceuticals. Less than 2% of the total daily mass load of the examined pharmaceuticals was discharged with sludge. These results indicate that biotransformation is the main removal mechanism of the selected compounds during biological wastewater treatment. Anaerobic co-digestion of WAS and OFMSW achieved removal efficiencies from 30 to 60%. Clearly, more research is needed to assess the efficiency of AcoD for the removal of pharmaceuticals when these are present in higher concentrations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2012.06.059.

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Supplementary data for:

Tracing pharmaceuticals in an integrated municipal plant for wastewater and organic waste treatment

Table S1. List of the target compounds

Therapeutic groups	Compounds
Nonsteroidal anti-inflammatory agents/analgesics	Ketoprofen
	Naproxen
	Indomethacine
	Diclofenac
	Mefenamic acid
Phenazone type analgesics	Phenazone
Lipid modifying agents	Bezafibrate
	Fenofibrate
	Gemfibrozil
	Mevastatin
	Pravastatin
	Atorvastatin
Psycholeptics and antiepileptics	Diazepam
	Lorazepam
	Carbamazepine
Metabolite	Epoxy-Carbamazepine
Drugs for peptic ulcer and gastro-oesophageal reflux disease	Ranitidine
	Famotidine
	Cimetidine
Macrolide antibiotics	Erythromycin
	Roxithromycin
	Clarithromycin
	Josamycin
	Azithromycin
Sulfonamide antibiotics	Sulfamethazine
Other antibiotics	Trimethoprim
	Chloramphenicol
	Metronidazole
Beta-blocking agents	Atenolol
	Sotalol
	Metoprolol
	Timolol
	Nadolol
	Pindolol
Beta-2-adrenoreceptor agonists	Clenbuterol
	Salbutamol
Barbiturates	Butalbital
Intestinal anti-inflammatory/anti-infective agents	Nifuroxazide
Angiotensin converting enzyme (ACE) inhibitor	Enalapril
Diuretics	Hydrochlorothiazide
	Furosemide
Antidiabetic	Glibenclamide

Table S2. Average daily mass loads of pharmaceuticals discharged from the WWTP through wastewater effluent and treated sludge

ATC Code	Compound	Effluent	Treated sludge
		mg/(d*1000PE)	mg/(d*1000PE)
M01	Ketoprofen	93	/
	Naproxen	68	/
	Diclofenac	136	2.1
C10	Bezafibrate	40	/
	Gemfibrozil	19	/.
	Atorvastatin	26	1.2
N05	Diazepam	2.0	/
	Lorazepam	31	1.5
N03	Carbamazepine	56	1.5
Matebolite	Epoxy-CBZ	12	/
A01	Ranitidine	6.7	/
J01	Trimethoprim	27	0.6
	Metronidazole	49	/
	Roxithromycin	1.5	/
	Clarithromycin	98	2.5
	Azithromycin	45	7.5
C07	Atenolol	166	0.7
	Sotalol	33	/
R03	Salbutamol	4.6	/
C03	Furosemide	127	6.0
	Hydrochlorthiazide	189	1.5
C09	Enalapril	2.0	/
A10	Glibenclamide	8.4	0.9

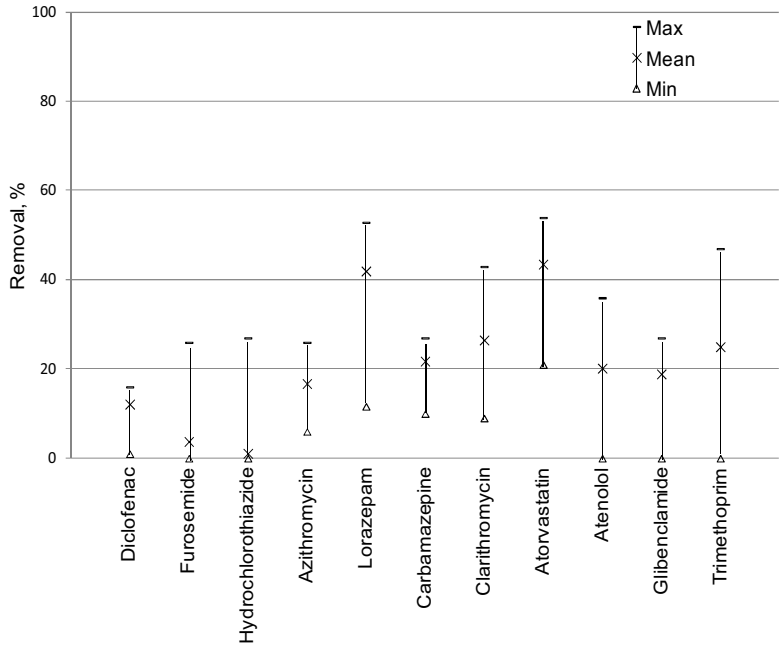
Table S3. EC50 (mg/L) values and calculated RQ for the pharmaceuticals detected in the influent and the effluent of WWTP A. The EC50 data were compiled from: Choi et al., 2008; Cleuvers, 2005; Grung et al., 2008; Haap et al., 2008; Halling-Sørensen et al., 2000; Hernando et al., 2007; Isidori et al., 2005; López-Serna et al., 2012; Montforts, 2005; Sanderson et al., 2004; Stuer-Lauridsen et al., 2000; Winter et al., 2008; Yang et al., 2008.

COMPOUNDS	Acute toxicity EC50 (mg/L)			RQ					
				INFLUENT			EFFLUENT		
	Fish	Green algae	*Daphnia magna*	Fish	Green algae	*Daphnia magna*	Fish	Green algae	*Daphnia magna*
Naproxen	190	96.6	126	3.20E-03	6.30E-03	4.90E-03	4.40E-04	8.60E-04	6.60E-04
Furosemide	138	5.94	82.7	5.10E-03	1.20E-01	8.50E-03	1.30E-03	3.10E-02	2.20E-03
Ketoprofen	258	124	169	2.20E-03	4.60E-03	3.40E-03	5.00E-04	1.00E-03	7.60E-04
Atenolol	774	620	313	6.30E-04	7.90E-04	1.60E-03	2.40E-04	3.00E-04	5.90E-04
Hydrochlorothiazide	1745	2.51	477	2.40E-04	1.70E-01	8.70E-04	1.40E-04	9.50E-02	5.00E-04
Metronidazole	375	561	1.68	8.00E-04	5.40E-04	1.80E-01	1.90E-04	1.20E-04	4.20E-02
Diclofenac	532	14.5	22	4.60E-04	1.70E-02	1.10E-02	3.20E-04	1.20E-02	7.80E-03
Clarithromycin	280	2.08	25.7	8.50E-04	1.10E-01	9.30E-03	4.60E-04	6.20E-02	5.00E-03
Bezafibrate	6	18	30	3.70E-02	1.20E-02	7.40E-03	1.10E-02	3.70E-03	2.20E-03
Azithromycin	19.8	1.97	3.07	4.30E-03	4.30E-02	2.80E-02	2.90E-03	2.90E-02	1.90E-02

The Table continues on the next page.

COMPOUNDS	Acute toxicity EC50 (mg/L)			RQ					
				INFLUENT			EFFLUENT		
	Fish	Green algae	*Daphnia magna*	Fish	Green algae	*Daphnia magna*	Fish	Green algae	*Daphnia magna*
Carbamazepine	35.4	85	11.9	2.50E-03	1.00E-03	7.40E-03	2.00E-03	8.30E-04	5.90E-03
Trimethoprim	110	16	123	8.70E-04	6.00E-03	7.80E-04	3.10E-04	2.10E-03	2.70E-04
Enalapril	122	6.22	39.4	5.90E-04	1.20E-02	1.80E-03	3.60E-05	7.10E-04	1.10E-04
Atorvastatin	0.09	0.22	0.09	8.90E-01	3.60E-01	8.80E-01	3.90E-01	1.60E-01	3.90E-01
Lorazepam	19.7	0.54	12.8	4.30E-03	1.60E-01	6.70E-03	2.00E-03	7.40E-02	3.20E-03
Ranitidine	1076	66	650	6.90E-05	1.10E-03	1.10E-04	1.40E-05	2.30E-04	2.40E-05
Sotalol	1029	13.4	65.1	7.10E-05	5.40E-03	1.10E-03	3.50E-05	2.70E-03	5.60E-04
Gemfibrozil	7.09	4	10.4	1.00E-02	1.80E-02	6.90E-03	4.90E-03	8.60E-03	3.30E-03
Glibenclamide	500	735	100	1.10E-04	7.70E-05	5.60E-04	2.10E-05	1.40E-05	1.00E-04
Salbutamol	31.5	36	30	7.20E-04	6.30E-04	7.50E-04	1.70E-04	1.50E-04	1.80E-04
Diazepam	28	16.5	14.1	2.00E-04	3.40E-04	4.00E-04	8.70E-05	1.50E-04	1.70E-04
Roxitromycin	288	3.3	7.1	2.40E-05	2.10E-03	9.60E-04	7.20E-06	6.30E-04	2.90E-04
			$RQ_{ij(sum)}$	**0.97**	**1.04**	**1.16**	**0.42**	**0.48**	**0.48**

Figure S1. Removal of pharmaceuticals during the short-cut nitrification/denitrification in a demonstration sequential batch reactor (SBR)



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RESEARCH ARTICLE

New indexes for compound prioritization and complexity quantification on environmental monitoring inventories

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Abstract
Introduction Lists of compounds resulting from environmental monitoring may be conveniently represented in a very general way using Pareto distributions, after ranking them on descending order according to their concentration or hazard quotient expressed as percentages, depending on whether the objective of the monitoring is focussed on mass load occurrence or risk assessment respectively.
Materials and methods Ranked distributions are characterized using appropriate indexes, such as *h* (Hirsch), well known in other disciplines like bibliometry. Furthermore, to such ordered distributions, simple numerical power type equations relating rank order and occurrence probability can be fitted, following the so-called power or Zipf law. Both *h* indices and the characteristic power law exponents are interpreted as measures of complexity of the overall mixture. On the other hand, compounds included within the *h* index may be seen as the most relevant in the mixture, thus providing a reasonable indication of what is worth analyzing. These concepts have been applied, as case study, to the characterization of the pharmaceutical compounds found in the input and output streams of wastewater treatment plants.
Results and discussion Whereas both the concentration load and ecotoxicity of pharmaceuticals in WWTPs obviously decrease in the output of the treatment (influent>effluent, sludge), complexity quantified using the proposed indexes does not follow the same trend, being this behaviour common to the three plants examined.
Conclusion The joint combination of *h* compounds of the three plants studied allowed optimizing the list of compounds to be analyzed, which must be considered the key ones for the scenario under study.

Keywords Chemical complexity · Environmental monitoring · Pareto statistics · Power law · Hirsch index · Pharmaceuticals · Wastewater treatment

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1 Introduction

Current use of chemicals by our technological society can be estimated in ca. a hundred of thousands of compounds (most of them organics), and this number is continuously growing (Muir and Howard 2006). Hence, depending on their properties, mode and extent of use, this large amount of different chemicals can potentially reach the environment, being their environmental and health effects unpredictable in long term. This has become a matter of major concern and constitutes the underlying reason for new regulations related to the safety of chemicals are being promoted. Thus, for instance, the recently

approved European Union regulation REACH Regulation EC No. 1907/2006 has an aim to regulate chemicals used in commerce and consumer products, including a list of 30,000 compounds. Similar regulatory processes have been currently undertaken in some other developed countries, such as USA, Canada (The Montebello Agreement) or Japan (Chemical Risk Information Platform, CHRIP).

On the other hand, a simultaneous and huge progress on the analytical capabilities has taken place, mostly associated to the development of multi-residue analytical methods based on chromatographic techniques (GC and LC) coupled to mass spectrometry (MS), capable to identify and quantify compounds at trace levels of nanograms or picograms per litre (Barrek et al. 2009; Baugros et al. 2008; Carvalho et al. 2008; Covaci et al. 2007; Díaz-Cruz et al. 2009; Feo et al. 2010 ; Gervais et al. 2008; Vulliet et al. 2008; Wick et al. 2010). GC–MS and LC–MS are thus powerful tools in the hands of environmental chemists, and consequently, their use in the environmental field has become extensive. In the last years, this has allowed to enlarge the list of controlled environmental contaminants from those more classical and mostly covered by the existing regulations to a group of the so-called emerging contaminants, such as pharmaceuticals, endocrine disruptors, personal care products, perfluorinated compounds, flame retardants, etc (Daughton 2004; Petrovic et al. 2010; Richardson 2010). These micro-contaminants have been often continuously released into the environment in low quantities; however, they can pose a significant risk to both human health and ecosystems due to their biological activities and suspected or unknown effects after long exposure periods.

Different approaches making use of multiple criteria have been developed in order to identify and rank compounds of environmental concern for monitoring purposes. Kumar and Xagoraraki (2010) proposed a comprehensive ranking system for prioritizing the monitoring of pharmaceuticals and personal care products (PPCP) and endocrine disrupting compounds (EDC) in US stream water/source water and finished drinking water using four criteria (occurrence, treatment in drinking water treatment plants, ecological effects and health effects), identifying 20 priority compounds within pharmaceuticals, PCPs, EDCs, antibiotics and teratogenic compounds. Murray et al. (2010) established a list based on calculation of consumption rate posing health risk that allowed for comparison of the risk posed by maximum and average concentrations in the freshwater environment. Muñoz et al. (2008) used life cycle impact assessment, a feature of the life cycle assessment methodology, to quantify the potential environmental impacts on ecotoxicity and human toxicity of wastewater containing priority and emerging pollutants. In order to do this, so-called characterisation factors are obtained for 98 frequently detected pollutants, and out of around 80 substances found in the wastewater samples, only 16 have a significant contribution, 10 of these substances being PPCPs. Arnot and Mackay (2008) used a holistic mass balance modelling approach integrating persistence, bioaccumulation, toxicity and quantity information for a specific substance to assess chemical exposure, hazard and risk allowing comparisons of exposure, hazard and risk for priority setting. All the foregoing approaches are intended to produce "absolute" lists (ranks) of priority compounds of general validity based on the intrinsic properties and effects of the compounds considered independently if they are present or not on a particular case. Whereas for many purposes, that point of view is adequate (for instance, regulatory) if one is concerned with specific scenarios or cases, it may be no so useful since relevant compounds are restricted to those that are actually present (independently of their rank on a general lists). Therefore, an alternative approach focusing only on the compounds occurring on a particular scenario is worth to be considered. Such ad hoc prioritization perspective is complementary (i.e., it does not invalidate) on the previous one and constitutes the purpose of the present exercise.

Environmental monitoring is facing a complex panorama in which the available analytical possibilities must be directed towards target compounds since not all measurable compounds are worth to be measured (this question is particularly relevant when routine monitoring control has to be implemented). Furthermore, complex additive, interactive and even synergetic effects caused by exposure to the mixture of different chemicals (as they actually occur in the environment) are often postulated, but they are seldom evidenced. Within the said context, the purpose of the present article specifically addresses to (a) how to select and prioritize compounds to be monitored on a given scenario and (b) how to characterize environmental mixtures of compounds, as obtained from monitoring campaigns, using appropriate indices.

Real environmental monitoring usually yields inventories or lists of compounds and concentrations. If our interest is focused on mass load, direct addition of concentration is acceptable. However, if we are aiming to characterize environmental risk, it is necessary to consider the ecotoxicity of the whole mixture of components. Mixture toxicity is a difficult question and has been subject of active research (van Gestel et al. 2010). Up to now, two models have been proposed to approximate mixture toxicity, namely, concentration addition (CA) (Loewe and Muischnek 1926) and independent action (IA) (Bliss 1939). CA is based on the assumption of a similar mode of action for all the substances present in the mixture. This implies that all components contribute to the final effect even at low concentrations or said in other words that concentrations can be added. Conversely, IA assumes that each compound acts separately by its own mechanism of action, and it will contribute to the

overall toxicity only if its concentration is relevant from the ecotoxicologic point of view. Whereas both models have been proven acceptable if the corresponding mechanistic assumptions are fulfilled, since exact modes of action are often unknown for many compounds, both CA and IA must be regarded as two special extreme cases (Backhaus 2011; Vighi et al. 2003). In practice, both models have been more or less successfully applied, being the results obtained with not very different, with CA tending to overestimate and IA to underestimate toxicity in controlled experiments (Junghans et al. 2006). Therefore, taking into consideration the precautionary principle, there is a wide consensus on accepting CA as the general purpose model of choice for a first approach to mixture toxicity estimation (Backhaus 2011).

Therefore, upon that basis, the proposed characterization of chemical inventories can be set up as follows: The starting step is to transform the original list of monitored compounds in a *ranked or descending ordered list*. As mentioned, criteria used for setting such ranks will vary depending on the purpose pursued in the monitoring. Whereas mass load expressed as concentrations poses no special problem, ecological risk associated to a pollutant can be adequately characterized using the so-called hazard quotients. Hazard quotients (*hq*) are defined as the ratio between predicted or (in our case) measured environmental concentrations (PEC or MEC, respectively) to their chronic toxicity, usually expressed as non-observed effect concentrations (NOEC) or predicted non-effect concentrations (PNEC) values (Bound and Voulvoulis 2006; Castiglioni et al. 2004; Cooper et al. 2008; Gros et al. 2010). When NOEC values are not available, EC50 or LC50 values from standard ecotoxicological tests can be used, eventually after correction by an assessment factor. These *hq* values are determined for every compound present or predicted on the environment and can be aggregated by simple addition (upon the CA assumption) on an overall hazard quotient (Ginebreda et al. 2010). Under this framework, if HQ equals to unity, there is a potential environmental risky situation (i.e., $HQ \geq 1$ is interpreted as environmental risk, while $HQ < 1$ should indicate no risk).

The second step is to characterize the ranked list by its properties that can be either simple indexes directly determined from the empirical data (such as Hirsch or *h* type indexes) or by previous fitting to the empirical data an appropriate mathematical distribution, being amongst the most common power (Zipf) law, though other distributions like the exponential can be equally tested. Particularly the former one has been found a wide and heterogeneous domain of applications, which include word frequency on a given language, citations of scientific papers, wealth of richest people, ecological networks, web hits, number telephone calls, population of cities, intensity of wars, among others. The subject has extensively reviewed by Newman (2005), where the interested reader is addressed, and its foundations seem very general (Corominas-Murtra and Solé 2010).

Along the present paper, we will provide the necessary background concepts and examine the possible application of the foregoing proposed methodology to chemical inventories as usually obtained from environmental monitoring campaigns. The occurrence of pharmaceuticals in the different input and output streams of some Waste Water Treatment Plants in Catalonia (NE Spain) has been used as representative case study.

2 Materials and methods

2.1 Scenario description, sample collection and instrumental analysis

Pharmaceuticals analyzed, basic monitoring conditions, site description and results have been previously reported by our group elsewhere (Jelic et al. 2011).

In summary, samples (i.e. influent and effluent wastewater and sewage sludge) were obtained from three full-scale wastewater treatment plants (WWTPs) in the region of Catalonia (Spain). Even though they obviously do not pretend to cover all the available typologies of sewage treatment technologies, they can be considered representative of typical conventional wastewater plant configurations existing in the region, deployed in order to fulfil the requirements of Directive 91/271/EC. They essentially consist on a secondary treatment step, eventually followed by an appropriate nutrient elimination step or other adequate tertiary treatment. The three plants investigated also differ on the relative contribution of industrial vs. urban effluents treated. Thus, whereas WWTP1 receives predominantly municipal wastewater, WWTP2 and especially WWTP3 have significant industrial contributions. All the samples were collected in eight sampling campaigns between July 2007 and March 2009, in campaign intervals of 2 to 3 months. Composite wastewater samples were collected at the entrance of the treatment plant i.e. influent wastewater and at the exit of the plants i.e. effluent wastewater (after the secondary treatment at WWTP2 and WWTP3, and after the tertiary one in WWTP1). The analyzed samples of sludge were collected at the final phase of the process, i.e. treated sewage sludge. In Table 1 are summarized some characteristics of the three investigated wastewater treatment plants (source: Annual report of Catalan Water Agency for 2008). The collection, handling, preparation and analysis of the samples using high-performance liquid chromatography coupled to a hybrid triple quadrupole-linear ion trap mass spectrometer (HPLC-QLIT-MS/MS) were carried out according to the previously

Table 1 Characteristics of the studied wastewater treatment plants (WWTPs)

	Type of treatment	HRT (h)	SRT (days)	Designed treatment capacity (m^3 day)	Average flow (m^3 day)	Population Equivalent	Sludge treatment	Disposal of sludge	Sludge production (t/year)	Dry matter (t/year)	Organic matter (%)
WWTP1	Biological + Tertiary	26–40	10	47,500	25,000	74,000	Composting	Disposal to soil; Agricultural usage	9,000	1,800	75
WWTP2	Biological	20	6	35,000	26,000	170,000	Anaerobic digestion	Disposal to soil; I nceneration	8,500	2,000	65
WWTP3	Biological with P and N removal	40	16	25,000	21,000	400,000	Anaerobic digestion + Drying	Controlled disposal to landfill	11,400	2,900	53

developed multi-residual methodologies for analysis of pharmaceuticals in wastewater and sludge samples (Gros et al. 2009; Jelic et al. 2009).

2.2 Ecotoxicological data and hazard quotients

In the present study, PNEC values were estimated respect daphnids as representative aquatic organisms from existing data on acute toxicity. Specifically, dividing EC50 values by an arbitrary safety factor, in this case equal to 1000, *PNEC* were derived (Sanderson et al. 2003). EC50 values used in this study were collected from the literature and are summarized in Table S1 (Supplementary material). It is worth noting that when more than one EC50 value was found, the lowest one was chosen. When no experimental values were available, we used EC50 estimated with ECOSAR, (see Table S1, Supplementary Material).

Hazard quotient hq_{ij} associated to compound j in the i measurement is given by the following expression:

$$hq_{ij} = \frac{c_{ij}}{\mathrm{PNEC}_j} \tag{1}$$

where c_{ij} is the concentration of compound j in sample i and PNEC_j the Predicted Non Effect Concentration for compound j, as described above:

$$\mathrm{PNEC}_j = \frac{\mathrm{EC50}_j}{1000} \tag{2}$$

Since more than one compound is usually present, their joint effects must be somehow considered. Hazard quotients can be aggregated for the different compounds present in the sample, through simple addition (concentration addition). Therefore, the overall hazard quotient for sample i is:

$$\mathrm{HQ}_i = \sum_j hq_{ij} \tag{3}$$

in order to fit a power law as it was convenient to express the contribution of each compound within every single HQ_i in terms of its percentage. This is carried out by normalization of hq_{ij} to 100, using the following expression:

$$hq'_{ij}(\%) = \frac{hq_{ij}}{\sum_j hq_{ij}} \times 100 = \frac{hq_{ij}}{\mathrm{HQ}_i} \times 100 \tag{4}$$

(where i and j stand as above)

The so normalized hq's can be modelled fitting a power law, on the same manner as concentrations.

Hazard quotients were only calculated for the liquid streams (i.e. influent and effluent). Sludge was left aside for two reasons: first of all, due to the lack of data on ecotoxicity on solid matrices and second because sludge obtained from WWTPs is not disposed onto the aquatic media.

2.3 Background and index definitions

2.3.1 Pareto distributions and h-indexes

In the present article, we consider a set of n compounds, of which the ith has a concentration equal to X^*_i. Let us consider a monitoring collection of results expressed as $\{X^*_i\}$ $i=1,\ldots,n$ in a rank order, so that $X^*_1 \geq X^*_2 \geq \ldots \geq X^*_n$. We assume that they can be described according to a Pareto distribution (named after the famous Italian economist Vilfredo Pareto, who applied it to the distribution of wealth) (Pareto 1896).

The well-known h index was originally introduced by Hirsch (2005) to measure the scientific production of a scientist in terms of publications and respective citations ("A scientist has a index h if h is the largest number of his/her papers having received at least h citations each"). Glänzel (2006) has formalized the notion in more rigorous mathematical terms. According to this author, h *index* is thus defined as $h \leq X_h$, provided that $h > X_{h+1}$. To cope with the case $X_h = X_{h+1} = h$, he proposes a slightly modified definition, mathematically formulated as $h = \max\{j: X_j \geq j\ , j = 1 \ldots n\}$.

However, since we are dealing with a set of chemical compounds $i=1,\ldots,n$ (instead of a set of papers) and their respective properties X^*_i (instead of their citations), in order to properly apply the notion of h index, both quantities must be commensurable. Some kind of scaling of concentrations is thus required. Though this can be done in several ways, we have chosen expressing them in percentage according to Eq. 5, mostly because their meaning and interpretation are straightforward:

$$X_i = \frac{X^*_i}{\sum_{i=1,n} X^*_i} \times 100 \qquad \text{with} \qquad \sum_{i=1,n} X^*_i = 100 \tag{5}$$

Note that any additive property associated to every single compound normalizable as in Eq. 1 can be used, for instance, concentrations or hazard quotients.

Like in the context of bibliometry, h index applied to chemical inventories provide an interesting measure of the more relevant compounds to be taken into consideration. Of course, besides h index, other indices equally based on Pareto distributions can be readily defined. For instance, the index P_Z ($0 < Z \leq 100$) defined as "the minimum number of compounds that account for Z% of the total load", whose mathematical definition is $P_Z = \min\{j: \Sigma X_j \geq Z\%, j = 1 \ldots n\}$. In the present work, we have used P_{90} for comparison purposes with respect to the h index. Both h and P_Z indexes provide similar type of information and can be equally used. However, from practical experience, h is sometimes preferred since it appears less arbitrary and case dependent than P_Z. Said in other words, there is no clear indication about how to choose Z (and thus P_Z), and different cases would require setting different Z as most appropriate.

2.3.2 h Compounds and H sets

For a given inventory, we introduce the set of h compounds (H) as those compounds that are included in the h index. Interpretation of H sets is quite obvious since it provides the list of the most relevant compounds within the inventory (in the same way as h papers are the most relevant ones of an author's production). H sets might be seen as a representation of the inventory, containing its crucial elements. From the practical point of view, they may provide a valuable indication of what is worth to include on a monitoring campaign.

Different H sets can be compared and "combined" through inspection of the list of h compounds contained in each one, using elemental set theory operations, such as union ($H_A \cup H_B$) or intersection ($H_A \cap H_B$), which, respectively, yield the joint lists of compounds (union) or common list of compounds (intersection), or any appropriate combination. The option of choice will be strongly case dependent.

2.3.3 Power (Zipf) law

Pareto distributions are usually displayed in bar diagrams, representing the ranked set of items (in our case chemical compounds) with their frequencies expressed according Eq. 5. An example for a real case (which will be examined later with more detail) is shown in Fig. 1a. Whereas Pareto distributions, as originally defined, are continuous probability distributions they may be also applied to discrete variables. They are typically represented by a power law that relates the frequency or probability of occurrence of an item or event and its rank k through an inverse dependence relationship. Originally formulated by the linguist Zipf, it has found applications in many domains of both social and natural sciences (for instance, the number of times a word occurs in a given language). In its more simple form, and for the purposes of the present work, it can be expressed as Eq. 6:

$$X_k = X_1 \cdot \left(\frac{1}{k}\right)^{\alpha} = X_1 \cdot k^{-\alpha} \tag{6}$$

Where X_k is the frequency (or probability) of occurrence of k element, k is the rank and α a power parameter. In our case, X_k is identified either with the percent of concentration or the normalized hazard quotient.

Since α exponent actually governs the shape of the curve (i.e., how steep or flat it is), it can be also interpreted as an alternative measure of "complexity". In the context of Eq. 6 and taking into consideration that α is comprised between 0 and ∞, the term complexity must be understood in the following sense: minimum complexity (maximum simplicity) corresponds to a 'mixture' of only one compound,

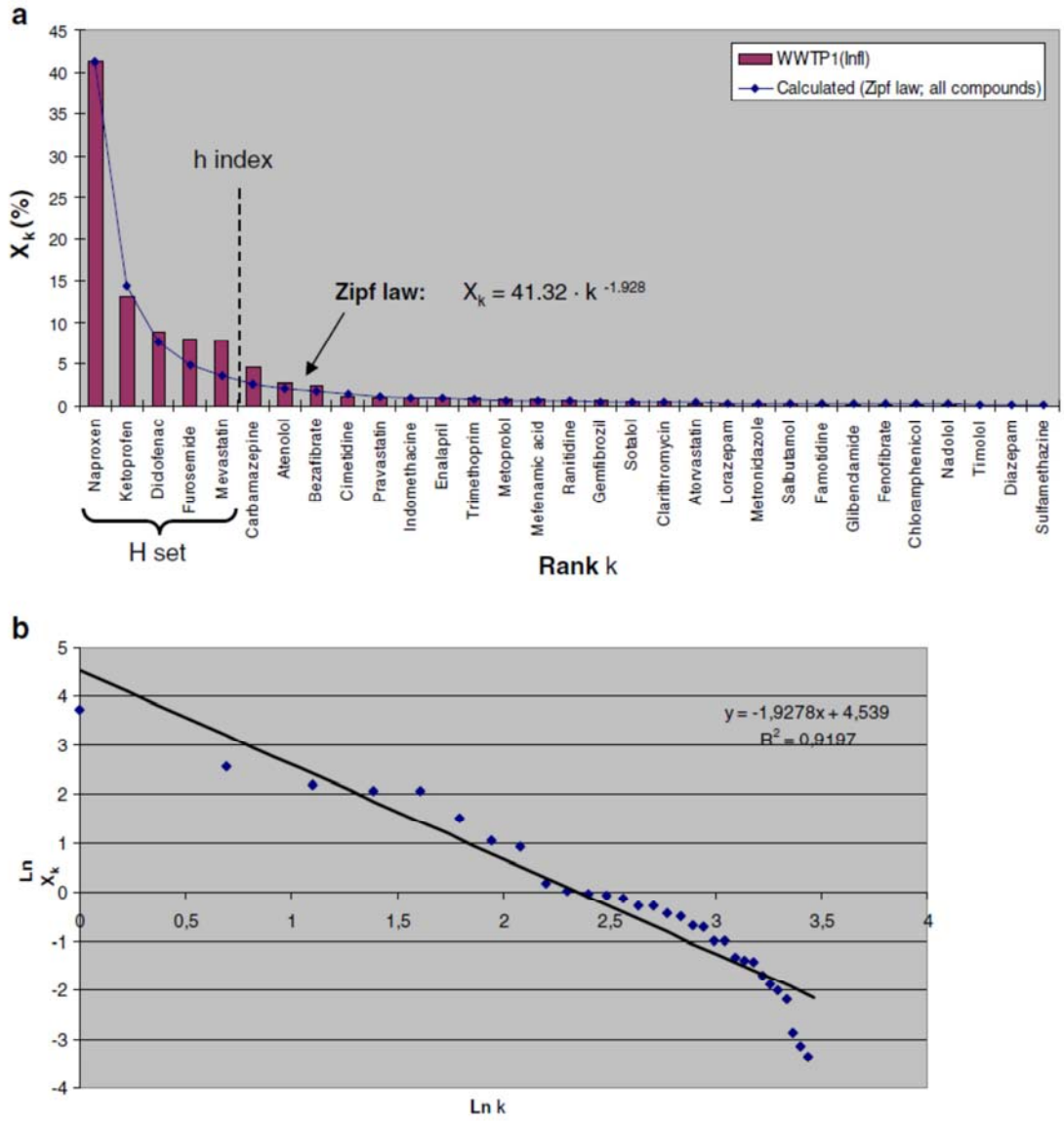

Fig. 1 Example of Pareto distribution corresponding to WWTP1 Influent concentrations showing **a** Pareto histogram, Zipf equation *h* index and *H* set **b** log–log plot

which occurs when $\alpha=\infty$ (from Eq. 6, it is straightforward to see that X_k vanishes for all $k>1$, since $k^{-\infty}=0$; only if $k=1$ then $k^{-\infty}=1^{-\infty}=1$); by contrast, the opposite situation ($\alpha=0$) can be thus qualified as 'maximum complexity'. In that case, $X_1=X_2=\ldots=X_k$, since $k^{-0}=1$ for all k. Lower exponents are thus indicative of flatter curves and hence of a more even distribution of compounds, or in other words, they would denote more complex mixtures in the above sense.

When dealing with empirical data, their fitting to a power law can be easily checked by a log–log plot of frequency vs. rank. A real example of adjusting a Zipf power distribution to empirical data and log–log plot is shown in Fig. 1b.

3 Results and discussion

In the previously referred study (Jelic et al. 2011), we have reported the occurrence of 43 pharmaceutical compounds in the influent, effluent and sludge of three wastewater treatment plants. The selected pharmaceuticals were representative of different therapeutic groups (i.e. non-steroidal anti-inflammatory drugs, lipid modifying agents (fibrates

and statins), psychiatric drugs (benzodiazepine derivative drugs and antiepileptics), histamine H2-receptor antagonists, antibacterials for systemic use, beta blocking agents, beta-agonists, diuretics, angiotensin converting enzyme inhibitors and anti-diabetics). The aim of the study was to obtain more in-depth information on their fate during conventional wastewater treatment.

In Table 2 are shown the measured concentrations of the pharmaceutical compounds detected in wastewater and sludge from the studied WWTPs. Out of 43 analyzed pharmaceutical compounds, 32 were detected in influent, 29 in effluent and 21 in sludge samples. The analysis of samples from different campaigns of a given plant showed variation in concentration levels, which was attributed to changes of the composition of influent waters in different seasons, weather conditions and operational conditions of the plant. For easier interpretation of the results, the concentrations of each pharmaceutical are given as the median and relative standard deviation values (RSD %) of concentrations measured for eight sampling campaigns. Concentrations lower than the method detection limit (m.d.l.) are marked with n.d. (i.e. not detected) and are set equal to half that value (m.d.l. × 0.5) for numerical calculations, which is an accepted common practice on multivariate chemometric analysis (Farnham et al. 2002). As explained below, only for the calculation of power law exponents these points were excluded.

PNEC values using *Daphnia* as reference organism for the compounds monitored are reported in Table S1 (Supplementary material). Among them, those showing highest ecotoxicity (lowest PNEC) values are atorvastatin, mevastatin, chloramphenicol, sulfamethazine, salbutamol and metronidazole. Hazard quotients for the influent and effluent of the three wastewater plants studied are given in Fig. 2 and Table S2 (Supplementary material). Even though no single compound was shown to exceed the unity value, the total hazard quotient for WWTP1 and WWTP2 influents did so. Overall plant performance can be easily seen comparing the influent and effluent streams in terms of either concentration or hazard quotient reduction. All the three plants studied showed depletion ratios ranging from 82% to 55% and 69% to 26% for concentration and hazard quotient respectively (Table 4). Among the three plants studied, the two having tertiary treatment (WWTP1 and WWTP3) and longer hydraulic and sludge retention times exhibited better performance than WWTP2 which has only secondary.

3.1 Compound prioritization and complexity measures

For every stream (i.e. influent, effluent and sludge) in every WWTP, the ranked or ordered inventories of both the concentrations and hazard quotients expressed as percentage were issued and the corresponding h indexes calculated (Table 3). All the h indices obtained were comprised between 4 and 7. They give an idea of the number of most relevant compounds to take into consideration in the mixture, thus providing at the same time a measure of the chemical complexity of each inventory in the foregoing explained sense. In general, h indexes are higher for concentrations (h=4 to 7) than for hazard quotients (h=4). Comparing streams for a given plant, concentration based h indexes tend to increase in the output (effluent and sludge) respect influent (Table 3, concentrations) while they remain constant for hazard quotients (Table 3, hazard quotients). As far as P_{90} indices are concerned, even though they have higher values than h indices (they are comprised between 9 to 14 for concentrations and 4 to 8 for hazard quotients), a similar trend is observed, thus providing essentially the same information.

As mentioned above, h indexes allow defining H sets or h compounds, i.e., those compounds embodied within the h index that can be truly qualified as the most relevant (Table 3). H sets were derived for all the studied streams (influent, effluent and sludge for concentrations and the former two for hazard quotients). As mentioned, all are comprised between four and seven compounds, holding for 56% to 79% and 83% to 90% of the total concentration and hazard quotient load, respectively.

In Table 3 are shown the H sets characteristic for every stream in each wastewater treatment plant. As far as concentrations are concerned, influent H sets are dominated by the non-steroidal anti-inflammatories (NSAID) naproxen, ketoprofen and diclofenac as well as by the diuretic compound furosemide, which are present in all the three H sets, being atenolol and mevastatin also present in one case. Sludge H sets are characterized by diclofenac (NSAID), furosemide, hydrochlortiazide (diuretic), clarythromycin (macrolid antibiotic) and carbamazepine (anticonvulsant), all of them included in the three sludge H sets, and in less extent by atorvastatin (two cases out of three), trimethoprim and glibenclamide (one case out of three). Effluent H sets are somewhat in-between Influent and Sludge. Thus, for instance, NSAIDs are represented (naproxen, diclofenac, ketoprofen) like in Influent H sets; it is also carbamazepine, which appears typically associated to Sludge H sets. In contrast, hazard quotient-based H sets show much less variation.

Such H sets have been combined using set theory operations, in order to obtain common patterns. In Table 4 are presented as representative examples the union $\{H_{\mathrm{WWTP1}} \cup H_{\mathrm{WWTP2}} \cup H_{\mathrm{WWTP3}}\}$ and the intersection $\{H_{\mathrm{WWTP1}} \cap H_{\mathrm{WWTP2}} \cap H_{\mathrm{WWTP3}}\}$ sets for concentrations and hazard quotients, which are readily obtained from the single H sets given in Table 3 (concentrations and hazard quotients, respectively), for each stream and plant. Joint

Table 2 Method detection limits, median concentrations and relative standard deviations (RSD, %) of the concentrations of the pharmaceuticals detected in wastewater influent, effluent and sewage sludge from the studied wastewater treatment plants (WWTP1, WWTP2 and WWTP3) during eight sampling campaigns

Compounds	MDL			WWTP 1			WWTP2			WWTP3		
	c, ng/L		c, ng/g	c, ng/L (RSD, %)		c, ng/g (RSD, %)	c, ng/L (RSD, %)		c, ng/g (RSD, %)	c, ng/L (RSD, %)		c, ng/g (RSD, %)
	Influent	Effluent	Sludge	Influent	Effluent	Sludge	Influent	Effluent	Sludge	Influent	Effluent	Sludge
Ketoprofen	3.9	2.1	0.39	2244 (42)	160 (167)	n.d.	2270 (52)	690 (81)	n.d.	1100 (55)	70 (176)	n.d.
Naproxen	6.3	0.9	0.27	7129 (40)	455 (102)	n.d.	4802 (32)	1126 (50)	n.d.	4161 (53)	105 (91)	n.d.
Diclofenac	1.2	1.2	0.6	1532 (62)	456 (65)	61 (45)	1090 (47)	785 (39)	23 (49)	385 (70)	336 (72)	40 (47)
Indomethacine	0.9	0.6	0.3	166 (115)	74 (178)	n.d.	175 (156)	128 (193)	n.d.	74 (122)	43 (174)	n.d.
Mefenamic acid	4.8	1.5	0.12	130 (85)	58 (103)	19 (110)	68 (148)	20 (50)	15 (98)	50 (265)	10 (85)	29 (65)
Bezafibrate	1.2	0.12	0.12	436 (101)	80 (73)	17 (36)	503 (59)	441 (26)	18 (46)	204 (55)	48 (53)	3.1 (51)
Fenofibrate	0.15	0.15	0.75	27 (92)	n.d.	6.7 (138)	5.2 (133)	n.d.	22 (51)	9.1 (165)	n.d.	16 (105)
Gemfibrozil	0.9	0.3	0.51	107 (137)	378 (106)	2.6 (145)	42 (265)	139 (170)	4.2 (90)	283 (186)	31 (93)	2.3 (106)
Atorvastatin	1.2	0.6	0.75	65 (54)	15 (104)	20 (81)	79 (59)	53 (120)	34 (64)	22 (82)	5.4 (155)	33 (54)
Pravastatin	7.5	2.7	0.72	179 (75)	22 (124)	n.d.	123 (74)	68 (143)	n.d.	49 (100)	19 (144)	n.d.
Mevastatin	0.6	0.6	1.4	1353 (265)	476 (265)	n.d.	208 (165)	287 (265)	n.d.	135 (265)	n.d.	n.d.
Diazepam	0.9	0.36	1.2	7.4 (103)	3.5 (103)	6.5 (52)	3.7 (76)	5.1 (48)	6.1 (71)	3.2 (100)	5.2 (51)	4.5 (30)
Lorazepam	2.1	1.2	1.5	64 (50)	50 (48)	11 (24)	93 (70)	64 (90)	10 (89)	49 (111)	31 (93)	7.8 (64)
Carbamazepine	0.6	0.6	0.06	782 (58)	539 (51)	29 (48)	664 (30)	509 (28)	23 (43)	327 (67)	367 (49)	31 (29)
Clarithromycin	1.5	1.2	2.1	86 (80)	33 (62)	55 (26)	57 (131)	50 (70)	40 (60)	44 (136)	30 (109)	44 (86)
Cimetidine	0.18	0.12	0.06	210 (70)	10 (265)	6.9 (69)	140 (40)	80 (37)	6.9 (63)	41 (83)	14 (110)	5.6 (57)
Ranitidine	0.9	0.6	0.09	113 (75)	9 (160)	7.8 (62)	221 (107)	179 (43)	4.6 (55)	127 (68)	34 (63)	4.6 (78)
Famotidine	0.3	0.21	0.03	42 (174)	n.d.	13 (19)	19 (175)	16 (174)	6.6 (46)	14 (193)	n.d.	7.5 (61)
Sulfamethazine	0.6	0.3	0.24	6.1 (137)	3.2 (150)	n.d.	10 (151)	1.7 (122)	n.d.	20 (37)	12 (166)	12 (53)
Trimethoprim	0.3	0.12	0.18	155 (60)	42 (76)	30 (66)	176 (99)	101 (59)	12 (105)	56 (193)	29 (79)	12 (35)
Metronidazole	1.8	0.21	1.7	47 (98)	30 (95)	n.d.	104 (101)	118 (84)	n.d.	15 (181)	13 (165)	n.d.
Chloramphenicol	0.6	0.18	0.06	23 (265)	17 (102)	n.d.	4 (173)	4 (91)	n.d.	n.d.	2.3 (173)	n.d.
Atenolol	2.7	2.7	0.21	490 (75)	129 (96)	9 (82)	1195 (43)	383 (52)	7.8 (74)	382 (71)	63 (57)	4.3 (73)
Sotalol	1.5	0.6	0.12	88 (201)	49 (159)	12 (127)	72 (83)	63 (49)	9.4 (106)	86 (185)	42 (90)	9.3 (112)
Metoprolol	0.6	0.6	0.36	131 (105)	82 (119)	n.d.	15 (63)	10 (75)	n.d.	n.d.	3.6 (140)	n.d.
Timolol	0.09	0.09	0.27	10 (160)	5 (94)	n.d.	11 (59)	6.8 (43)	n.d.	7.4 (173)	4.2 (91)	n.d.
Nadolol	0.24	0.06	0.09	20 (65)	5.4 (50)	2 (89)	22 (61)	13 (93)	3.2 (101)	8 (53)	n.d.	6.4 (132)
Salbutamol	0.09	0.06	0.09	44 (84)	5.8 (176)	n.d.	45 (53)	19 (49)	n.d.	12 (75)	6.7 (109)	n.d.
Enalapril	1.5	1.2	1.1	165 (53)	1.2 (211)	n.d.	344 (102)	6.5 (234)	n.d.	214 (80)	8.5 (265)	n.d.
Glibenclamide	1.2	0.6	0.3	32 (90)	18 (105)	20 (86)	60 (106)	58 (196)	19 (59)	10 (143)	11 (160)	34 (53)
Furosemide	3.9	1.8	0.15	1371 (54)	142 (79)	36 (59)	1374 (36)	865 (22)	24 (66)	865 (27)	212 (47)	43 (80)
Hydrochlorthiazide	1.5	0.21	0.12	n.d.	n.d.	37 (22)	n.d.	n.d.	39 (30)	n.d.	n.d.	32 (54)

n.d. not detected

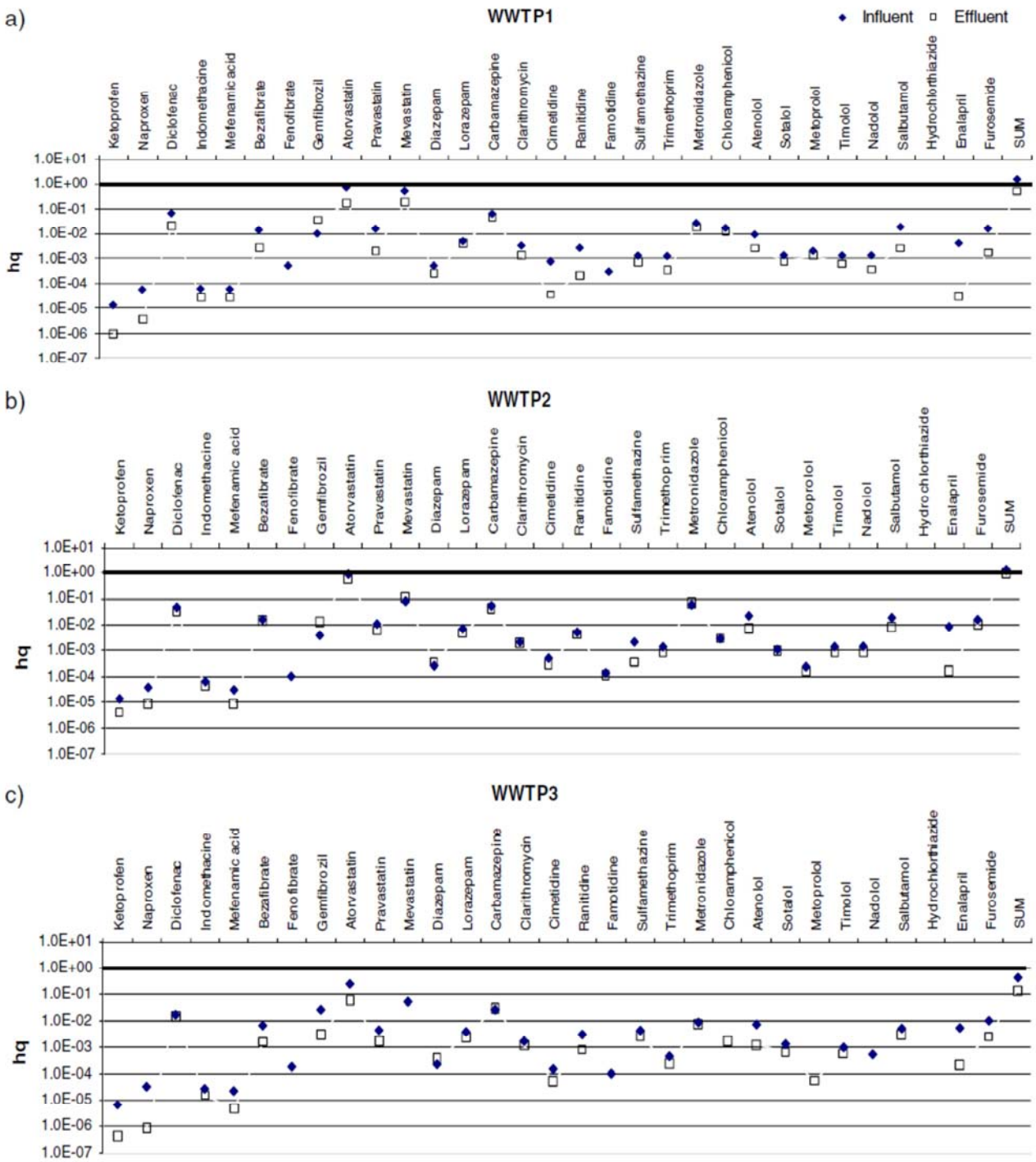

Fig. 2 Influent and effluent hazard quotients (*daphnia*) for a WWTP1, b WWTP2 and c WWTP3

sets include 14 and 6 compounds for concentrations and hazard quotients, respectively. Contrastingly, in the case of the intersection sets only one common compound, the NSAID diclofenac, is found in all the nine concentration based *H* sets, whereas for hazard quotients, the intersection has three compounds (atorvastatin, mevastatin and carbamazepin). Since in our case study, this second option tends to excessively cut off and reduce the number of selected compounds, the joint or union would be preferred to reasonably identify a combined set of priority compounds.

Table 3 Chemical complexity characteristic parameters for the different input and output streams (influent, effluent and sewage sludge) from the studied wastewater treatment plants (WWTP1, WWTP2 and WWTP3)

	WWTP 1			WWTP2			WWTP3		
	Influent	Effluent	Sludge	Influent	Effluent	Sludge	Influent	Effluent	Sludge
Concentrations									
Total concentration (ng/L or ng/g)	17257	3349	405	13996	6290	331	8754	1558	384
h index	5	5	6	5	6	6	4	4	7
H compounds	Naproxen Ketoprofen Diclofenac Furosemide Mevastatin	Carbamazepine Mevastatin Diclofenac Naproxen Gemfibrozil	Diclofenac Clarithromycin Hydrochlorthiazide Furosemide Trimethoprim Carbamazepine	Naproxen Ketoprofen Furosemide Atenolol Diclofenac	Naproxen Furosemide Diclofenac Ketoprofen Carbamazepine Bezafibrate	Clarithromycin Hydrochlorthiazide Atorvastatin Furosemide Carbamazepine Diclofenac	Naproxen Ketoprofen Furosemide Diclofenac	Carbamazepine Diclofenac Furosemide Naproxen	Clarithromycin Furosemide Diclofenac Glibenclamide Atorvastatin Hydrochlorthiazide Carbamazepine
h content (%)	79.0	70.2	68.8	76.7	70.2	55.8	74.4	65.5	67.6
P90	9	12	14	10	13	14	9	13	14
α (n)[a]	1.928 (31)	1.825 (29)	1.022 (20)	2.054 (31)	1.884 (30)	0.856 (20)	1.960 (29)	1.529 (27)	1.046 (21)
Hazard quotients									
Hazard quotient	1.588	0.519		–	1.294		0.958	–	
h index	4	4		4	4		4	4	
H compounds	Atorvastatin Mevastatin Diclofenac Carbamazepine	Mevastatin Atorvastatin Carbamazepine Gemfibrozil		Atorvastatin Mevastatin Metronidazole Carbamazepine	Atorvastatin Mevastatin Metronidazole Carbamazepine		Atorvastatin Mevastatin Carbamazepine Gemfibrozil	Atorvastatin Carbamazepine Diclofenac Metronidazole	
h content (%)	90.0	86.1		86.3	87.9		81.4	83.0	
P90	4	5		5	5		8	8	
α (n)[a]	2.803 (31)	3.224 (29)		2.894 (31)	3.142 (30)		2.739 (29)	2.894 (27)	

[a] Zipf equation exponent (in parentheses number of compounds used) $X_k = X_1 \cdot k^{-\alpha}$

Table 4 Combined (priority) compound lists based on H sets (compounds included in h indexes) for the WWTPs studied, defined as the total joint H set $\{H_{WWTP1} \cup H_{WWTP2} \cup H_{WWTP3}\}$ and intersection H set $\{H_{WWTP1} \cap H_{WWTP2} \cap H_{WWTP3}\}$ for concentrations and hazard quotients

Concentrations	Hazard quotients
$\{H_{WWTP1} \cup H_{WWTP2} \cup H_{WWTP3}\}$	
Atenolol	
Atorvastatin	Atorvastatin
Bezafibrate	Carbamazepine
Carbamazepine	Diclofenac
Clarithromycin	Gemfibrozil
Diclofenac	Metronidazole
Furosemide	Mevastatin
Gemfibrozil	
Glibenclamide	
Hydrochlorthiazide	
Ketoprofen	
Mevastatin	
Naproxen	
Trimethoprim	
$\{H_{WWTP1} \cap H_{WWTP2} \cap H_{WWTP3}\}$	
Diclofenac	Atorvastatin
	Carbamazepine

Interestingly, there are some coincidences in the concentration and hazard quotient-based union lists, being the compounds shared atorvastatin, mevastatin, carbamazepin, diclofenac and gemfibrozil. These compounds must be thus regarded as the most relevant ones from a double point of view: concentration load and ecotoxicological impact.

Finally, we have attempted fitting potential curves to the experimental results upon the basis of Zipf law compliance. All log–log plots show acceptable inverse correlations (r=−0.824 to −0.964, p<0.05). A representative example corresponding to concentrations of WWTP1 influent is shown in Fig. 1b. As it can be realized from that example, points in the 'tail' of the plot (i.e. very low values) tend to separate from the line. This is a known behaviour probably due to their inherent uncertainty (Newman 2005) and for that reason not detected values were excluded for the calculation of α exponents (i.e., the slope of the regression line). The corresponding exponents together with the number of compounds used are given in Table 3. As mentioned before, the exponents of the curves actually govern their "shape" (i.e., how steep or flat they are), lower exponents indicating flatter curves and hence more even distribution of compounds. In our case study, if one compares Zipf α exponents for concentrations (Table 3a) and hazard quotients (Table 3b) for the same plants and streams, the former are usually lower, thus showing that mass load is more evenly "distributed" among more number of compounds than hazard quotient (i.e., hazard quotient, and hence environmental risk, is accumulated in fewer compounds than mass load). When the different streams are compared for the same plant, concentration exponents generally decrease in the order Influent-Effluent-Sludge though the differences are not very high, while in the case of hazard quotients both influent and effluent based distributions show fairly similar exponents. According to our interpretation of exponents based on Eq. 6, this behaviour is consistent with the foregoing results found using h and P_{90} indexes.

4 Conclusion

Along the present paper, we have discussed the application of Pareto distributions, well known in many scientific domains, as a convenient approach to characterize chemical inventories typically obtained from environmental monitoring. Pareto distributions can be conveniently applied after proper ordering of the compound list according to its occurrence expressed as concentration or hazard quotient percentage. Appropriate indexes, such as h (Hirsch) or other alike, can be thus defined and calculated. Furthermore, such ordered distributions might be represented according to simple numerical equations, relating rank order and occurrence probability, of power type constituting an example of the application of the so-called Zipf law. Both h indices and the characteristic exponents of the Zipf equations are straightforward interpreted as measures of complexity of the overall mixture.

On the other hand, compounds included within the h index may be seen as the most representatives in the mixture, thus providing a reasonable indication of what is worth analyzing in the scenario under study.

The foregoing concepts have been applied as case study to the characterization of the pharmaceutical compounds found in the input and output streams of three urban wastewater treatment plants. Comparing input and output streams, it can be stated that whereas WWTPs efficiently reduce contamination expressed in terms of total concentration of pollutants (in our case pharmaceutical compounds) or its associated ecotoxicological risk, chemical complexity does not follow the same trend, being increased in the effluent and sludge with respect to influent in the case of concentrations, or remain the same in the case of hazard quotients. This behaviour has shown to be common to the three plants.

Determination of h compounds (H sets) in the streams of the sewage plants studied have permitted to establish the most relevant compounds to be taken into consideration (in the same form that h papers are the most relevant for a

given author's production) and have allowed to compare the different streams and plants.

The outlined concepts are of general application to any ordered list or inventory of chemical compounds and can provide an interesting and alternative way to characterize mixtures and to identify priority compounds for the scenario under study. We have shown through a practical example that the proposed approach has not merely a simple theoretical interest, but it can be advantageously used in practical cases, such as the characterization of WWTP streams and the selection of relevant compounds, which can be of value in the optimization of monitoring effort. Further work on the application of above topics is in progress.

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Supplementary material for:

New indexes for compound prioritization and complexity quantification on environmental monitoring inventories

Table S1. EC50 acute toxicity and PNEC values for *Daphnia*. (PNEC = EC50/1000)

Compounds	Daphnia EC50 mg/L	PNEC(w) mg/L	Reference
Ketoprofen	1.69E+05	1.69E+02	ECOSAR 2009
Naproxen	1.26E+05	1.26E+02	ECOSAR 2009
Diclofenac	2.20E+01	2.20E-02	Grung et al., 2008
Indomethacine	2.72E+03	2.72E+00	ECOSAR 2009
Mefenamic acid	2.22E+03	2.22E+00	ECOSAR 2009
Bezafibrate	3.00E+01	3.00E-02	Hernando et al., 2007
Fenofibrate	5.00E+01	5.00E-02	Hernando et al., 2007
Gemfibrozil	1.04E+01	1.04E-02	Hernando et al., 2007
Atorvastatin	8.60E-02	8.60E-05	ECOSAR 2009
Pravastatin	1.10E+01	1.10E-02	ECOSAR 2009
Mevastatin	2.50E+00	2.50E-03	ECOSAR 2009
Diazepam	1.41E+01	1.41E-02	2000
Lorazepam	1.28E+01	1.28E-02	ECOSAR 2009
Carbamazepine	1.19E+01	1.19E-02	ECOSAR 2009
Clarithromycin	2.57E+01	2.57E-02	Isidori et al., 2005
Cimetidine	2.71E+02	2.71E-01	Kim et al., 2007
Ranitidine	4.16E+01	4.16E-02	ECOSAR 2009
Famotidine	1.37E+02	1.37E-01	ECOSAR 2009
Sulfamethazine	4.66E+00	4.66E-03	ECOSAR 2009
Trimethoprim	1.23E+02	1.23E-01	Grung et al., 2008
Metronidazole	1.68E+00	1.68E-03	ECOSAR 2009
Chloramphenicol	1.32E+00	1.32E-03	ECOSAR 2009
Atenolol	5.14E+01	5.14E-02	ECOSAR 2009
Sotalol	6.51E+01	6.51E-02	ECOSAR 2009
Metoprolol	6.39E+01	6.39E-02	Grung et al., 2008
Timolol	7.52E+00	7.52E-03	ECOSAR 2009
Nadolol	1.46E+01	1.46E-02	ECOSAR 2009
Salbutamol	2.29E+00	2.29E-03	ECOSAR 2009
Hydrochlorthiazide	4.77E+02	4.77E-01	ECOSAR 2009
Enalapril	3.94E+01	3.94E-02	ECOSAR 2009
Furosemide	8.27E+01	8.27E-02	ECOSAR 2009

Table S2. Hazard quotients (*hq*) in wastewater influent and effluent for the studied wastewater treatment plants (WWTP1, WWTP2 and WWTP3).

Compounds	WWTP 1		WWTP2		WWTP3	
	hq		hq		hq	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
Ketoprofen	1.33E-05	9.47E-07	1.34E-05	4.09E-06	6.51E-06	4.14E-07
Naproxen	5.65E-05	3.61E-06	3.81E-05	8.93E-06	3.30E-05	8.33E-07
Diclofenac	6.97E-02	2.07E-02	4.95E-02	3.57E-02	1.75E-02	1.53E-02
Indomethacine	6.13E-05	2.72E-05	6.44E-05	4.72E-05	2.74E-05	1.60E-05
Mefenamic acid	5.85E-05	2.63E-05	3.06E-05	8.86E-06	2.25E-05	4.52E-06
Bezafibrate	1.45E-02	2.67E-03	1.68E-02	1.47E-02	6.78E-03	1.61E-03
Fenofibrate	5.30E-04	n.d.	1.04E-04	n.d.	1.82E-04	n.d.
Gemfibrozil	1.03E-02	3.63E-02	4.04E-03	1.34E-02	2.72E-02	2.97E-03
Atorvastatin	7.52E-01	1.74E-01	9.16E-01	6.14E-01	2.55E-01	6.34E-02
Pravastatin	1.63E-02	1.99E-03	1.12E-02	6.15E-03	4.45E-03	1.74E-03
Mevastatin	5.42E-01	1.91E-01	8.34E-02	1.15E-01	5.42E-02	n.d.
Diazepam	5.27E-04	2.49E-04	2.64E-04	3.62E-04	2.26E-04	3.67E-04
Lorazepam	5.02E-03	3.92E-03	7.29E-03	5.01E-03	3.85E-03	2.45E-03
Carbamazepine	6.58E-02	4.54E-02	5.59E-02	4.29E-02	2.75E-02	3.09E-02
Clarithromycin	3.36E-03	1.28E-03	2.20E-03	1.96E-03	1.70E-03	1.17E-03
Cimetidine	7.73E-04	3.51E-05	5.15E-04	2.94E-04	1.50E-04	5.33E-05
Ranitidine	2.72E-03	2.16E-04	5.31E-03	4.31E-03	3.05E-03	8.11E-04
Famotidine	3.06E-04	n.d.	1.40E-04	1.19E-04	1.02E-04	n.d.
Sulfamethazine	1.30E-03	6.93E-04	2.22E-03	3.68E-04	4.34E-03	2.68E-03
Trimethoprim	1.26E-03	3.45E-04	1.43E-03	8.23E-04	4.51E-04	2.36E-04
Metronidazole	2.80E-02	1.78E-02	6.21E-02	7.05E-02	9.00E-03	7.54E-03
Chloramphenicol	1.75E-02	1.25E-02	3.02E-03	3.02E-03	n.d.	1.74E-03
Atenolol	9.54E-03	2.51E-03	2.33E-02	7.46E-03	7.44E-03	1.22E-03
Sotalol	1.36E-03	7.49E-04	1.11E-03	9.64E-04	1.32E-03	6.38E-04
Metoprolol	2.05E-03	1.29E-03	2.41E-04	1.60E-04	n.d.	5.66E-05
Timolol	1.33E-03	6.14E-04	1.45E-03	9.10E-04	9.81E-04	5.63E-04
Nadolol	1.35E-03	3.68E-04	1.49E-03	8.92E-04	5.33E-04	n.d.
Salbutamol	1.93E-02	2.54E-03	1.97E-02	8.23E-03	5.21E-03	2.93E-03
Hydrochlorthiazide	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Enalapril	4.19E-03	3.01E-05	8.73E-03	1.65E-04	5.43E-03	2.16E-04
Furosemide	1.66E-02	1.71E-03	1.66E-02	1.05E-02	1.05E-02	1.35E-04

n.d. : not detected

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2.2.3. Issues related to the accurate estimation of the occurrence, removal and fate of pharmaceuticals during wastewater treatment

Pharmaceuticals have been detected in natural waters for more than forty years, but with improvements in analytical instrumentations and sample preparation procedures, the number of scientific publications on the issue has increased significantly (252). These studies have contributed to estimations of the magnitude of pharmaceuticals that reach the environment and better understanding of their removal and fate during wastewater treatment. These points have become increasingly interesting since the primary route of pharmaceuticals for human use to the environment is via WWTP, where households present the major source point for most of the over-the-counter and prescription drugs (253). It is worth noting that there are many uncertainties affecting the concentration values, and even more the mass loads, that are reported, which arise from the nature of disposal of pharmaceuticals, sewer system and sampling protocols (109, 176). First of all, it is impossible to predict when one pharmaceutical compound, in its parent compound form and/or as metabolite, will be flushed to sewer system. This toilet flushing event lasts only few seconds at the source, which is then extended to some minutes at the influent of a WWTP depending on hydraulic conditions and flow distances. The number of flushes that contain pharmaceuticals depends on the number of people related to the excretion/topical application. If there are only few such flushes daily, then it is obvious that only a high sampling frequency can capture them (109). The load pattern of pharmaceuticals is mainly influenced by the number of wastewater pulses entering the sewer, the pulses' initial length determined by the sanitary appliance and the transformation in the house connection (254). Another issue is related to the inlet and WWTP process. Biological wastewater treatment process is very complex and it involves a large variety of biological and chemical reactions occurring in parallel via a large consortium of microorganisms. Then, influent wastewater varies substantially both in composition and flow rate, with time scales ranging from hours to months. Also, discrete events (e.g. rainfalls) may occur from time to time, and internal disturbances may be generated due to inadequate operations. WWTP are hardly ever in steady state but rather subject to transient behaviour all the time (255). Therefore, in order to obtain accurate experimental results (e.g. concentrations or mass loads of pharmaceuticals) not only high frequency of sampling, but also flow-proportional sampling, depending on the characteristics of the sewer system, would be an experimental setup of choice. Nevertheless, it is not always feasible for economic, technical or organizational (e.g. WWTP management policy) reasons. For instance, we did not have a direct access to the sampling activities for the first study (scientific publication Nº2), and thus we had to rely on the information we received from the staff that performed the sampling campaigns. On the other hand, in the latter study

(scientific publication Nº3), we had full access to the wastewater treatment facility, and we could carry out the sampling as it was suggested elsewhere (109, 176), and as the conditions allowed (e.g. availability of portable sampling equipment and measurement tools). Here, we could calculated the overall uncertainties associated with our results from the individual uncertainties of sampling, flow measurements and chemical analysis, using the uncertainty values estimated for different sampling procedures by Ort et al (176) and information obtained from the technical staff at the WWTP, and from the data obtained during chemical analysis.

Apart from the issue related to the feasibility of capturing all the pharmaceuticals introduced into sewer system, determination of the presence of these compounds in wastewater and sludge will depend only on the sample preparation and instrumental analysis applied. Once the instrumentation is available, data quality of chemical analysis can be controlled with different quality assurance techniques such as analytical replicates, matrix spikes, surrogate/internals standard spikes, detection limits determination etc. On the other hand, removal and fate of pharmaceuticals during full-scale wastewater treatment seem to be a rather complicated topic. When we speak about the removal of pharmaceuticals, we think about the “disappearance” of the parent compound of interest. Such “removal” comprises all the losses of a parent compound produced by different mechanisms of chemical and physical transformation, biodegradation and sorption to solid matter. Only in lab-scale experimental conditions, we could control different physical/chemical/biological removal mechanisms and study which mechanism contributes mostly to the transformation of a parent compound. And the degree of the transformations can be only defined by studying the transformation products or end products of mineralization. Therefore, under “removal” we consider only the degree to which the parent compound was lost, without defining which degradation mechanism prevails.

Pharmaceuticals belonging to same therapeutic groups can have different chemical structures (Table 2.1). Biodegradation and sorption depend on the chemical structures of these compounds. Additionally, many pharmaceuticals undergo a structural transformation within the human body resulting in metabolites. These compounds, both parent compounds and their metabolites, can undergo different structural changes by a variety of biotic and non-biotic (oxidation, hydrolysis, photolysis) processes in sewer system and wastewater treatment. Such structural transformations result in changes in the physico-chemical properties that may have a significant impact on polarity, solubility, and other properties that govern their fate during wastewater treatment and in the environment (256). Additionally, operation factors can influence the removal of pharmaceuticals during wastewater treatment. Among them are the composition of wastewater, technical setup (e.g. conventional activated sludge treatment or MBR), existence of anoxic and anaerobic compartments, SRT and sludge concentrations, operating temperature and pH (257). Therefore, it is

practically impossible to observe general trends in pharmaceuticals behavior and removal during wastewater treatment. And this is exactly the conclusion of our studies and all the similar ones found in the literature on this topic: a wide variation in removal efficiencies is observed also for individual compounds, and across therapeutic classes and treatment processes. None of the analyzed pharmaceutical compounds was found to be entirely removed during biological wastewater treatment. Thus, conventional WWTP cannot be expected to be the only mechanism for controlling the entry of pharmaceuticals into the environment because they were neither designed nor can provide their complete removal. Beside development of new designs and strategies for the improvement of existing wastewater treatments, more appropriate management of wastewaters before they enter WWTPs (e.g. pre-treatment of industrial wastewater; flow split options for subsequent smaller and specifically designed treatments) should also be considered. Additionally, the amount of pharmaceuticals entering WWTP should be more consciously and efficiently controlled via source control strategies (i.e. prevention) to effectively reduce their impact to the environment (1).

2.3. Conclusions

The work presented in this chapter represents one small step towards a better understanding of the occurrence, removal and fate of pharmaceuticals during conventional wastewater treatment. The obtained results are useful for the estimations of (a) the magnitude of pharmaceuticals that reach the environment via either effluent or sludge and (b) the efficiency of the currently applied wastewater treatments regarding the elimination of pharmaceuticals. Nevertheless, more information on chronic toxicity of pharmaceuticals and on the occurrence and toxicity of their transformation products are definitely welcome especially when attempting to reuse wastewater and dispose sludge to agricultural areas and landfills.

The following specific conclusions can be drawn from the performed studies:

- Applied wastewater treatments have been shown to be only moderately effective in removing the selected pharmaceuticals. Since the removal of pharmaceuticals depends on their phisico-chemical properties, as well as on wastewater characteristics, operational conditions and employed treatment technology, high variations in removal efficiencies were expected and observed, without clear conclusion on the removal of any particular compound. No statistically significant difference in removal of pharmaceuticals from full-strength (Nº2) or diluted wastewater (Nº3) was observed. It's worth noting that the removal estimated as difference of influent and outlet (including both effluent wastewater and sludge) mass loads of parent compounds may easily be underestimated for at least two reasons: a) occurrence of transformation products was not determined, and b) sampling was not perfectly suitable for the analysis.
- The pharmaceuticals detected at the effluent of the plants cover a wide range of physico-chemical properties and biological activities. Estimated risk quotients (calculated from maximum detected concentrations and literature data for the acute toxicity of detected pharmaceuticals) indicated no risk of adverse effects for the aquatic environment when wastewater was diluted by groundwater infiltration (Nº3), but also when the detected pharmaceuticals were present in higher concentrations in full-strength wastewater (Nº4). In both cases, chronic toxicity effects of such "mixtures" are unknown and thus the real risk that they may pose to the environment could not be fully assessed, but the presence of pharmaceuticals must not be ignored.
- Considered from a double point of view, i.e. from detected concentrations and from estimated ecotoxicological impact, diclofenac, carbamazepine and atorvastatine were found to be the key compounds to be analyzed in wastewater treatment plants (Nº4).

- Although the analyzed compounds are polar molecules, some were still detected in sludge samples. This proves that also mechanisms other than hydrophobic partitioning are important for the sorption of pharmaceuticals to sludge. Nevertheless, these interactions were not strong enough, and the selected pharmaceuticals were generally found in aqueous phase. Estimated (or literature) K_d values and mass balances indicated that biotransformation is the main removal mechanism of the selected compounds during biological wastewater treatment.

Chapter III

Degradation of the anticonvulsant carbamazepine during biological and advanced oxidation processes in lab-scale reactors

Degradation of the anticonvulsant carbamazepine during biological and advanced oxidation processes in lab-scale reactors

As noted in the previous chapter, disappearance of a parent compound during wastewater treatment does not mean either that the compound was eliminated or that the applied treatment was really efficient. Instead, it can indicate that the parent compound was transformed to products that can be also pharmaceutically or biological active or/and can be toxic. For that reason, identification and quantification of transformation products formed during (wastewater) treatments, as well as the estimation of (eco)toxicity that could arise as a consequence of the transformation, are desirable and worthwhile for a comprehensive risk assessment regarding pharmaceuticals in the wastewater treatment, and in the environment, in general.

This chapter summarizes the results of two studies on the degradation of the antiepileptic carbamazepine (CBZ) in the following (independent) sets of experiments:

a) biological degradation performed in lab-scale air-pulsed fluidized reactor using white-rot fungus Trametes Versicolor, and
b) chemical degradation in the lab-scale batch reactors applying TiO_2 photocatalysis via simulated solar irradiation (SSI) and UVA (PC), and sonophotocatalysis (SPC).

In the following lines, we will give a brief introduction to white-rot fungi and the AOPs employed for the degradation studies, their application and potentials for wastewater treatment. Carbamazepine was chosen for the degradation experiments because of its persistence to conventional wastewater treatment demonstrated in many research studies, and because it was among three “key compounds” to be analysed in wastewater according to the results of the previously noted publication Nº4. The effects of the white-rot fungus and the AOPs (photocatalysis and sonophotocatalyis) on CBZ, in order to study their elimination potential in the aqueous phase, will be explained in two **scientific publications:**

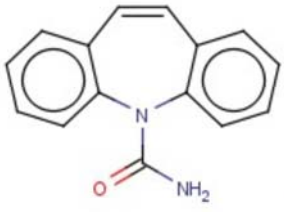
Nº5: Degradation of carbamazepine by Trametes versicolor in an air pulsed fluidized bed bioreactor and identification of intermediates (Water Research (2012) 46 (4) 955-964)

Nº6: Photocatalytic and sonophotocatalytic degradation of the anticonvulsant carbamazine and identificationof transformation products (submitted)

3.1. Why carbamazepine?

Carbamazepine is one of the most widely prescribed and very important drugs for the treatment of epilepsy, trigeminal neuralgia and some psychiatric diseases (258). The drug was discovered by chemist Walter Schindler at J.R. Geigy AG (now part of Novartis) in Basel,Switzerland, in 1953 (259). Few years after it was introduced in the treatment of epilepsy, and since 1962 it has been used for the treatment of trigeminal neuralgia (260).

CBZ, 5H-dibenzo[b,f]azepine-5-carboxamide, is an iminostilbene derivative with a tricyclic structure similar to phenytoin. CBZ is a neutral lipophilic that is practically insoluble in water and ether, but soluble in acetone, alcohol, chloroform and propylene glycol. Table 3.1 summarizes some physic-chemical properties of CBZ.



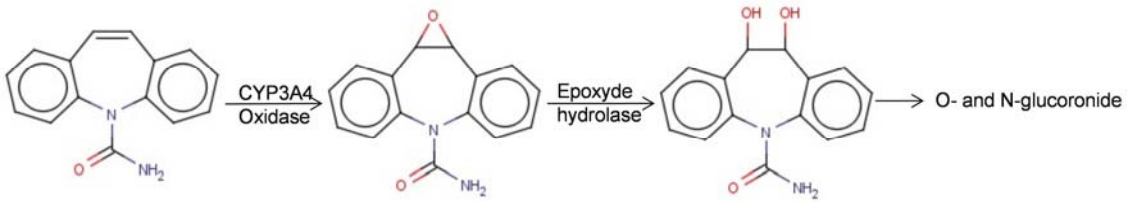
$C_{15}H_{12}N_2O$

Mw	236.27 g/mol
Names	Tegretol, Carbamazepen, Finlepsin, Carbazepine, Tegretal, Carbamezepine, Neurotol, Biston, Epitol
Water solubility	17.7 mg/L (25 °C)
logP	2.77
Henry´s Law Constant	1.09 x 10-5 Pa m3 mol-1 (25°C)
pKa	neutral
Elimination half-life in water	25-65 h
Dosage	800-1200 mg/day
Excretion	72% excreted in urine, 28% in feces

* Zhang et al, 2008; Chemaxon platforms; PubChem

Table 3.1. Physico-chemical properties of CBZ

Metabolites accounting for over 70% of the administered dose are recovered from human urine and over 30 different compounds were identified after enzymatic hydrolysis of conjugate (261). The major metabolic pathway of CBZ is the formation of the stable and active 10,11-dihydro-10,11-epoxycarbamazepine (epoxy-CBZ) by cytochrome P-450 isozyme CYP3A4. Only minimal amounts (ca. 1%) of epoxy-CBZ, however, are found as such, since it is further converted into inactive trans-10,11–dihydro-10,11–dihydroxy-CBZ (Diol-CBZ) by an epoxide hydrolase promoted hydrolysis. The Diol-CBZ and the corresponding glucuronide are therefore the main metabolites of carbamazepine (262). Several other minor metabolites have also been identified with CBZ. Early studies on the urinary metabolites of CBZ in epileptic patients identified the secondary amine linked glucuronide of CBZ, the *O*-glucuronide of *trans*-10,11-Diol-CBZ, and isomeric *O*-glucuronides of hydroxy (C-2, C-3, and others), dihydroxy, and hydroxymethoxy CBZ (263). In addition, several dihydrodiols, other oxygenated products, and four trace nmetabolites isomers of methylsulfoxyhydroxy CBZ have been also described but not quantified (264-265).



According to the Spanish Agency of Medicines and Helthcare Products (Agencia Española de Medicamentos y Productos Sanitarios) (23), consumption of CBZ in Spain was 1.3 DDD per 1000 Inhabitants per day. Its global consumption was estimated to be approximately 1000 tons per year (97). %). This antiepileptic is one of the most studied pharmaceuticals detected in the environment. It is hardly or no degraded during wastewater treatment and many studies have found it ubiquitous in various environmental matrices (soil, surface and ground water) (97, 115, 266). According to its degradability constant, k_{biol}<0.1 L g_{SS}^{-1} d^{-1} (119), CBZ has been classified in a group for which "no substantial removal by degradation" in a typical nutrient-removing municipal wastewater treatment was observed (<20%). Measured CBZ concentrations in wastewater effluents ranged from ng L^{-1} up to µg L^{-1} (60-61, 68). It was also detected in drinking water, though at low ng L^{-1} concentrations (267). Due to its persistence and ubiquitous occurrence it was proposed as a suitable marker for anthropogenic influences in the aquatic environment (115). It was found that CBZ bioaccumulates through food contamination in the aquatic environment (268), and, according to the criteria established by Council Directive 92/32/EEC (European Commission, 1992), it was classified as potentially harmful for aquatic organisms because the acute toxicity data were below 100 mg/L (269-270).

Degradation of CBZ has become a topic of concern given the fact that it is recalcitrant to biological attack and it is neither removed during conventional biological wastewater (<10%) nor membrane bioreactor treatments (<20%) (71, 92, 97, 143, 159, 271). Physicochemical processes such as coagulation-flocculation and flotation did not give better results concerning its elimination (20–35 %) (113, 160). The use of white-rot fungi has become an interesting, and a very important alternative for pharmaceuticals remediation processes due to their non-specific ligninolytic enzymatic system that includes manganese peroxidase (MnP), lignin peroxidase (LiP), versatile peroxidase (VP) and laccase (272-275). Although CBZ is not a substrate for laccase, repeated treatments with this enzyme and a redox mediator 1-hydroxybenzotriazole (HBT) led to degradation values of 60% after 48 h (276). MnP and VP produced by *Pleurotus ostreatus* have also been shown to oxidize CBZ to a significant level (98%) (277). Besides ligninolytic enzymes, inhibition experiments indicated that the cytochrome P450 (CYT P450) enzyme system also play an important role in CBZ degradation (277). In addition, a novel strategy based on the induction of hydroxyl radicals in *Trametes versicolor* using the quinone redox cycling resulted in a high percentage of CBZ degraded (80%) in 6 h (278). Also advanced oxidation

processes as ozonization (279), UV/H_2O_2 induced photolytic degradation (280) , photocatalytic degradation with TiO_2 (281), or direct photolysis (282-283), resulted in high percentages of CBZ degradation (>90%) but the main limitation is the formation of undesirable and sometimes toxic by-products (284). Therefore, the identification of the by-products, and the understanding of the degradation mechanisms is essential to assess their possible environmental impact. Although there have been an increasing number of scientific reports on the topic (285-287), the knowledge is stills scarce and more research is needed.

3.2. White-rot fungi

Lignocellulose is a renewable organic material and the major structural component of all plants. Lignocellulosic wastes are produced in large amounts by many industries including those of forestry, pulp and paper, agriculture, and food (288). Lignocellulose consists of three major components: cellulose, hemicellulose and lignin. Cellulose (45% d.w. of wood) is linear macromolecule consisting of several hundred to over ten thousand D-glucose molecules connected by β-1,4-glycosidic bonds, and represents the most abundant carbon source in the earth. Hemicelluloses (25-30% d.w. of wood) are heterogeneous macromolecules composed of pentoses (including xylose and arabinose), hexoses (mainly mannose, less glucose and galactose) and sugar acids . Lignin is a heterogeneous biopolymer composed of guaiacyl- (G), syringyl- (S) and *p*-hydroxyphenyl (H) subunits (289). It is the only naturally synthesized polymer with an aromatic backbone that provides plant support, impermeability and resistance to microbial attack. Because of the oxidative coupling of the lignin aromatic alcohol monomers, it is highly recalcitrant to degradation (290). Although lignin resists attack by most microorganisms, basidiomycetes white-rot fungi, are able to degrade lignin efficiently under aerobic conditions (290-291). The white-rot fungi (e.g. *(Phanerochaete chrysosporium*, *Trametes versicolor*, *Pleurotus ostreatus*, *Bjerkandera fumosa*, *Thelephora* sp. etc) are the only microorganisms able to mineralize lignin producing CO_2 and water by selective delignification and simultaneous rot. The name “white-rot” has been traditionally used to describe forms of wood decay where lignocellulose is broken down, leaving a light, white, rather fibrous residue completely different from the brown powder left by brown rot fungi (292).

White-rot fungus - *Trametes versiolor*

Due to their powerful degrading capabilities towards recalcitrant biopolymers, white-rot fungi have long been studied for bioremediation applications (293). Lignin-degrading enzymes, extracellularly excreted by the white-rot fungi, are responsible for

the powerful degrading capabilities of white-rot fungi. Two types of extracellular oxidative enzymes are responsible for lignin degradation: peroxidases and laccases (phenol oxidases). The peroxidases are heme-containing enzymes which require the presence of hydrogen peroxide to oxidize lignin and lignin-related compounds. The peroxidases fall into two different types based on their very different substrate spectra. Their mechanism of action entails mono-electronic processes and transforms substrates in radicals, which subsequently evolves non-enzymatic reactions. One type is manganese peroxidase (MnP), for which the optimal conditions for the secretion are the use of a substrate with low carbon and nitrogen content, supplemented with Mn^{2+}. MnP is unique in its ability to oxidize Mn^{2+}, resulting in the formation of diffusible oxidants (Mn3+) capable of penetrating the cell wall matrix and oxidizing mainly phenolic substrates (290). The other is lignin peroxidase (LiP) that oxidizes nonphenolic and aromatic compounds similar to lignin. It is unique in its ability to oxidize substrates of high redox potential, e.g. three-four rings PAHs, PCBs and dyes (up to 1.4 V); it shows the strongest oxidizing properties among peroxidases that enables it to catalyze the oxidation of non-phenolic aromatic compounds without participation of mediators, forming radical aromatic cations) (290). Versatil peroxidase is considered a hybrid between MnP and LiP. It can oxidize not only Mn^{2+} but also aromatic compounds with a high molecular weight by manganese-independent reactions (290, 294). Laccases are multicopper phenol oxidases and they oxidize phenols and aromatic amines. Rather than H_2O_2, these enzymes utilize dioxygen as an oxidant, reducing it by four electrons to water (293). Laccase shows low substrate specificity and the oxidation reaction of phenolic moieties is usually accompanied by demethylation, decarboxylation and can also result in ring cleavage (294).

Because of non-specific activity of these enzymes, and radical-based degradation mechanism, these fungi are capable of degrading a mixture of various pollutants at low concentration levels (295-297).The number of compounds known to be degraded by white-rot fungi continues to increase with the ongoing research. The mechanism of degradation is still not clear and it is rather complex, involving oxidation, reduction, methylation and hydroxylation, and numerous low molecular weight cofactors that can serve as redox mediators (293). The fungi (and their enzymes) were used for biobleaching (298-299), biodecolorization (300-301), and bioremediation (302-304). White-rot fungi could also be used for the elimination of several EDCs and the associated estrogenic activity (305). Recently, these fungi and their enzymes have been successfully applied for degradation of pharmaceutical compounds as well (274-276, 306-307).

For its wide distribution, and white rot type biochemistry, *Trametes versiolor* (also: *Coriolus versicolor*, *Polyporus versicolor*, Turkey Tail, Yun Zhi, Kawaratake) is a good candidate for bioremediation studies for the biodegradation of recalcitrant organic pollutants. A group from the Department of Chemical Engineering of the

Autonomous University of Barcelona (Departament d'Enginyeria Química, Escola d'Enginyeria, Universitat Autònoma de Barcelona (UAB)) has demonstrated many times over the potential of this fungus in degradation of pharmaceuticals, both in liquid media and sewage sludge (274, 278, 308-311).

In collaboration with the UAB group (Project TECNO), we studied the capability of Trametes verisolor to degrade CBZ in bioreactor operated in batch and continuous operation modes. We also identified transformation products formed in the time-course experiments and carried out an acute toxicity bioassay of the final (treated) aqueous media in both systems. The results of the study are presented in the **scientific publication Nº5**, titled: Degradation of carbamazepine by *Trametes versicolor* in an air pulsed fluidized bed bioreactor and identification of intermediates.

3.3. Advanced oxidation processes

Advanced Oxidation Processes (AOPs) have gained much scientific attention in last twenty years because of their ability to remove various organic pollutants from water. There are different reaction systems, but all of them follow the basic principle: production of hydroxyl radicals (·OH), or enhancement of the formation of reactive moieties, which attack organic molecules, mineralizing them or oxidizing to less complex intermediates. AOPs include homogeneous and heterogeneous photocatalysis based on UV, near-UV or solar visible irradiation, ozonation, electrolysis, the Fenton's reagents, ultrasound and wet air oxidation, and less conventional processes like ionizing radiation, microwaves, pulsed plasma and the ferrate reagent. Depending on the untreated water characteristics and the treatment objective itself, AOPs can be employed either alone or coupled with other physicochemical and biological processes (312). The most popular AOPs applied in combination are: H_2O_2/UV, O_3/UV, H_2O_2/O_3, H_2O_2/O_3/UV, UV/TiO_2/H_2O_2, ultrasound/Fenton's reagents, UV/Fenton's reagent, wet air oxidation/H_2O_2 and electrolysis/Fenton's (313). They have been widely studied for the removal of various organic contaminants, including pharmaceuticals, from aqueous solution in bench and pilot-scale reactors. In general, the advanced oxidation technologies have been shown to be very effective in removing pharmaceuticals from water (312). Generally, ·OH mediated reactions increase the number of functional groups and the polarity of molecules, two crucial features for the intended properties of pharmaceuticals, leading to disappearance of their original medicinal activity that should be proved by the analysis of oxidation products (314).

In comparison with chemical and biological processes, AOPs might be more environmentally-friendly because they neither transfer pollutants between phases as in adsorption or chemical precipitation, nor produce sludge as in bio-chemical processes (315). Nevertheless, majority of the AOPs fail to mineralize complex organic pollutants, especially in real wastewater; for the complete oxidation of the pollutants, the operating costs of AOPs remain relatively high compared to biological treatment (316). Although not mineralized, organic pollutants are oxidized to less complex intermediates which can positively affect the biodegradability of wastewater that can be then treated by conventional biological treatments (317). Thus, solar AOPs, as preliminary treatment, followed by biological treatment seem to be economically and environmentally attractive options. Technology selection and the combination of AOP/biodegradation will depend upon water characteristics, organic compound properties, and economic costs.

3.3.1. TiO_2 photocatalysis under UV-A (PC) and simulated solar irradiation (SSI)

Among the AOPs, heterogeneous photocatalysis has been investigated mostly because of practical interest in contaminated water remediation and air-pollution control. While further research in this field may make it applicable for full-strength wastewater treatment, at present it was successfully applied for the removal of pharmaceuticals only in lab-scale and pilot-scale reactors (281, 287, 318).

A key parameter in heterogeneous photocatalysis is the selection of the proper catalyst. As from the discovery of the photocatalytic splitting of water on a TiO_2 electrode under ultraviolet (UV) light by Fujishima and Honda in 1972 (319), various catalysts have been studied, such as TiO_2, CdS, SnO_2, WO3, SiO_2, ZnO, ZnS, Nb_2O_3, Fe_2O_3, $SrTiO_3$, CeO_2, Sb_2O_4 etc (320). In the last decades titanium dioxide (TiO_2) has been a preferred choice in environmental applications due to its availability, photocorrosion resistance, catalytic efficiency, low toxicity and cost (321-322). TiO_2 Degussa P25, commercially available, consists of two forms of TiO_2: 75 % anatase and 25 % rutile. Photocatalysis using Degussa P25 shows very good performances, and it has been used in many studies as a standard TiO_2-catalyst (323-324). Various organic pollutants in water can be completely decomposed and mineralized at the surface of UV-excited TiO_2 photocatalysts. In order to avoid the use of TiO_2 powder, which complicates later separation from water, it can be used in “immobilized” form, e.g. thin film form (320). While only UV light and O_2 are necessary for the reactions, many factors such as light intensity, pH, ions, photocatalysts, kinds and concentrations of substrates, etc., have a great influence on the efficiency of the mineralization process (325). Further research in photocatalytic efficiency improvement and suitable form of catalysis are needed for practical applications of TiO2 photocatalysis from the economic point of view(326).

The basic mechanism of TiO_2 phocatalytic oxidation involves the formation of electron–hole pairs induced by UV-irradiation, whose charge carriers react with chemical species such as H_2O, OH^-, and O_2 to produce hydroxyl radicals (·OH), superoxide radical anions ($O_2{\cdot}^-$), and H_2O_2 which contribute to decomposition of organics at the TiO_2 surface(Figure 3.1.) (326). A number of studies showed that the contribution of $O_2{\cdot}^-$ to the oxidation process is quite smaller than that of hole (h^+) or ·OH. Nevertheless, the reaction between $O_2{\cdot}^-$ and h^+ produces singlet oxygen ($^1O_2$), a strong oxidant, which contributes to TiO_2 photocatalysis in the fast initial processes (327). Tatsuma et al. (328) observed the remote oxidation of methylene blue at a distance of 0.5 mm from TiO_2 film and postulated that ·OH should participate in the remote photocatalytic reactions. They suggested a possible remote oxidation mechanism that includes: (1) diffusion of H_2O_2 in gas phase, (2) photolysis of H_2O_2 into ·OH radicals in the gas phase under UV irradiation, and (3) oxygenation and decomposition of organics (328).

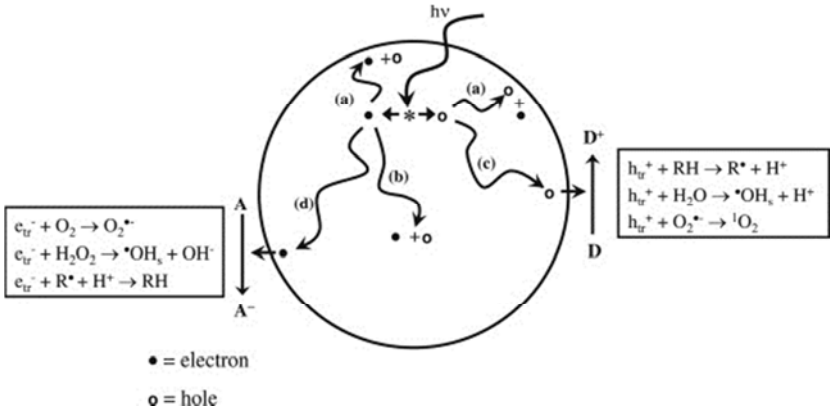


Figure 3.1. Processes occurring on exposed TiO_2 particle after UV-excitation (325)

The application field of TiO2 photocatalysis is restricted by the fact that, due to their wide band-gaps, pristine anatase and rutile TiO_2 photocatalysts mainly absorb UV-photons. Since mercury lamps as UV-source should be avoided due to their environmental risk, new techniques of irradiation has been studied recently, e.g. UV-LED (329), excimer lamp (330) and solar light (331). Heterogeneous photocatalysis using semiconductors excitable by solar light is the most attractive, but solar light only contains a small amount of UV-photons (about 5%), and room light lamps emit mainly visible photons. Therefore, starting one decade ago, much effort has been made for extending the spectral response of pure TiO_2 material to visible light, including doping TiO_2 with metal impurities, coupling TiO_2 with narrow band-gap semiconductors, preparing oxygen-deficient TiO_2, and doping TiO_2 with non-metal atoms (anion doping). Especially in the recent years, non-metal doping has become the most attractive; various non-metallic elements such as N, C, S, B, P, and F, were reported to shift the absorption threshold of TiO_2 into the visible range and provide the TiO_2 material with photocatalytic activity under visible illumination (325). Solar energy

(near-UV wavelengths) has been successfully applied in a series of experiments performed at Plataforma Solar de Almeria using parabolic reactors and TiO2 slurry photocatalysis (331). Both solar and UV induced TiO_2-photocatalysis have been successfully used to remove organic pollutants and bacteria on wall surfaces, water and air (332).

3.3.2. Sonophotocatalysis (SPC)

Another rapidly developing field in AOPs for applications in water and wastewater treatment and environmental remediation is the use of ultrasound irradiation (sonolysis) to destroy or accelerate the destruction of anthropogenic hazardous contaminants in liquid-phase (333), decontamination of sediment (334-335), surface and ultrafiltration membrane cleaning for membrane fouling control (336). Ultrasound occurs at a frequency range of 20 kHz to 500 MHz. When high-intensity, low-frequency ultrasound is introduced to a medium (e.g., water) the medium is subjected to a series of compression and rarefaction cycles (337), which result in the formation and subsequent collapse of cavitation bubbles (acoustic cavitation) (333, 338). The collapse of bubbles generates a variety of physical and chemical phenomena including high-velocity fluid movement, localized high temperature (up to 5000 K) and pressure (up to 1000 atm), resulting in the generation of highly reactive species such as ·OH, hydrogen (·H) and hydroperoxyl (·HO_2) radicals and hydrogen peroxide due to the thermal dissociation (pyrolysis) of organic compounds and water vapor present in the cavities during the compression phase (Figure 3.2) (339-341).

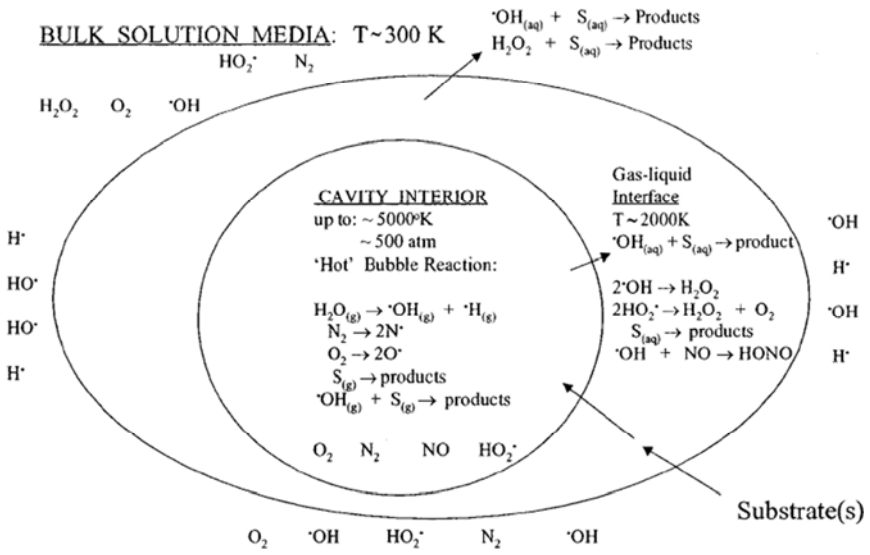


Figure 3.2. Three reaction zones in the cavitation process (341)

The concentration of ·OH at a bubble interface can be as high as 10^8 or 10^9 higher than that in the other AOP (342). Most studies involving the ultrasound-mediated contaminant degradation have adopted the “hot spot” model to explain experimental results. According to this model, sonochemical reaction is a highly heterogeneous reaction in which reactive species and heat are produced from a well-defined micro-reactor, comprised of three regions where chemical reactions occur: (i) the inside of the cavitation bubble known as the hot gaseous nucleus; (ii) the interfacial region between the gas-phase cavitation bubble and the liquid-phase bulk solution, a region with radial gradient in temperature and local radical density; and (iii) the bulk solution at ambient temperature (333, 343).

Ultrasound has been used for the waste water treatment of various pollutants such as aromatic compounds, chlorinated aliphatic compounds, explosives, herbicides and pesticides, organic dyes, organic and inorganic gaseous pollutants, organic sulfur compounds, oxygenates and alcohols, pharmaceuticals, personal care products, pathogens and bacteria in water (344). Ultrasound-mediated pollutant degradation combines simultaneous oxidation, thermolysis, shear degradation of shock waves, micro-jets pitting (345), and enhanced mass transfer and mixing together REF. Its use in industry is limited because of the inefficient conversion of energy in producing ultrasonic cavitation burdened with high operating costs. Nevertheless, it may be attractive because the operating costs may be off-set by reducing or eliminating other process costs. The use of ultrasound may enable operation at milder operating conditions (e.g., lower temperatures and pressures), eliminate the need for extra costly solvents, and reduce the number of synthesis steps while simultaneously increasing end yields, permit the use of lower purity reagents and solvents, and/or increase the activity of existing catalysts. Therefore, use of ultrasound seems to be a promising alternative for pharmaceuticals and high-value chemicals.

Of particular interest is the possibility of coupling or combining ultrasound irradiation with other AOPs (333). Such novel techniques have proven to provide treatment efficiencies that are greater than the sum of efficiencies that could be achieved by the individual processes applied alone (346-347). Combination of ultrasound with different AOPs was also found to be economically more attractive than the use of ultrasound alone for waste water treatment (344). For instance, simultaneous use of ultrasound and heterogeneous photocatalysis, i.e. sonophotocatalysis, lead to enhanced degradation of various organic pollutants (348-351). Sonophotocatalysis is a good way to take advantage of the benefits of the single technique avoiding the corresponding drawbacks (352). Ultrasound enhances reaction rates because of the formation of highly reactive radical species formed during acoustic cavitation. The influence of ultrasonic energy on chemical activity may involve any or all of the following: production of heat, promotion of mixing (stirring) or mass transfer, promotion of intimate contact between materials, dispersion of

contaminated layers of chemicals, and production of free-chemical radicals (353). When combined with TiO2 photocatalysis, the physical effects of ultrasound can enhance the reactivity of the catalyst by enlarging the surface area or accelerate a reaction by proper mixing of reagents (353). Sonophotocatalysis appears to be a very promising process for commercially feasible decontamination process. A number of research studies have examined SPC for degradation of organic contaminants such as phenols (354), chlorinated aromatic compounds (355-356), dyes (357-359), herbicides (360-361), and pharmaceuticals (348-349). Nevertheless, most of the experimental works performed were carried out in lab-scale systems with single model compounds and only few have dealt with complex synthetic or actual effluents (354, 362-363). It would be of great importance to test the system with real wastewater, and with sunlight irradiation, as this would drastically reduce the costs of application of the treatment.

In the **scientific publication Nº6**, titled: *Photocatalytic and sonophotocatalytic degradation of the anticonvulsant carbamazine and identification of transformation products,* we discuss the results of a study on degradation of CBZ by UV and solar induced TiO2 photocatalysis and sonophotocatalysis in water. This study was carried out in collaboration with a group of Environmental Engineering Department (GAIA group) of the University of Cyprus (Project PHAREM).

3.4. Identification of transformation products of CBZ

In the recent years, there was a notable increase in the use of TOF-MS and QqTOF-MS for structural elucidation and compound confirmation. These instruments provide increased resolution capacity (typically around 10000), which allows precise empirical formula assignments for unknowns, as well as extra confirmation for positive identifications in quantitative analysis. The combination of tandem MS and accurate mass measurement in QqTOF is a very powerful tool for compound identification, and thus it found important application especially in pharmaceuticals, EDCs and pesticide degradation products analysis.

We used a Waters/Micromass QqToF-system interfaced with a Waters ACQUITY UPLC system (Waters/Micromass, UK) for the qualitative analysis of TPs of CBZ formed in time-course experiments a) using Trametes versicolor and b) applying photocatalysis and sonophotocatalysis. In order to identify the molecular ions of the TPs of CBZ, first full scan MS data were collected for each time-point of the experiments, followed by acquisition of the product ion spectra of the tentatively assigned TPs at different collision energies, employing accurate mass measurements with internal mass calibration (<5 ppm mass error) for all experiments. From the accurate mass measurements, the elemental compositions of TPs were calculated

using the MassLynx software, with a tolerance set at 5 ppm, taking into account the correct analyte-lock mass (Tyrosine-valine-tyrosine,$[M + H]^+$ = *m/z* 380.2185) signal ratio. The combination of exact mass, retention time, and MS/MS data allowed a tentative identification of TPs. The mass spectra of the putative TPs were compared with the mass spectra of CBZ, to check if there were similarities in structure with the parent compound, with chemical standards, when available, and with literature data. When TPs were confirmed with corresponding chemical standards, quantitative analysis was performed using HPLC-QqLIT-MS/MS; otherwise, degradation curves were made from relative peak response areas (semi-quantitative).

3.5. Results and discussion

Scientific publications:

Nº5: Degradation of carbamazepine by *Trametes versicolor* in an air pulsed fluidized bed bioreactor and identification of intermediates, 2012, Water Research, 46 (4) 955-964

Nº6: Photocatalytic and sonophotocatalytic degradation of the anticonvulsant carbamazine and identification of transformation products (submitted)

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Degradation of carbamazepine by *Trametes versicolor* in an air pulsed fluidized bed bioreactor and identification of intermediates

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ABSTRACT

The paper describes the aerobic degradation of carbamazepine (CBZ), an anti-epileptic drug widely found in aquatic environment, from Erlenmeyer flask to bioreactor by the white-rot fungus *Trametes versicolor*. In Erlenmeyer flask, CBZ at approximately 9 mg L^{-1} was almost completely eliminated (94%) after 6 d, while at near environmentally relevant concentrations of 50 μg L^{-1}, 61% of the contaminant was degraded in 7 d. Acridone, acridine, 10,11-dihydro-10,11-dihydroxy-CBZ, and 10, 11-epoxy-CBZ were identified as major metabolites, confirming the degradation of CBZ. The degradation process was then carried out in an air pulsed fluidized bioreactor operated in batch and continuous mode. Around 96% of CBZ was removed after 2 days in batch mode operation, and 10,11-dihydro-10,11-epoxycarbamazepine was found as unique metabolite. In bioreactor operated in continuous mode with a hydraulic retention time of 3 d, 54% of the inflow concentration (approx. 200 μg L^{-1}) was reduced at the steady state (25 d) with a CBZ degradation rate of 11.9 μg CBZ g^{-1} dry weight d^{-1}. No metabolite was detected in the culture broth. Acute toxicity tests (Microtox) indicated that the final culture broth in both batch and continuous mode operation were non toxic, with 15 min EC50 values of 24% and 77%, respectively.

1. Introduction

Carbamazepine (CBZ), 5H-dibenzazepine-5-carboxamide, an iminostilbene derivative with a tricyclic structure, is one of the most widely prescribed and very important drug for the treatment of epilepsy, trigeminal neuralgia and some psychiatric diseases (Fertig and Mattson, 2008). In 2002 the annual consumption in Spain was approximately 25 tons, which increased up to 32 tons in 2006 (De la Fuente and García, 2007), and its global consumption was estimated to be approximately 1000 tons per year (Zhang and Geiβen, 2010).

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This antiepileptic is one of the most studied pharmaceuticals detected in the environment. It is hardly or no degraded during wastewater treatment and many studies have found it ubiquitous in various environmental matrices (soil, surface and ground water) (Clara et al., 2005; Joss et al., 2005; Zhang et al., 2008). Measured CBZ concentrations in wastewater effluents ranged from ng L^{-1} up to μg L^{-1} (Camacho-Muñoz et al., 2010; Gros et al., 2010; Jelic et al., 2011; Kasprzyk-Hordern et al., 2009). It was also detected in drinking water, though at low ng L^{-1} concentrations (Benotti et al., 2009).

Degradation of CBZ has become a topic of concern given the fact that it is recalcitrant to biological attack and it is neither removed during conventional biological wastewater (<10%) nor membrane bioreactor treatments (<20%) (Miao and Metcalfe, 2003; Clara et al., 2004; Joss et al., 2005; Radjenovic et al., 2007; Zhang et al., 2008). Physicochemical processes such as coagulation-flocculation and flotation did not give better results concerning its elimination (20–35 %) (Carballa et al., 2004; Suárez et al., 2008). On the other hand, advanced oxidation processes (AOP) as ozonization (Ternes et al., 2002), UV/H_2O_2 induced photolytic degradation (Vogna et al., 2004), photocatalytic degradation with TiO_2 (Doll and Frimmel, 2005), or direct photolysis (Chiron et al., 2006), resulted in high percentages of CBZ degradation (>90%) (Esplugas et al., 2007) but the main limitation is the formation of undesirable and sometimes toxic by-products (Negrón-Encarnación and Arce, 2007).

To date, the only microorganisms able to degrade CBZ are white-rot fungi (Marco-Urrea et al., 2009; Hata et al., 2010; Zhang and Geißen, 2010; Golan-Rozen et al., 2011). This group of microorganisms possesses a high capability to degrade a wide range of xenobiotics and recalcitrant pollutants due to their non-specific ligninolytic enzymatic system that includes manganese peroxidase (MnP), lignin peroxidase (LiP), versatile peroxidase (VP) and laccase (Tanaka et al., 1999; Durán and Esposito, 2000). *In vitro* experiments using LiP from *Phanerochaete chrysosporium* showed limited CBZ degradation (<10%) (Zhang and Geißen, 2010). Although CBZ is not a substrate for laccase, repeated treatments with this enzyme and a redox mediator 1-hydroxybenzotriazole (HBT) led to degradation values of 60% after 48 h (Hata et al., 2010; Marco-Urrea et al., 2009). MnP and VP produced by *Pleurotus ostreatus* have also been shown to oxidize CBZ to a significant level (98%) (Golan-Rozen et al., 2011). Besides ligninolytic enzymes, inhibition experiments indicated that the cytochrome P450 (CYT P450) enzyme system also play an important role in CBZ degradation (Marco-Urrea et al., 2009; Golan-Rozen et al., 2011). In addition, a novel strategy based on the induction of hydroxyl radicals in *Trametes versicolor* using the quinone redox cycling resulted in a high percentage of CBZ degraded (80%) in 6 h (Marco-Urrea et al., 2010a).

Aside CBZ, there is an increasing list of pharmaceuticals that are degraded by white-rot fungi, that make these organisms an interesting catalysts to be taken into account for pharmaceutical remediation processes (Blánquez and Guieysse, 2008; Rodríguez-Rodríguez et al., 2009; Marco-Urrea et al., 2009; Hata et al., 2010; Marco-Urrea et al., 2010b,c,d). However, studies applying fungus in lab-scale bioreactors are very scarce in the literature, and they commonly deal with endocrine disrupting compounds and dyes (Blánquez and Guieysse, 2008; Cabana et al., 2007; Pakshirajan et al., 2011). Due to the poor removal of CBZ in wastewater treatment plants we here evaluated the capability of *T. versicolor* to degrade this recalcitrant compound applying two different strategies of operation in bioreactor, i.e. batch and continuous. In addition, we identified transformation products formed in the time-course experiments. Finally, acute toxicity bioassay was carried out to evaluate the risk involved with the emission of the treated aqueous medium in both systems.

2. Materials and methods

2.1. Fungus and chemicals

T. versicolor (ATCC#42530) was from the American Type Culture Collection and was maintained by subculturing on 2% malt extract agar slants (pH 4.5) at room temperature. Subcultures were routinely made every 30 days.

Pellets production was done as previously described by Font et al. (2003). Pellets formed by this process were washed with sterile deionized water.

All the pharmaceutical standards were of high purity grade (>97%). CBZ, acridine, acridone and 10,11-dihydro-10,11-epoxycarbamazepine (CBZE) were purchased from Sigma–Aldrich (Barcelona, Spain). The solvents, HPLC grade methanol, acetonitrile, water (Lichrosolv) and formic acid 98% were provided by Merck (Darmstadt, Germany). Glucose, ammonium tartrate dibasic and 2,2-dimethylsuccinic acid 99% were purchased from Sigma–Aldrich (Barcelona, Spain).

2.2. Experimental procedures

2.2.1. Degradation experiments in Erlenmeyer flasks

Degradation experiments were performed in 250 mL Erlenmeyer flasks containing appropriate amounts of mycelial pellets (0.48 g dry weight) in a total volume of 50 mL of Kirk medium (pH 4.5) (Kirk et al., 1978). CBZ, from a stock solution in ethanol, was added into the flasks to give the desired final concentration (9 mg L^{-1} and 50 μg L^{-1}). After CBZ addition, flasks were incubated under constant shaking (135 rpm) at 25 °C. To avoid the possible influence of light on CBZ stability, all the experiments were carried out in the dark. A whole flask was sacrificed for analysis for each time point: at 2–3 h intervals over the first 8 h, then twice a day during next 4 days, and daily until the end of the experiment (15 days).

Degradation of CBZ in time-course experiments was evaluated by comparing its concentration in the heat-killed control flasks and in the experimental flasks. The heat-killed controls consisted of autoclaved cultures (121 °C for 30 min) that were processed under the same conditions as the experimental cultures. The amount of adsorbed CBZ was determined using the heat-killed controls.

In time-course degradation experiments, where CBZ was added at high concentrations (approx. 9 mg L^{-1}), the entire flask contents were collected at selected intervals during the experiment, and filtered through 0.45 μm glass fiber filter from Whatman (Barcelona, Spain). Subsequently, 1 mL-sample was withdrawn to be analyzed by high performance liquid

chromatography with UV detection (HPLC-UV). Also, glucose and laccase production were measured. The remaining part of the samples was used for the identification of the transformation products.

When CBZ was added at low concentration (50 μg L^{-1}), we proceeded as previously explained, but the entire flask content was pre-concentrated by solid phase extraction (SPE) and afterward analyzed by HPLC-UV. The experiment was performed in triplicate. The target compound in the liquid medium was extracted in one step by solid phase extraction with Oasis HLB cartridges (60 mg adsorbent, Waters, Barcelona, Spain) (Gros et al., 2006). Briefly, the cartridges were preconditioned sequentially with 5 mL of methanol and 5 mL of deionized water adjusted at sample pH. After that, the sample was passed through the cartridge and dried under vacuum. Then, the adsorbed compounds were eluted with methanol (2 × 2 mL) and subsequently concentrated to dryness under a gentle nitrogen stream. The extracts were reconstituted with 0.5 mL 25:75 (v/v) acetonitrile-water. Extraction efficiency of carbamazepine, evaluated by recovery experiments, was 98.5%.

2.2.2. *Degradation experiments in bioreactor*

A glass fluidized bioreactor with a volume of 1500 mL was used for the degradation experiments (Blánquez et al., 2007). Fluidized conditions were maintained by air pulses generated by an electrovalve. The electrovalve was controlled by a cyclic timer (1 s open, 5 s close) and the air flow was 12 L h^{-1}. Temperature was maintained stable at 25 °C and pH in the bioreactor was controlled at 4.5. Approximately 3.8 g dry weight pellets were inoculated. Glucose and nitrogen (as ammonium tartrate) were added at a rate of 0.879 g glucose g^{-1} dry weight pellets d^{-1} and 1.98 mg ammonium tartrate g^{-1} dry weight pellets d^{-1}, respectively.

The batch reactor medium contained 10 ml L^{-1} and 100 ml L^{-1} of micro and macronutrient solution, respectively, and 4 antifoam drops. The medium was sterilized at 121 °C for 30 min. After it was sterilized, CBZ, from a stock solution in ethanol, was added into the medium to the final concentration of approx. 200 μg L^{-1}.

The start-up of the experiments in the bioreactor, when operated in continuous mode, was the same as in the batch reactor. Medium containing CBZ at 200 μg L^{-1} was fed into the reactor at a flow rate of 114 ml h^{-1} for 10 min every hour (automatic timer), to provide a hydraulic retention time (HRT) of 3 days. Glucose and ammonium tartrate were fed from their stock solution (300 g L^{-1} and 169 mg L^{-1} respectively) at a flow rate of 21.7 mL h^{-1} for 1.2 min every hour. The biomass, in pellet form, was retained in the bioreactor throughout the experiment with no loss in the effluent and no extra addition of biomass was needed.

Samples of the liquid phase from both experiments (12.5 mL) were collected once a day until the end of the experiments and pre-concentrated as described in Section 2.2.1.

2.3. *Analytical procedures*

2.3.1. *Analysis of CBZ*

Analysis of CBZ was performed using a Dionex 3000 Ultimate HPLC (Barcelona, Spain) equipped with a UV detector at 230 nm. The column temperature was 30 °C and a sample volume of 20 μL was injected from a Dionex autosampler (Barcelona, Spain). Chromatographic separation was achieved on a GraceSmart RP 18 column (250 mm × 4 mm, particle size 5 μm). The mobile phase consisted of 6.9 mmol L^{-1} acetic acid in water adjusted to pH 4 (by NaOH) with 35% v/v acetonitrile. It was delivered isocratically at 1 mL min^{-1} as described elsewhere (Stafiej et al., 2007). The detection limit was >0.1 mg L^{-1}.

2.3.2. *Identification and quantification of metabolites*

2.3.2.1. *UPLC/ESI-QqToF-MS analysis.* Accurate mass measurements of CBZ and its biotransformation products formed in time-course degradation experiment (Section 2.2.1.) were carried out in full-scan and product ion scan mode using a Micromass QqToF-system interfaced with a Waters ACQUITY UPLC system (Micromass, Manchester, UK). Samples from the biodegradation experiments were separated on a Waters ACQUITY BEH C18 column (50 × 2.1 mm, 1.7 μm particle size) equipped with precolumn (5 × 2.1 mm) of the same packing material. The mobile phases were (A) formic acid 0.05% in water and (B) acetonitrile/methanol, 2/1. After 1 min isocratic conditions at 90% A, the portion of A was linearly decreased to 5% within 10 min. This condition was held for 2 min and then the initial mobile phase composition was restored within 1 min and maintained for column regeneration for another 2 min. The flow rate was 300 μL min^{-1}. The injection volume was 10 μL. The MS analysis was performed with an electrospray ionization (ESI) interface in the positive ion mode applying a capillary voltage of +3500 V. The nebulizer gas flow was set to 600 L h^{-1} and the drying gas flow to 50 L h^{-1} with a temperature of 350 °C. The ToF analyzer operated at a resolution of 5000 (FWHM) and ESI mass spectra were recorded in 1-s intervals with automatic switching of the dual-sprayer every 10 s for infusion of the internal calibrant for a duration of 1 s. Tyrosine-valine-tyrosine served as internal lock mass with $[M+H]^+ = m/z$ 380.2185. All MS data acquisition and processing was done using the software package MassLynx V4.1.

2.3.2.2. *HPLC/ESI-QqLIT-MS analysis (low concentration experiments).* The quantitative analysis of CBZ and its transformation products (for which their chemical standards were available) was performed using Symbiosis Pico™ (SP104.002, Spark, Holland), equipped with an autosampler and connected in series with a 4000 QTRAP Hybrid Triple Quadrupole - Linear Ion Trap mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA, USA). More information on the analytical methods is shown in the Supplementary information (SI).

2.3.3. Vibrio fischeri *luminescence reduction test (Microtox test)*

Microtox system was used for toxicity assessment. *V. fischeri* is a marine luminescent bacterium that liberates energy in the form of visible light (maximum intensity at 490 nm). Toxicity data were based on a 15 min exposure of bacteria to a filtered solution (pH 7) at 25 °C. Effluent toxicity was expressed in units of EC50. The experimental samples tested were collected from time-course degradation experiments in both batch and continuous bioreactor.

2.3.4. Other analyses

Laccase activity was assayed in 100 mM sodium phosphate buffer, at pH 5, using 10 mM of 2,6-dimethoxyphenol (DMP) as substrate and measuring the production of coerulignone as described elsewhere (Martinez et al., 1996). The molar extinction coefficient of DMP was 24.8 mM^{-1} cm^{-1} (Wariishi et al., 1992).

For determining mycelial dry weight, the cultures were vacuum filtered over preweighed glass-fiber filters (Whatman, Barcelona, Spain). The filters containing the mycelial mass were dried at 100 °C to constant weight.

Glucose concentration was measured with an YSI 2000 enzymatic analyzer from Yellow Springs Instrument and Co. (Yellow Springs, OH, USA).

3. Results and discussion

3.1. Degradation of CBZ by T. versicolor in Erlenmeyer flasks

Time-course degradation experiments performed in Erlenmeyer flasks showed that CBZ added at 9 mg L^{-1} was almost completely degraded (94%) by *T. versicolor* after 6 d of incubation (Fig. 1). In a previous report, the percentage of degradation of CBZ by this fungus was considerably lower (57%) even for a longer incubation period (7 d) (Marco-Urrea et al., 2009). This was explained by the depletion of oxygen that may have occurred in the sealed microcosms used in the study. The experiments performed at mg L^{-1} concentration range simplified the analytical procedure, but CBZ is typically found at much lower concentration in municipal wastewaters. Therefore, additional experiments were carried out at a concentration of approx. 50 μg L^{-1} in order to assess the capability of *T. versicolor* to degrade the contaminant at near environmentally relevant concentrations. As can be observed in Fig. 2, 61% of CBZ was degraded within 7 d. In all the experiments, only 17% of CBZ was removed due to adsorption in the biomass as observed from the difference in CBZ concentration between the heat-killed controls and the uninoculated ones.

Previous experiments showed that purified laccase did not significantly degrade CBZ, although the addition of a redox mediator HBT facilitated and improved the degradation (60% after 48 h) (Marco-Urrea et al., 2009; Hata et al., 2010). It is known that white-rot fungi produce lignin-related phenols or unsaturated fatty acids in the mycelium hyphae that can act as natural mediators expanding the oxidative potential of laccase for degradation of xenobiotics (Cañas and Camarero, 2010). Thus, the role of laccase on CBZ degradation cannot be underestimated. As shown in Figs. 1 and 2, after 7 d of experiments, extracellular laccase activity was approximately 100 U L^{-1} and 200 U L^{-1} in the experiments at high (mg L^{-1}) and low (μg L^{-1}) concentrations of CBZ, respectively. However, since a steep decrease of CBZ was observed during the first hours of the experiment, while laccase was still not detected (Figs. 1 and 2), no conclusive correlation between extracellular laccase activity and degradation of CBZ could be drawn.

In addition to laccase, other ligninolytic enzymes have been assumed to be involved in CBZ degradation by white-rot fungi. The addition of crude LiP from *P. chrysosporium* to CBZ resulted in degradation percentage below 10% (Zhang and Geißen, 2010), but *T. versicolor* does not produce this enzyme. Crude enzyme MnP from *Bjerkandera* sp. strain BOS55 did not have any significant effect on CBZ oxidation (Marco-Urrea et al., 2009). However, Golan-Rozen et al. (2011) reported high degradation rates of CBZ (up to 99%) in glucose peptone (GP) medium with Mn^{2+} and suggested the involvement of MnP on CBZ removal since this medium expressed genes encoding MnP in *P. ostreatus*. In absence of Mn^{2+}, the degradation of CBZ by *P. ostreatus* was attributed to the activity of another enzyme VP, on the basis of the high enzymatic activity of this enzyme (Golan-Rozen et al., 2011). Another enzymatic mechanism involved in degradation of CBZ by white-rot fungi is the CYT P450 system. Its capability of degrading CBZ in liquid medium was demonstrated when the degradation rates were compared in the presence of CYT P450 inhibitors in *T. versicolor* and *P. ostreatus* and without them (Marco-Urrea et al., 2009; Golan-Rozen et al., 2011). In our study, rather negligible levels of laccase and MnP (data not shown) were detected during the first hours in the time-course degradation experiments, which could indicate that this intracellular system was involved in CBZ oxidation at the beginning of the incubation period (Figs. 1 and 2). The fact that ligninolytic enzymes and the CYT P450 system influence CBZ

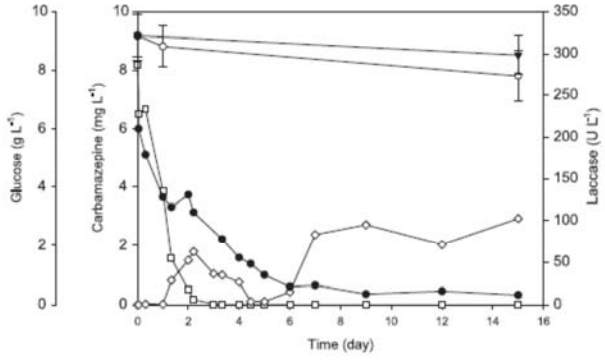

Fig. 1 – Time course of carbamazepine degradation added at 9 mg L^{-1} by *T. versicolor* pellets in Erlenmeyer flask. Symbols: uninoculated controls (▼), experimental cultures (●), heat-killed (○), glucose (□) and laccase activity (◇).

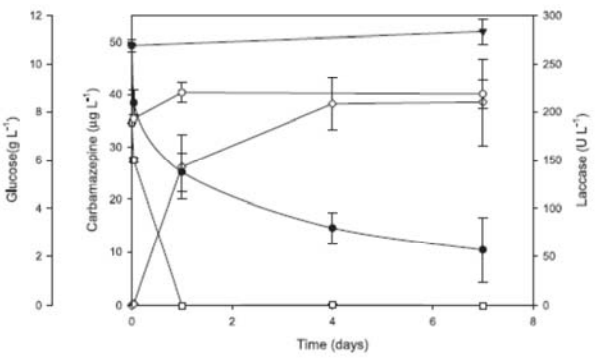

Fig. 2 – Time course of carbamazepine degradation added at 50 μg L^{-1} by *T. versicolor* pellets in Erlenmeyer flask. Symbols: uninoculated controls (▼), experimental cultures (●), heat-killed (○), glucose (□) and laccase activity (◇).

degradation indicated the applicability of whole cells of white-rot fungi in the removal of this pharmaceutical in aqueous media.

3.2. *Identification of the transformation products of CBZ*

In order to identify the molecular ions of the transformation products of CBZ, to propose empirical formulas and to elucidate their chemical structures, first full scan MS data were collected on a QqToF-MS instrument followed by acquisition of the product ion spectra of the tentatively assigned metabolites. Table 1 shows the results of these high-resolution measurements along with the relative mass errors for the proposed elemental compositions. As observed, four major transformation products from CBZ were identified when exposed to *T. versicolor*: CBZE, 10,11-dihydro-10,11-dihydroxycarbamazepine (CBZD), acridine, and acridone.

As regards the parent compound CBZ (6.95 min, in Fig. 1 – SI), it formed a protonated molecule at *m/z* 237 under (+) ESI conditions with a minor contribution of the sodium adduct at *m/z* 259. Upon collision-induced dissociation of the protonated CBZ molecule, fragment ions were detected at *m/z* 220 and *m/z* 194 corresponding to the neutral loss of NH_3 and HNCO (43 Da), respectively (Fig. 3). In search of transformation products in the samples from the biodegradation experiments, full-scan chromatograms were recorded over a mass range from *m/z* 70 to 800. This allowed discerning the emergence of five major peaks, four of which were attributed to CBZ-related metabolites having molecular ions of *m/z* 180, 196, 253 and 271 (Fig. 3).

The mass spectrum of the most intense peak in turn (3.55 min, in Fig. 1 – SI) was characterized by a number of peak clusters across the entire mass range (data not shown). At least three series of peaks with repeating and alternating *m/z* units could be discerned in the full-scan spectrum while their product ion spectra revealed a similar fragmentation pattern with the characteristics of oligomeric products. As the signal at 3.55 min was also observed in the chromatographic analysis of the test medium from a parallel study, dealing with the degradation of another pharmaceutical compound by *T. versicolor*, no further attempts were made to elucidate the structure of these apparently endogenous fungal metabolites.

With respect to the two degradates with ion masses lower than that of the parent compound, the molecular ion of the product at *m/z* 196 showed an accurate mass of *m/z* 196.0767 suggesting the elimination of C_2H_3N from CBZ. In the product ion spectrum (Fig. 3), the only detected fragment ion was at *m/z* 167 while the characteristic loss of 43 Da observed for CBZ was absent. This fragment ion was attributed to the loss of $HCO^{\cdot}$ upon formation of a radical cation as indicated by the integer double-bond equivalent (DBE). The structure of this compound was proposed to correspond to acridone, which was corroborated by the match in retention time and mass spectral fragmentation with an authentic standard. The accurate mass data obtained for the molecular ion of the breakdown product at *m/z* 180 suggested an elemental composition of $C_{13}H_{10}N$ (−3.9 ppm). Fragmenting the $[M+H]^+$ ion with a collision energy of 33 eV yielded signals at *m/z* 152 ($-C_2H_4$) and 128 ($-C_4H_4$) yet the precursor ion proved fairly stable. At more energetic conditions in the collision cell the dissociation process led to complex spectra of many peak clusters with ions of similar abundance. Based on the likely molecular formula and the fragmentation scheme, the metabolite at *m/z* 180 was proposed to correspond to the aromatic nitrogen heterocycle acridine. Analysis of a commercially available standard of acridine under identical UPLC-MS conditions corroborated the metabolite identity.

The extracted ion chromatogram of *m/z* 253, i.e. the ion mass of possible monooxygenation products, displayed a dominant peak at a retention time of 5.59 min (Fig. 1 – SI). Accurate mass measurements on the QToF-MS instrument were in line with the postulated incorporation of an oxygen atom into the CBZ structure ($C_{15}H_{13}N_2O_2$ for the $[M+H]^+$). The product ion spectrum of *m/z* 253 resembled the CBZ spectrum in that protonated molecule underwent loss of ammonia (17 Da) or HNCO (43 Da) to produce the ions at *m/z* 236 and 210,

Table 1 – Accurate mass measurements of the biodegradation products of CBZ as determined by UPLC–(+)ESI-QqToF-MS. Data for (pseudo)-molecular ions correspond to acquisitions in full-scan mode, those of fragment ions to product ion spectra of the protonated molecules.

Retention time [min]	Compound	Ion	Measured mass [*m/z*]	Elemental composition	Calculated mass [*m/z*]	Relative error [ppm]	Double-bond equivalents
3.05	Acridine	$[M+H]^+$	180.0806	$C_{13}H_{10}N$	180.0813	−3.9	9.5
		152	152.0581	$C_{11}H_6N$	152.0500	53.2	9.5
		128	128.0518	C_9H_6N	128.0500	14.1	7.5
4.59	CBZD	$[M+Na]^+$	293.0909	$C_{15}H_{14}N_2O_3Na$	293.0902	2.4	9.5
		$[M+H]^+$	271.1096	$C_{15}H_{15}N_2O_3$	271.1083	4.8	9.5
		253	253.0986	$C_{15}H_{13}N_2O_2$	253.0993	3.6	10.5
		236	236.0700	$C_{15}H_{10}NO_2$	236.0712	−5.1	11.5
		210	210.0947	$C_{14}H_{12}NO$	210.0909	8.3	9.5
5.59	CBZE	$[M+H]^+$	253.0985	$C_{15}H_{13}N_2O_2$	253.0977	3.2	10.5
		236	236.0711	$C_{15}H_{10}NO_2$	236.0712	−0.4	11.5
		210	210.0918	$C_{14}H_{12}NO$	210.0919	−0.5	9.5
		180	180.0825	$C_{13}H_{10}N$	180.0813	6.7	9.5
5.65	Acridone	$[M+H]^+$	196.0763	$C_{13}H_{10}NO$	196.0762	0.5	9.5
		167	167.0743	$C_{12}H_9N$	167.0735	4.8	9.0
6.95	CBZ	$[M+H]^+$	237.1027	$C_{15}H_{13}N_2O$	237.1028	−0.4	10.5
		194	194.0975	$C_{14}H_{12}N$	194.0970	2.6	9.5

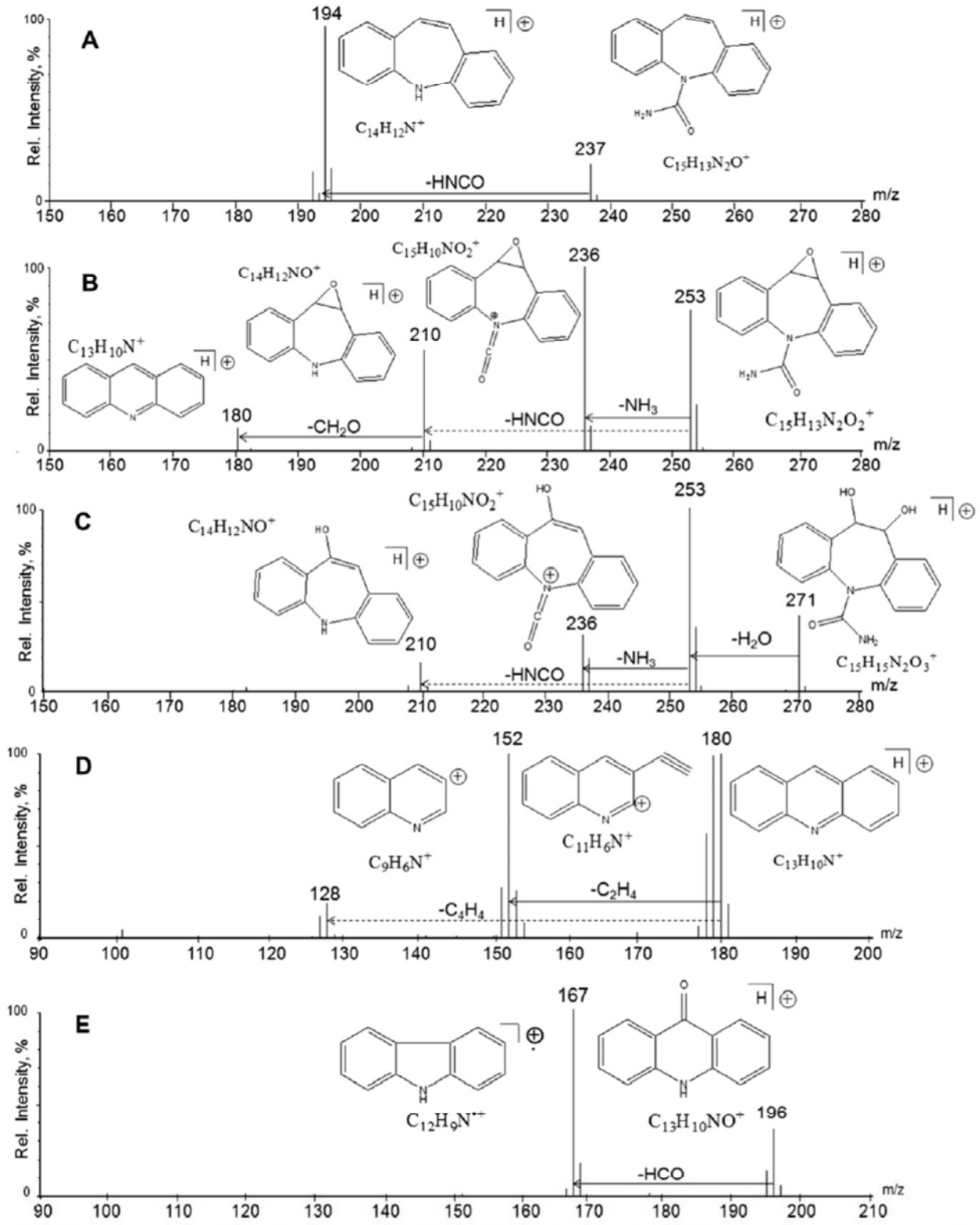

Fig. 3 – UPLC-(+)ESI-QqToF-MS product ion spectra of (A) CBZ, $[M + H]^+ = m/z$ 237 (CE 14 eV); (B) CBZE, $[M + H]^+ = m/z$ 253 (CE 8 eV); (C) CBZD $[M + H]^+ = m/z$ 271 (CE 5 eV); (D) acridine, $[M + H]^+ = m/z$ 180 (CE 33 eV); (E) acridone, $[M + H]^+ = m/z$ 196 (CE 35 eV).

respectively. The ion *m/z* 180 was rationalized to originate from *m/z* 210 by loss of formaldehyde (−0.3 ppm) resulting in a stabilized fragment ion with the structure of the protonated acridine (cf. Fig. 3). Hydroxylation of the phenyl ring was ruled out because standard solutions of 2-hydroxy and 3-hydroxy-CBZ gave distinct mass spectra (data not shown). Epoxidation of the double bond in the central 7-membered ring was therefore proposed, which was further supported by the (+) ESI-MS^2 data of CBZE reported in Miao and Metcalfe (2003) where the same set of fragment ions was described. As far as the transformation product at 4.95 min is concerned, the full-scan mass spectrum showed beside the molecular ion $[M + H]^+$ at *m/z* 271 an abundant sodiated molecule at *m/z* 293 indicating the ease for coordinating the metal cation. The proposed elemental composition was $C_{15}H_{15}N_2O_3$ for *m/z* 271.1096 with a mass error of 4.8 ppm (Table 1). Generation of the product ion spectrum of the $[M + H]^+$ resulted in three fragment ions at *m/z* 253, 236 and 210 corresponding to dehydration followed by the loss of ammonia or HNCO (Fig. 3), respectively. This fragmentation pathway was consistent with CBZD, presumably the hydrolysis product of the aforementioned epoxide in the degradation pathway of *T. versicolor*. The fragmentation pattern of *m/z* 271 in the present study matched with the authentic standard described elsewhere (Miao and Metcalfe, 2003). The observation of the intense sodium adduct in the full-scan mass spectrum of the present study reflected the capacity of the analytes to complex the cation via the hydroxyl groups of the vicinal diol.

Fig. 4 depicts the decay of CBZ (added at approx. 9 mg L^{-1}) and the evolution of its transformation products during the experiment in Erlenmeyer flasks. About 94% of the initially present amount of CBZ was eliminated already after 6 d, and no further decrease of the concentration was observed. Three of four products, CBZE, acridone and acridine, were formed in the first few hours of the experiment, with different rates of production; whereas CBZD emerged after one day. After 2 d, all degradation products concentration remained constant along the time except acridine, which began to be removed from day 9.

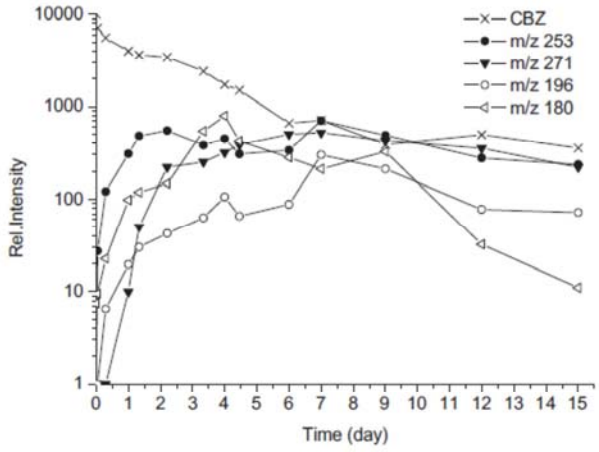

Fig. 4 – Plot of degradation of CBZ and evolution of its metabolites in Erlenmeyer flask experiment using *T. versicolor* (CBZ added at 9 mg L^{-1}).

CBZE was reported to be the major subproduct of the transformation of CBZ by the fungi *Cunninghamella elegans* and *Umbelopsis ramanniana* (Kang et al., 2008). Hata et al. (2010) also reported the formation of CBZE in the presence of laccase. Golan-Rozen et al. (2011) identified CBZE as major metabolite of CBZ degradation with whole cells of *P. ostreatus* and reported minor amounts of CBZD and 2- or 3-hydroxycarbamazepine. It is worth mentioning that the first step of the oxidative breakdown of CBZ in the CYT P450-mediated metabolism in humans is the oxidation to CBZE (Sillanpaa, 1996) and consecutively to CBZD and other hydroxylated compounds (Lertratanangkoon and Horning, 1982). The CYT P450 system has been shown to have a major role on CBZ degradation in white-rot fungi (see Section 3.1) and the identification of the metabolites in this and similar studies (Golan-Rozen et al., 2011) might serve as an additional proof of its involvement. Nevertheless, in the present study we cannot demonstrate that CBZD was formed by oxidation of CBZE. As for acridine, it was detected in the study of Hata et al. (2010) when the redox mediator HBT was added to the medium. Acridine was also found as major photodegradation intermediate (Chiron et al., 2006), and it was detected as a transformation product of CBZ exposed to a UV/H_2O_2 treatment (Vogna et al., 2004).

3.3. *Degradation of CBZ in bioreactor and evaluation of the toxicity of the culture broth*

The high degradability showed by white-rot fungi is in contrast with the negligible levels of CBZ elimination showed in conventional biological wastewater treatment systems. Therefore, the next step to assess the use of this technology is the application of white-rot fungi in bioreactors.

First, a batch bioreactor was used for the degradation of CBZ added at approx. 200 μg L^{-1}. As observed in Fig. 5A, almost complete removal of CBZ (95.6%) was obtained within 48 h. The higher degradation efficiency obtained here, in comparison with Erlenmeyer flasks, can be explained by the continuous addition of glucose, pH control and air pulses supplies that allow the fungus to thrive and obtain better degradation yields. Regarding the operational parameters, glucose was continuously consumed throughout the experiment, which indicates that *T. versicolor* was active until the end. Low activity values of laccase were detected during the process (up to 18.97 U L^{-1} at day 5) and the pH was maintained at 4.5. CBZE was found as unique metabolite in batch bioreactor treatment at a concentration of 127 μg L^{-1}, when CBZ was almost completely degraded within two days (Fig. 5A). After this point, CBZE tended to drop but at 5 d began to accumulate in the broth up to a concentration of 80 μg L^{-1}. The fact that no other metabolites were detected is attributed to the low concentration of the parent compound (CBZ) in this experiment that produced the minor metabolites at concentrations below the detection limit.

The next step in the experiments was to operate the bioreactor in continuous mode (Fig. 5B). Hydraulic retention time of 3 d was used with the aim to degrade CBZ but to avoid, as less as possible, the appearance of its transformation products. After 20 d of the experiment, when the steady state was reached, CBZ concentration in the outflow decreased to 54% of its inflow concentration, where the CBZ degradation rate was 11.9 μg CBZ g^{-1} dry weight pellets d^{-1}. Regarding the

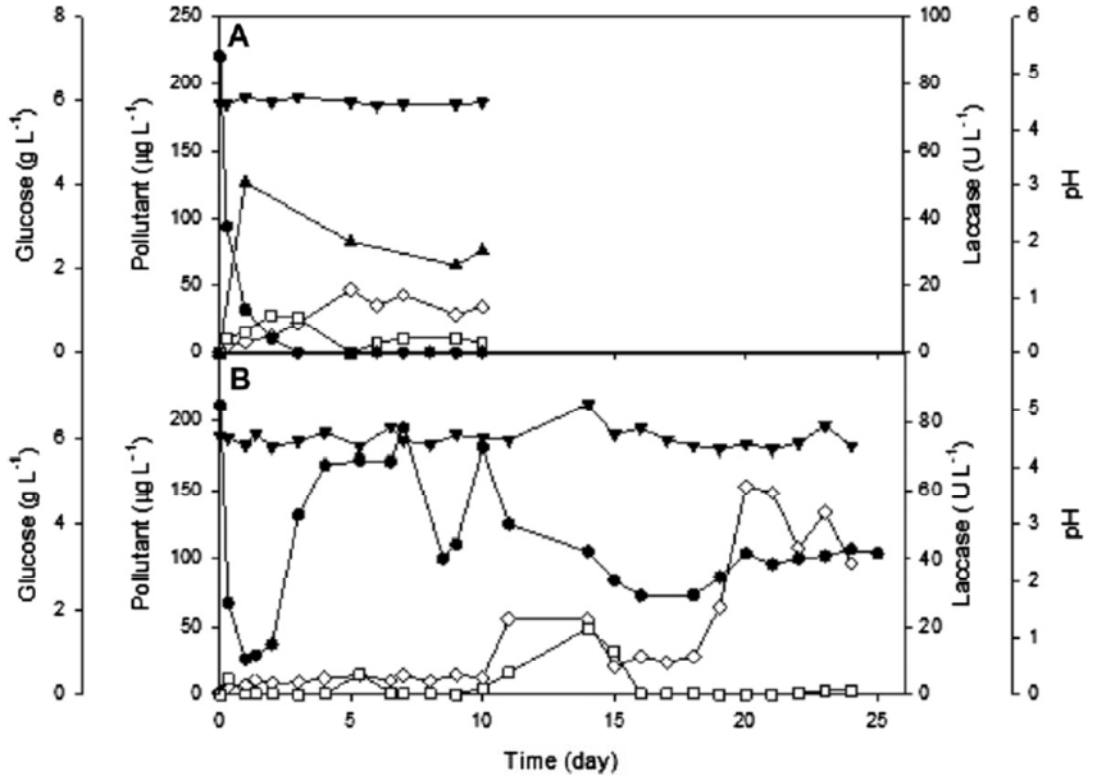

Fig. 5 – Concentration profiles of CBZ and its CBZE metabolite in degradation study using *T. versicolor* pellets in batch (A) and continuous (B) bioreactor treatment. In addition, analysis of glucose, laccase production and pH are included. Symbols: CBZ (●), CBZE (▲), glucose (□), laccase activity (◇) and pH (▼).

continuous addition of glucose, no accumulation was observed in the steady state, indicating that the fungus was active and therefore was not necessary to re-inoculate biomass at least during the first 25 d. The pH was maintained at 4.5. Samples taken on the 3rd, 10th and 25th days were analyzed to identify transformation products but no metabolites were detected, probably due to their low concentration, as stated above.

Finally, a standard bacterial bioassay (Microtox) was performed for assessing the toxicity of the treated aqueous medium. The control containing CBZ at 200 µg L^{-1} was analyzed by Microtox test and showed a 15 min EC50 of 95%. At the end of the batch bioreactor treatment (10 d), the measured acute toxicity, expressed as 15 min EC50, was 24.0%. In continuous mode, a 15 min EC50 of 77% was determined in the steady state (23 d). These results showed low toxicity in both bioreactor treatments. However, in both cases (bath and continuous modes) the results of the acute toxicity were below the EC50 of the control indicating that transformation products of CBZ may be more toxic than the parent compound.

4. Conclusion

The results of this study indicated that *T. versicolor* is capable of degrading CBZ in aqueous medium in an air pulsed fluidized bioreactor operated in batch and continuous mode. Acridone, acridine, CBZE and CBZD were identified as the major transformation products of CBZ degradation. In batch reactor, CBZ concentration decreased by 96% within 2 days. More than a half (54%) of CBZ fed to the bioreactor operated in continuous mode (HRT 3 d) was removed when the process reached the steady state. Acute toxicity test showed that the final culture broths in both batch and continuous mode operation were non toxic. Therefore, the applied treatment might be a good strategy for the degradation of CBZ. Nevertheless, further experiments are planned to study the degradation of CBZ by *T. versicolor* in bioreactor fed with real domestic wastewater, and then evaluate the possibility of full scale application of the process.

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Appendix. Supplementary information

Supplementary material related to this article can be found online at doi:10.1016/j.watres.2011.11.063.

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Supplementary information

Degradation of carbamazepine in bioreactor by *Trametes versicolor* and identification of intermediates

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A) Additional information on the HPLC/ESI-QqLIT-MS analysis used: The quantitative analysis of CBZ and its transformation products was performed using Symbiosis Pico™ (SP104.002, Spark, Holland), equipped with an autosampler and connected in series with a 4000 QTRAP Hybrid Triple Quadrupole - Linear Ion Trap mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA, USA). Selected samples were analyzed using an adoptedChromatographic separation was achieved with a Purospher Star RP-18 endcap-ped column (125mm x 2.0mm, particle size 5 mm) preceded by aC18 guard column (4 mm x 4 mm, particle size 5 mm), both supplied by Merck (Darmstadt, Germany).The mobile phases were (A) acetonitrile and (B) HPLC grade water with 0.1% formic acid. The gradient was as follows: isocratic for 5 min at 5 % eluent A, linear increase to 95 % A within 10 min, hold for 2 min, return to initial conditions in 2 min, equilibration for 3 min. The flow rate was 300 µL/min and the injection volume was 20 µL. For the analysis, the Turbo Ion Spray source was operated in the positive ion mode using the following settings for the ion source and mass spectrometer: curtain gas 30 psi, spraying gas 50 psi, drying gas 50 psi, drying gas temperature of 700 °C and ion spray voltage of 5500 V. The transitions for multiple reaction monitoring (q1: quantifier ion, q2: qualifier ion), declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) were as follows: for CBZ: *m/z* 237→194 (DP 76 V, CE 29 eV, CXP 19 V); for CBZE: q1 *m/z* 253→180 (36 V, 35 eV, 14 V) and q2 *m/z* 253→236 (36 V, 15 eV, 12 V); for acridine: q1 *m/z* 180→152 (56 V, 47 eV, 8 V), and for acridone: *m/z* 196→167 (60 V, 47 eV, 10 V).

B)

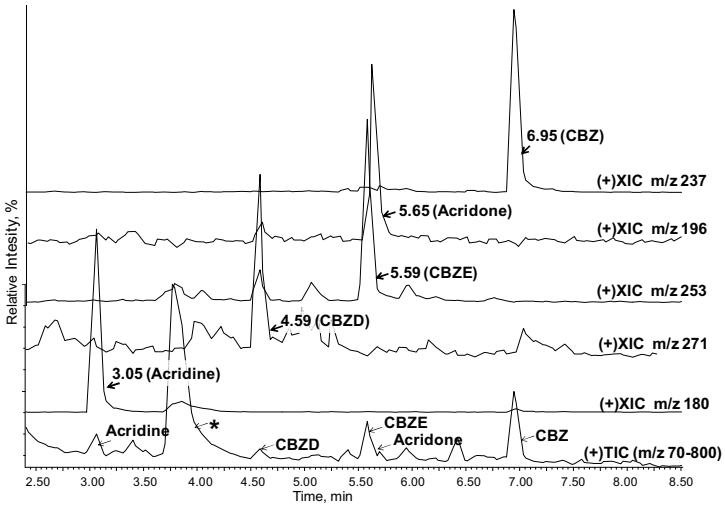

Figure 1 SI: UPLC- ESI-QqToF-MS chromatograms of a sample taken on the third day of the incubation of CBZ by *T. versicolor*: (+)XIC of m/z 237 (CBZ), (+)XIC of m/z 196 (acridone), (+)XIC of m/z 253 (CBZE), (+)XIC of m/z 271 (CBZD)), (+)XIC of m/z 180 (acridine) and (+)TIC (m/z 70-500).

Photocatalytic and sonophotocatalytic degradation of the anticonvulsant carbamazepine and identification of transformation products

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Abstract. Abstract. We studied the degradation of the antiepileptic carbamazepine (CBZ) in aqueous media during TiO2-photocatalysis under UV-A and simulated solar irradiation, and under the combined use of ultrasonic sound waves and UV-A irradiation (sonophotocatalysis). UV-driven photocatalysis and sonophotocatalysis have been shown to be very effective in degrading CBZ. About 95% of the initially present CBZ was transformed after 120 min of the experiments. On the other hand, only 10 % of CBZ was photodegraded during solar-driven photocatalysis. The mineralization of the substrates was found to be lower than the CBZ degradation, i.e. around 40 % in all the experiments, which indicates that a considerable organic load remained, attributed to the presence of the persistent oxidation products. Ultraperformance liquid chromatography coupled to a quadrupole-time-of- flight mass spectrometry was used for a tentative identification of the transformation products of CBZ formed during the conducted experiments. The studied photocatalytic processes yielded oxygenated transformation products, which were formed by hydroxylation and further oxidation of the molecule of CBZ. Acute toxicity test (*D.magna* immobilization test) showed an increase in toxicity of the treated samples during the experiments, which can be associated with the formation of CBZ-related transformation products.

Keywords: carbamazepine, photocatalysis, sonophotocatalysis, transformation products

1. Introduction

Advanced analytical methods have allowed for the measurement of pharmaceutically active compounds in wastewater effluent and surface waters, giving rise to a concern about their possible impact on the environment, and on human and animal health. It is clear now that conventional biological wastewater treatments have limited capability of removing pharmaceuticals from wastewater (Kasprzyk-Hordern et al., 2009; Wick et al., 2009). A representative example is the antiepileptic drug carbamazepine (CBZ) that has been frequently detected in various environmental compartments, including surface and ground water, sediment and soil (Clara et al., 2005; Joss et al., 2005; Zhang et al., 2008), and in drinking water (Benotti et al., 2009). According to its degradability constant (k_{biol}<0.1 L g_{SS}^{-1} d^{-1}(Joss et al., 2006), CBZ has been classified in a group for which “no substantial removal by degradation” in a typical nutrient-removing municipal wastewater treatment was observed (<20 %). Due to its persistence and ubiquitous

occurrence it was proposed as a suitable marker for anthropogenic influences in the aquatic environment (Clara et al., 2004). It was found that CBZ bioaccumulates through food contamination in the aquatic environment (Vernouillet et al., 2010), and, according to the criteria established by Council Directive 92/32/EEC (European Commission, 1992), it was classified as potentially harmful for aquatic organisms because the acute toxicity data were below 100 mg/L (Fent, 2008; Quinn et al., 2008).

In order to improve the efficiency of the conventional wastewater treatment for the removal of pharmaceuticals and other organic contaminants, various advanced treatment technologies, such as biological membrane filtration, UV irradiation and Advanced Oxidation Processes (AOPs), have been proposed as upgrade options. Among the AOPs, heterogeneous photocatalysis is an emerging technology that appears to be a promising tool for wastewater treatment as it has been successfully applied for the removal of pharmaceuticals and other micro-pollutants from water in lab-scale reactors (Coleman et al., 2004; Doll and Frimmel, 2005; Radjenović et al., 2009b). A key parameter in heterogeneous photocatalysis is the selection of the proper catalyst. In the last decades titanium dioxide (TiO_2) has been a preferred choice in environmental applications due to its availability, photocorrosion resistance, catalytic efficiency, low toxicity and cost (Dalrymple et al., 2007; Pirkanniemi and Sillanpää, 2002). Another rapidly developing field in AOPs for applications in environmental remediation is the use of ultrasound irradiation to destroy or accelerate the destruction of liquid-phase contaminants (Adewuyi, 2005). Of particular interest is the possibility of coupling or combining ultrasound irradiation with other AOPs (Adewuyi, 2005). Such novel techniques have proven to provide treatment efficiencies that are greater than the sum of efficiencies that could be achieved by the individual processes applied alone (Augugliaro et al., 2006; Comninellis et al., 2008). For instance, simultaneous use of ultrasound and heterogeneous photocatalysis, i.e. sonophotocatalysis, lead to enhanced degradation of various organic pollutants (Davydov et al., 2001; Madhavan et al., 2010a; Peller et al., 2003; Stock et al., 2000). Sonophotocatalysis is a good way to take advantage of the benefits (e.g. mass transfer resistance is eliminated, higher rate of generation of radicals etc.) of the single technique avoiding the corresponding drawbacks (e.g. mass transfer limitations and fouling of the catalyst) (Gogate and Pandit, 2004).

As regards the application of AOPs for the degradation of CBZ, ozonization (Ternes et al., 2002), UV/H_2O_2 induced photolytic degradation (Vogna et al., 2004), photocatalytic degradation with TiO_2 (Doll and Frimmel, 2005), and direct photolysis (Calza et al., 2012; Chiron et al., 2006), all resulted in high percentages of CBZ degradation. The main limitation of the AOPs is the formation of undesirable and sometimes toxic by-products as a result of non selective free radical reaction in liquid media (Negrón-Encarnación and Arce, 2007; Radjenović et al., 2009). Therefore, the identification of the by-products, and the understanding of the degradation mechanisms is essential to assess their possible environmental impact. Although there have been an increasing number of scientific reports on the topic (Medana et al., 2008; Pérez-Estrada et al., 2007; Radjenović et al., 2009a), the knowledge is stills scarce and more research is needed.

The current study focuses on the detailed mass spectrometric characterization and identification of the TPs resulting from the TiO_2 photocatalytic degradation of CBZ under UV-A (PC), solar irradiation (SSI) and combined ultrasound and UVA irradiation (SPC) in aqueous matrices by using liquid chromatography quadrupole-time-of-flight mass spectrometry (LC-QqToF-MS). A comparison among the treatment processes applied is also provided regarding the CBZ degradation and DOC removal. In addition the acute toxicity of CBZ and its oxidation products generated during the processes was assessed.

2.1. Materials and Methods

2.1. Chemicals

All the pharmaceutical standards were of high purity grade (>97%). CBZ was purchased from Sigma Aldrich (Munich, Germany), and 2-hydroxy CBZ and 3-hydroxy CBZ were obtained from Toronto

Research Chemicals Inc. (Ontario, Canada). Acetonitrile, methanol and water used for the LC-MS analysis were Chromasolv LC grade solvents provided by Sigma Aldrich's Riedel de Häen (Steinheim, Germany). The catalyst Aeroxide® TiO_2 P25 (anatase/rutile, 80/20, 21nm particle size, $50m^2/g$ BET area) was supplied by Evonik Industires AG.

2.2. Water matrices

The PC and SSI experiments were performed in three different matrices: ultrapure - demineralized water (DW), groundwater (GW) and effluent wastewater (WW). The wastewater was collected after the tertiary treatment (i.e. sand filtration and chlorination) of an urban activated sludge wastewater treatment plant ($WWTP_{Eff}$). Groundwater (GW) was collected from a pumping well by the Geological survey department of Cyprus.

The physico-chemical characterization of the wastewater and groundwater is summarized in Tables 1 and 2, respectively. All the values are given as average of triplicate analysis. The analysis of groundwater was carried out by the Geological survey department of Cyprus, and the analysis of wastewater used for the experiments was performed by the WWTP laboratory staff.

Table 1. Characteristics of the wastewater used in the experiments, before addition of CBZ

Parameter	Temperature °C	Conductivity µS/cm	DO mg/L	pH --	Turbidity NTU	TOC mg/L	SST mg/L	COD mg/L	Total-N mg/L	Total-P mg/L	Chlorides mg/L	NH_4-N mg/L
Value	19.7	1424	4	8.22	1.9	11	4.6	6	3.7	2.96	196	0.16

Table 2. Characteristics of the groundwater used in the experiments, before addition of CBZ

Parameter	Sample depth M	Water level M	Total hardness mg/L	Sulphates mg/L	Chlorides mg/L	Boron mg/L
Value	207	181	180	1.25	50	0.1

2.3. Experimental procedures

2.3.1. Dark experiment

The experiments in dark were performed to study the adsorption capacity of the catalyst at various catalyst loadings. The experiments were carried out at two concentrations of CBZ, i.e. 5 and 10 mg/L, and with Aeroxide® ranging from 50 to 800 mg/L. Before the irradiation, the suspension was stirred in the dark for 30 min to reach the adsorption/desorption equilibrium. The experiment was performed in triplicate. The substrate conversion was measured by UV-Vis spectroscopy.

2.3.2. Photolysis

Photolysis experiments were performed to determine the contribution of this effect to the overall photocatalytic process. The experiments were performed at neutral pH in ultrapure water containing 5 and 10 mg/L of CBZ. Samples were exposed to direct UV-A (PC) or simulated solar radiation (SSI) and continuously stirred during the photolysis experiments. The samples were analysed as triplicates. The substrate conversion was measured by UV-Vis spectroscopy.

2.3.3. TiO_2-photocatalysis under UV-A (PC) and simulated solar irradiation (SSI)

Photocatalytic experiments were performed in an immersion well, batch type, laboratory scale photochemical reactor purchased from ACE Glass Inc. (Vineland, NJ, USA) which is described in detail

elsewhere (Fotiadis et al., 2007). The reactor was charged with 350 mL of CBZ solution (10 mg L^{-1}) and the catalyst loading ([TiO_2]=100 mg L^{-1}) and the resulting suspension was continuously stirred. The initial concentration of CBZ, although considerably higher than the typically found in environmental samples, was chosen so as to allow the sufficient UV-light absorption of CBZ and the accurate determination of residual concentrations with the UV/Vis spectrophotometric system and to credibly elucidate the TPs with the analytical techniques employed in this work. At the beginning, the suspension was stirred for 30 minutes in the dark to ensure complete equilibration of the adsorbed substrate on the catalyst surface. After that period, the solution was irradiated by a 9 W UV-A lamp, 3.16 W m^{-2} (Radium Ralutec lamp, 9 W/78, λ=350-400 nm) and this was taken as ''time zero'' for the reaction. The photon flux of the lamp was frequently determined actinometrically using the potassium ferrioxalate method (Kuhn et al., 2004) and it was 3.37 x10^{-6} einstein s^{-1}. During the photocatalytic experiments, the temperature was maintained constant at 25±2°C using a temperature control unit.

In the case where the experiments were conducted under artificial sunlight conditions, solar irradiation was provided by a Phillips xenon lamp (Xe-OP) of 1000 W nominal power in a Newport (91113) solar simulator. The irradiation intensity of the simulator was determined using a radiometer (Newport 70260) and it was found 272.3 W m^{-2}.

2.3.4. Sonolysis (US) and sonophotocatalysis (SPC)

Sonochemical (or sonolysis) experiments were carried out using a digital Sonifier 450 Branson (Emerson Electric Co. US) device equipped with a titanium horn tip, operating at 20 kHz. In a typical run, 350 mL of an aqueous CBZ solution (10 mg L^{-1}) were loaded in a cylindrical water-jacketed vessel for temperature control and the horn was immersed at 2/3 of its height inside the liquid. The reaction solution was continuously sparged with air through a tube from the top of the vessel, thus ensuring good mixing of the reactor contents. For sonocatalytic (TiO_2+US) experiments, 100 mg L^{-1} of TiO_2 were also added to the reaction system.

Sonophotocatalytic experiments were carried out in a cylindrical Pyrex glass vessel that housed both the ultrasound probe and an UV-A lamp. The digital sonifier described previously was employed for sonication while the irradiation was provided by a 9 W UV-A lamp as described above. This experimental setup was suitable to study the effects of the individual or combined use of ultrasound and light avoiding any modifications in the reactor geometry. The experimental procedure was similar to that described in section 2.3.3. All runs were performed in triplicate and mean values are quoted as results. The uncertainty in this assay, quoted as the standard deviation of three separate measurements, was never larger than 10%.

2.4. Analytical methods

After the reaction mixture was irradiated, in all three cases: PC, SS and SPC, the samples were withdrawn from reactors after every 15 minutes for 120 min. The samples were centrifuged to remove catalyst particles, filtered with 0.2 μm filters, and then analyzed for i) dissolved organic carbon (DOC) and ii) residual CBZ concentration, iii) CBZ transformation products, and iv) CBZ concentration.

I) ***DOC analysis.*** To monitor the total mineralization of the substrates, DOC was measured on a Shimadzu 5050A TOC analyzer, whose operation is based on the catalytic combustion/non-dispersive infrared gas analysis. The calibration was done by injecting standard solutions of potassium hydrogen phthalate. DOC analysis was run in duplicate.

II) ***Quantitative analysis of CBZ by UV spectroscopy.*** The degradation (conversion) of CBZ during the course of experiments was monitored by UV-Vis Jasco V-530 spectrophotometer. The absorbance was measured at 284 nm (UV_{284}), the wavelength that corresponds to the maximum of absorbance in the visible region for CBZ. The linearity between absorbance and concentration was tested using solutions at various concentrations of either substrate in the range 0-20 mg/L and the

response was found to be linear over the whole range of concentrations under consideration. In this study, the term "conversion" refers to the degradation of the parent compound, but also to that of the CBZ oxidation intermediate products, which contribute to the absorption at the characteristic wavelength of the parent compound (284 nm), calculated by the decrease of UV absorption at 284 nm. Therefore, the obtained concentrations used to compute reaction conversions and rates may account for the residual substrate and possibly some of its reaction TPs. UV-Vis analysis analysis was run in triplicate.

III) ***Identification of TPs of CBZ by UPLC/ESI-QqToF-MS.*** A 10 mL aliquot of the irradiated solution, withdrawn every 15 min for 2 hours, was extracted by solid phase extraction (SPE) using Oasis® HLB cartridges (6 mL, 200 mg) (Waters Corp. MA, USA) placed in a filtration equipment (Supelco, Bellefonte, PA). The compounds were eluted by 2x3 mL of pure methanol, and the extract was concentrated by solvent evaporation with a gentle stream of nitrogen and reconstituted to a final volume of 1 mL in water/methanol (95:5, v: v) prior to instrumental analysis.

Accurate mass measurements of CBZ and its transformation products formed in time-course degradation experiments were carried out in full-scan and product ion scan mode using a Micromass QqToF-system interfaced with a Waters ACQUITY UPLC system (Micromass, Manchester, UK). Samples from the degradation experiments were separated on a Waters ACQUITY BEH C_{18} column (50 × 2.1 mm, 1.7 µm particle size) equipped with precolumn (5 × 2.1 mm) of the same packing material. The mobile phases were (A) formic acid 0.05% in water and (B) acetonitrile/methanol (2:1). After 1 min isocratic conditions at 90 % A, the portion of A was linearly decreased to 5 % within 7 min. This condition was held for 1 min and then the initial mobile phase composition was restored within 1 min and maintained for column regeneration for another 2 min. The flow rate was 300 µL/min. The injection volume was 5 µL. The MS analysis was performed with an electrospray ionization (ESI) interface in the positive ion mode applying a capillary voltage of +3500 V and the collision energies ranging from 10 to 40 eV. The nebulizer gas flow was set to 600 L/h and the drying gas flow to 50 L/h with a temperature of 350 °C. The ToF analyzer was operated at a resolution of 5000 (FWHM) and ESI mass spectra were recorded in 1-s intervals with automatic switching of the dual-sprayer every 10 s for infusion of the internal calibrant for a duration of 1 s. Val-Tyr- Val served as internal lock mass with $[M+H]^+$= *m/z* 380.2185. All MS data acquisition and processing was done using the software package MassLynx V4.1.

IV) **Quantitative analysis by HPLC/ESI-QqLIT-MS.** The quantitative analysis of CBZ was performed using Symbiosis Pico™ (SP104.002, Spark, Holland), equipped with an autosampler and connected in series with a 4000 QTRAP Hybrid Triple Quadrupole - Linear Ion Trap mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA, USA). Chromatographic separation was achieved with a Purospher Star RP-18 endcapped column (125 x 2.0 mm, particle size 5 m) preceded by a C_{18} guard column (4 x 4 mm, particle size 5 m), both supplied by Merck (Darmstadt, Germany). The mobile phases were (A) HPLC grade water with 0.1% formic acid and (B) acetonitrile. The gradient was as follows: isocratic for 5 min at 5 % eluent B, linear increase to 95 % B within 10 min, hold for 2 min, return to initial conditions in 2 min, equilibration for 3 min. The flow rate was 300 µL/min and the injection volume was 20 µL. For the analysis, the Turbo Ion Spray source was operated in the positive ion mode using the following settings for the ion source and mass spectrometer: curtain gas 30 psi, spraying gas 50 psi, drying gas 50 psi, drying gas temperature of 700 °C and ion spray voltage of 5500 V. The transition of CBZ for multiple reaction monitoring was *m/z* 237→194, declustering potential was 76 V, collision energy 29 eV and collision cell exit potential 19 V.

2.5. Ecotoxicity assessment

Toxicity measurements were carried out in samples taken at various times of the treatment processes applied using the Daphtoxkit F™ magna toxicity test. Toxicity analysis was conducted according to the standard testing protocol using the freshwater species *Daphnia magna* (ISO 6341). Daphtoxkit F™ toxicity test is based on the observation of the *D.magna* immobilization after 24 and 48 hours of

exposure to the treated samples. Tests in which mortality greater than 10% was observed were rejected and not used for statistical analysis. Each sample was run in duplicate and mean values are quoted as results with standard deviation never exceeding 1.1%.

3. Results and discussion

To obtain relevant information about the photocatalytic degradation of CBZ, it was necessary to carry out experiments from which adsorption on titania and direct photolysis of the substrate was excluded. Preliminary dark experiments were conducted to assess the extent of CBZ (10 mg L^{-1}) adsorption onto TiO_2 particles at catalyst loading of 100 mg L^{-1}. It was observed that equilibrium was reached within 30 min with no significant adsorption of CBZ (less than 9%) on the catalyst's surface; therefore further experiments were conducted in the presence of irradiation. Similar adsorption capacity was observed for other pharmaceuticals elsewhere (Méndez-Arriaga et al., 2008; Rizzo et al., 2009). Photolytic experiments (CBZ aqueous solutions were directly exposed to UV-A and solar irradiation) were carried out to determine its effect to the overall photocatalytic process. The photolytic experiments did not exhibit any considerable degradation of CBZ. The extent of photolytic degradation was marginal, *i.e.* only 7% after 120 min of reaction under both UV-A and solar irradiation conditions and this was accompanied by an insignificant DOC reduction of about 2% (data not shown). These tests indicated that neither TiO_2 chemical adsorption nor photolysis of CBZ were significant.

The experimental protocol that was followed to estimate the effect of each treatment process on CBZ degradation, involves first the application of photocatalysis under artificial UV-A and solar irradiation, followed by processes of increased complexity, by studying the substrate degradation by applying ultrasound irradiation (sonolysis) and finally a combination of photocatalysis under UV-A with ultrasound irradiation (sonophotocatalysis). At this point it should be mentioned that regarding the CBZ conversion and DOC removal during the processes applied, this article will mainly provide a comparison among these processes and then a detailed description on the elucidation of the TPs and the degradation pathways will be presented.

3.1. Photocatalytic degradation of CBZ solutions under UV-A (PC) and solar irradiation (SSI)

Heterogeneous photocatalysis experiments were performed using 100 mg L^{-1} TiO_2, the amount optimized for CBZ degradation for the photochemical reactors used in this study (Achilleos et al., 2010). It was observed that TiO2-photocatalysis of CBZ (10 mg/L) in presence of UV-A leaded to 75 % of conversion (in terms of absorbance) and 40% of DOC removal (Table 3) after 120 min. Similar values of CBZ conversion and DOC removal were observed during SSI experiments: 69% conversion of the substrate and 45% DOC removal within 120 min of treatment in ultrapure water. Additional experiments were also conducted to assess the contribution of bleaching in the presence of 1.4 mM H_2O_2 yielding an increase conversion of the substrate (PC: 99.8%, SSI: 78% within 120 min) due to the fact that the solution was enriched with hydroxyl radicals as well as peroxide prevents electron holes from recombining with electrons, a major cause for reduced photocatalytic activity (Konstantinou and Albanis, 2004). It should be noted that mineralization was found to be lower in comariosn with the conversion of CBZ substrate, a fact that clearly implies that a considerable organic load remained, attributed to the presence of the persistent oxidation products.

In order to evaluate the effect of water matrix on solution mineralization (in terms of DOC removal), WW and GW samples were spiked with 10 mg/L CBZ, and Aeroxide® was added in the range 500-3000 mg/L and subject to PC and SSI treatments. In both experiments, mineralization increased with increasing catalyst loading. A DOC reduction of 30% for WW and 38% for GW samples was achieved after 120 min of PC treatment with 3000 mg/L of catalyst. Under the same conditions, the CBZ conversion was 38% in WW samples, and 55% in GW samples. At the catalyst loading of 500 mg/L less than 10 % of DOC was removed from WW samples, and around 15 % from GW samples. After 120 min

of SSI treatment, the highest DOC removal (i.e. 25 % for WW and 40 % for GW) and CBZ conversion (i.e. 32 % in WW and 44% in GW) were observed for 3000 mg/L of the catalyst in the studied water matrixes. It could be noted that CBZ degradation is always faster than solution mineralization as it implies the formation of TPs that can be more resistant than the parent compound. In any case, DOC is not completely removed, indicating the formation of persistent oxidation products.

The matrix appears to have negative effect on mineralization, which can be explained in terms of: (i) increased initial carbon concentration – conversion decreases with increasing initial concentrations: the WW and GW samples already have had 11 and 2 mg/L of DOC, respectively, (ii) the presence of species like chlorides and carbonates/bicarbonates that may act as scavengers of ·OH radicals and other reactive moieties, and (iii) the increased solution pH (the wastewater sample is alkaline) (Achilleos et al., 2010). In GW and WW samples, PC treatment offers slightly higher substrate conversion and DOC removal, compared to SSI treatment (Table 3), that may be due to: (i) the spectrum of the xenon lamp consists of just about 5% UV irradiation and, consequently, the degree of photoactivation for the titania is expected to be limited, and (ii) reactor setups for UV-A and solar irradiation experiments are different. The former offers full and uniform illumination of the reaction mixture since the lamp is located inside the vessel, while the latter yields partial illumination since the source is outside the vessel. In general, however, all the parameters examined have exhibited similar effects during both types of irradiation.

3.3. Sonolytic and sonocatalytic degradation of CBZ

In a series of preliminary experiments, the effect of changing of ultrasound power on sonochemical degradation of CBZ was studied at 10 mg/L of initial CBZ concentration, as a function of the sonication time, under air sparging. The results showed the increase of CBZ conversion from 3 to 50 % with the increase in power density from 130 to 640 W/L under continuous air sparging (Figure 1). At the power of 640 W/L, the DOC removal was around 21% after 120 min of sonolysis. The beneficial effect of ultrasound power on conversion is attributed to increased cavitation activity at higher acoustic power, as it leads to the increasing number of collapsing bubbles and of the amount of $HO^{\cdot}$ generated, and thus enhanced degradation/transformation (Madhavan et al., 2010a). The subsequent experiments involving the use of ultrasound were performed at a 640 W/L density.

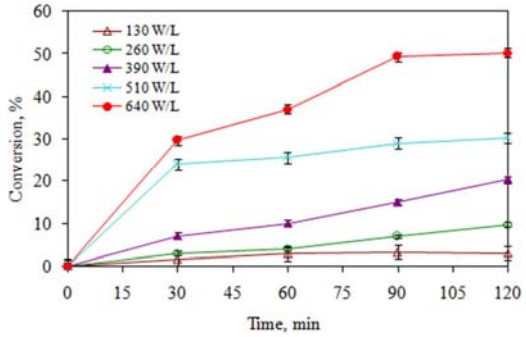


Figure 1: Effect of ultrasound power density on CBZ degradation during sonolysis (10 mg/L CBZ; 100 mg/L; air sparging)

In further experiments, the effect of adding H_2O_2 at various concentrations (0.07-1.4 mM) on CBZ conversion was studied (data not shown). CBZ conversion increased slightly, from 51 to 56 % with the increase in the peroxide concentration from 0.07 to 1.4 mM H_2O_2 after 120 min of treatment. The maximum DOC removal achieved was 25% in the presence of 1.4 mM H_2O_2. To verify that the CBZ conversion observed was due to combined action of ultrasound and peroxide rather than bleaching, a silent experiment was conducted showing that H_2O_2 alone does not practically contribute to degradation (data not shown).

When 100 mg/L of TiO2 was added to the defined sonolytic system (i.e. sonocatalysis), a few percents increase in the CBZ conversion amount and mineralization was observed, compared to sonolysis (Table 3). The increase may be due to additional cavitations from TiO2 suspended solids (Pandit et al, 2001), but it is within experimental error as well. In order to evaluate the effect of TiO_2 content and water matrix on solution mineralization during sonocatalysis, WW and GW were enriched with 10 mg/L of CBZ and TiO2 in concentrations from 500 to 3000 mg/L. As in the photocatalytic experiments, the highest DOC removal were observed for the catalyst loading of 3000 mg/L, and it was 10 and 15 % for WW and GW samples, respectively. Slightly better performance of sonocatalysis in the whole range of catalyst loading was observed for "cleaner" GW samples. As mentioned before, particulate matter in water matrices affects cavitation and thus the rate of sonochemical degradation. Although the particles can enhance degradation providing additional nuclei for bubble formation, they can also lead to reduced degradation due to sound attenuation (Adewuyi, 2001). Sonocatalytic experiments were also carried out in the presence of 0.07-3 mM H_2O_2. The addition of H_2O_2 did not practically improve the reduction of DOC (*i.e.* only about 3% greater than without peroxide), thus indicating the stability of the WW and GW matrix.

3.4. Sonophotocatalytic degradation of CBZ

After the sonolytic and photolytic experiments, and sonocatalytic and PC experiments, CBZ degradation by simultaneous ultrasound and UV-A irradiation in the presence of TiO_2 (SPC) was studied. The changes in CBZ initial concentration under the operational conditions used in previous (photocatalytic and sonocatalytic) experiments were employed: 10 mg/L of CBZ, 100 mg/L of TiO_2 and 640 W/L ultrasound power. Similar substrate conversion efficiencies were observed for SPC and PC treatments (78% for SPC, and 74% for PC) under the given conditions, but quite higher than during sonolysis/sonocatalysis (Table 3). Process efficiency regarding CBZ degradation and DOC removal, was found to follow the order: sonolysis < sonocatalysis < photocatalysis < sonophotocatalysis.

Table 3. Conversion of CBZ (adsorption based quantification), DOC removal and degradation of CBZ (quantified by HPLC-QqTRAP-MS/MS) in aqueous solutions during applied processes at the optimum conditions.

Treatment process	Conversion, %	DOC removal, %	Degradation, %
PC[1] (TiO_2+UV-A)	75	40	93
SSI[2] (TiO_2+solar irradiation)	69	45	10
Sonolysis[3] (US)	50	21	*nd*
Sonocatalysis[4] (TiO_2+US)	52	25	*nd*
SPC[5] (TiO_2+UV-A+US)	78	40	93

nd - not determined
[1] $[CBZ]_0$=10 mg L^{-1}, $[TiO_2]$=100 mg L^{-1}, 9W UV-A
[2] $[CBZ]_0$=10 mg L^{-1}, $[TiO_2]$=100 mg L^{-1}, Xenon lamp 1 kW
[3] $[CBZ]_0$=10 mg L^{-1}, 640 W L^{-1}, air sparging
[4] $[CBZ]_0$=10 mg L^{-1}, $[TiO_2]$=100 mg L^{-1}, 640 W L^{-1}, air sparging
[5] $[CBZ]_0$=10 mg L^{-1}, $[TiO_2]$=100 mg L^{-1}, 9W UV-A , 640 W L^{-1}, air sparging

3.5. Degradation kintetics

Most of photocatalytic reactions occurring at relatively low substrate concentrations can usually be desciribed by a pseudo-first order kinetic expression (Adewuyi, 2005; Gültekin and Ince, 2007; Madhavan et al., 2010a):

$$-\frac{dC}{dt} = k_{app}C \leftrightarrow \ln\frac{Co}{C} = k_{app}t$$

where k_{app} is an apparent rate constant, *Co* and *C* are the CBZ initial and final concentrations, respectively. Table 4 lists the values of the pseudo-first order degradation constants, k_{app}, and the linear regression coefficients (R^2) for SSI, PC, sonocatalysis and SPC of CBZ. According to the data, photodegradation of CBZ, in all the studied cases, fits well to the pseudo-first-order kinetic model. However, it should be noted that the degradation rate changes with the initial CBZ concentrations (Achilleos et al, 2011), and thus here presented kinetic data are only used to compare the rate of degradation under various experimental conditions, but at single CBZ concentration (10 mg/L).

As shown in Table 4, SPC offered faster degradation of CBZ than PC and sonocatalysis separately, under similar operating conditions. During the first 15 min, 17% of CBZ was degraded/transformed during PC, while and 52% during SPC (data not shown). Faster degradation after the coupling of photocatalysis with ultrasound may be attributed to several reasons such as increased production of hydroxyl radicals in the reaction, enhanced mass transfer of organics between the liquid phase and the catalyst surface, and increased catalytic activity due to ultrasound de-aggregating catalyst particles, thus increasing surface area (Berberidou et al., 2007, Gogate and Pandit, 2004; Madhavan et al., 2010b).

Table 4: Pseudo-first order rate constants corresponding to the photocatalytic, sonocatalytic and sonophotocatalytic degradation of 10 mg L^{-1} initial CBZ concentration.

Pseudo-first order kinetic constant	Kinetic parameters		
	($\times 10^3$ min^{-1})	R^2	$t_{1/2}$ (min)
$k_{UVA+TiO2}$	17.5 ($\pm$0.7)	0.9827	40
$k_{solar+TiO2}$	6.0 ($\pm$0.2)	0.9623	116
$k_{US+TiO2}$	3.0 ($\pm$0.1)	0.979	231
$k_{UVA+TiO2+US}$	46 ($\pm$0.9)	0.977	15.07

3.6. Identification of the TPs of CBZ in the SPC, PC and SSI experiments

Nine TPs of CBZ were detected in SPC and PC experiments, and eight in SSI experiment. In general, the TPs are oxygenated (hydroxy and keto) derivatives of CBZ. The fact that the same TPs were detected in these different experiments indicates similar reaction mechanisms (Schematics 1).

The chemical structures of the TPs of CBZ during SPC, PC and S treatments were tentatively proposed based on the MS/MS fragmentation and accurate mass information obtained by UPLC-(+)ESI-QqToF-MS. Table 5 shows the experimental and calculated masses of the molecular and fragment ions, the relative mass errors and the proposed elemental compositions for the TPs. The (+)ESI-MS/MS product ion spectra and putative assignments of characteristic fragment ions of CBZ and the CBZ-related TPs are shown in Figures 2-4.

As regards the parent compound (RT 5.0 min), it formed a protonated molecule at *m/z* 237 under (+) ESI conditions with a minor contribution of the sodium adduct at m/z 259. Upon collision-induced dissociation (CID) of the protonated CBZ molecule, fragment ions were detected at *m/z* 220 and *m/z* 194 corresponding to the neutral loss of NH_3 and HNCO (43 Da), respectively (Figure 2).

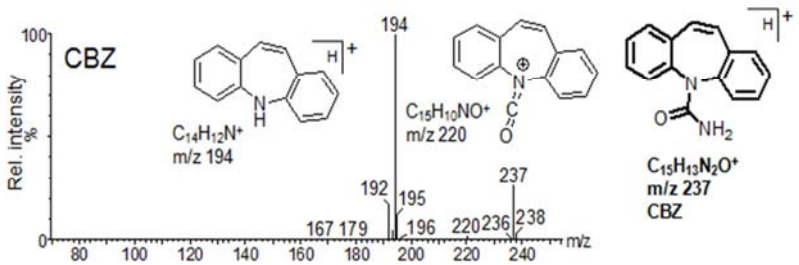


Figure 2. UPLC-(+)ESI-QqToF-MS product ion spectra of CBZ ($[M+H]^+$ = *m/z* 237)

To screen for TPs of CBZ in the samples from the three experiments, full-scan chromatograms were recorded over a mass range from *m/z* 70 to 600. In the time course PC and SPC experiments, the emergence of peaks having molecular ions $[M+H]^+$ at *m/z* 253, 251, 267, 271, 269, and 224 was observed, which we attributed to CBZ-related TPs (Figure 3 and Figure 4). In the case of solar irradiation experiment, the TP with m/z 224 was not detectable.

Table 5. Accurate mass measurements of the photodegradation products of CBZ as determined by UPLC–(+)ESI-QqToF-MS. Data for (pseudo)-molecular ions correspond to acquisitions in full-scan mode, those of fragment ions to product ion spectra of the protonated molecules (* confirmed by chemical standards)

Compounds	Ions	Elemental composition	Meas. Mass [m/z]	Calc. Mass [m/z]	Error (mDa)	Error (ppm)	DBE
CBZ*	$[M+H]^+$	$C_{15}H_{13}N_2O$	237.1035	237.1028	0.7	3	10.5
	$[M+Na]^+$	$C_{15}H_{12}N_2ONa$	259.085	259.0847	0.3	1.2	10.5
	$[M+H-NH_3]^+$	$C_{15}H_{10}NO$	220.0752	220.0762	-1	-4.7	11.5
	$[M+H-HNCO]^+$	$C_{14}H_{12}N$	194.0975	194.097	0.5	2.6	9.5
2-OH*	$[M+H]^+$	$C_{15}H_{13}N_2O_2$	253.0975	253.0977	-0.2	-0.8	10.5
	$[M+Na]^+$	$C_{15}H_{12}N_2O_2Na$	275.0787	275.0796	-0.9	-3.3	10.5
	$[M+H-NH_3]^+$	$C_{15}H_{10}NO_2$	236.0696	236.0712	-1.6	-6.8	11.5
	$[M+H-HNCO]^+$	$C_{14}H_{12}NO$	210.092	210.0919	0.1	0.5	9.5
3-OH*	$[M+H]^+$	$C_{15}H_{13}N_2O_2$	253.0971	253.0977	-0.6	-2.4	10.5
	$[M+Na]^+$	$C_{15}H_{12}N_2O_2Na$	275.0801	275.0796	0.5	1.8	10.5
	$[M+H-NH_3]^+$	$C_{15}H_{10}NO_2$	236.0718	236.0712	0.6	2.5	11.5
	$[M+H-HNCO]^+$	$C_{14}H_{12}NO$	210.0916	210.0919	-0.3	-1.4	9.5
TP 266	$[M+H]^+$	$C_{15}H_{11}N_2O_3$	267.0775	267.077	0.5	1.9	11.5
	[M+H-CO]+	$C_{14}H_{11}N_2O_2$	239.084	239.0821	1.9	8.1	10.5
	$[M+H-HNCO]^+$	$C_{14}H_{10}NO_2$	224.0719	224.0712	0.7	3.1	10.5
	$[M+H-2CO]^+$	$C_{14}H_{11}O_2$	211.0711	211.0759	-4.8	-22.8	9.5
	$[M+H-HNCO-CO]^+$	$C_{13}H_{10}NO$	196.0753	196.0762	-0.9	-4.6	9.5
	$[M+H-HNCO-2CO]^+$	$C_{12}H_{10}N$	168.0805	168.0813	-0.8	-4.9	8.5
TP 223	$[M+H]^+$	$C_{14}H_{10}NO_2$	224.0696	224.0712	-1.6	-7.1	10.5
	$[M+H-H_2]^+$	$C_{14}H_8NO_2$	222.0552	222.0555	-0.3	-1.4	11.5
	$[M-H2O]^+$	$C_{14}H_8NO$	206.0596	206.0606	-1	-4.9	11.5
	$[M+H-CO]^+$	$C_{13}H_{10}NO$	196.0759	196.0762	-0.3	-1.5	9.5
	$[M+H-CO-H_2O]^+$	$C_{13}H_8N$	178.0659	178.0657	0.2	1.1	10.5
	$[M-+H\ H2CO]^+$	$C_{12}H_8N$	166.0651	166.0657	-0.6	-3.5	9.5
TP 251	$[M]^+$	$C_{15}H_{11}N_2O_2$	251.0819	251.0821	-0.2	-0.8	11.5
	$[M+H-CO]^+$	$C_{14}H_{11}N_2O$	223.0869	223.0871	-0.2	-0.9	10.5
	$[M+H-HNCO]^+$	$C_{14}H_{10}NO$	208.0771	208.0762	0.9	4.3	10.5
	$[M+H-CN2O]^+$	$C_{14}H_{11}O$	195.0715	195.081	-9.5	-49	9.5
	$[M+H-HNCO-CO]^+$	$C_{13}H_{10}N$	180.0815	180.0813	0.2	1.1	9.5
TP 270	$[M+H]^+$	$C_{15}H_{15}N_2O_3$	271.1077	271.1083	-0.6	-2.2	9.5
	$[M+Na]^+$	$C_{15}H_{14}N_2O_3Na$	293.091	293.0902	0.8	2.7	9.5
	$[M+H-H2O]^+$	$C_{15}H_{13}N_2O_2$	253.0995	253.0977	1.8	7.1	10.5
	$[M+H-NH3-H2O]^+$	$C_{15}H_{10}NO_2$	236.0708	236.0712	-0.4	-1.7	11.5
	$[M+H+H2O-HNCO]^+$	$C_{14}H_{12}NO$	210.0898	210.0919	-2.1	-10	9.5
	$[M+H-H2O-HNCO-CO]^+$	$C_{13}H_{10}N$	180.08	180.0813	-1.3	-7.2	9.5
TP 268	$[M+H]^+$	$C_{15}H_{13}N_2O_3$	269.092	269.0926	-0.6	-2.2	10.5
	$[M+H-NH3]^+$	$C_{15}H_{12}N_2O_2$	252.0837	252.0899	-6.2	-24.5	11
	$[M+H-H2O]^+$	$C_{15}H_{11}N_2O_2$	251.0823	251.0821	0.2	1	11.5
	$[M+H-HNCO]^+$	$C_{14}H_{12}NO_2$	226.0847	226.0868	-2.1	-9.3	9.5
	$[M+H-NH3-CO]^+$	$C_{14}H_{10}NO_2$	224.0716	224.0712	0.4	2	10.5
	$[M+H-H_2O-HNCO]^+$	$C_{14}H_{10}NO$	208.0802	208.0762	4	19	10.5
	$[M+H-HNCO-CO]^+$	$C_{13}H_{10}NO$	196.0741	196.0762	-2.1	-10.7	9.5

Hydroxy-CBZ. Four peaks were observed in the extracted ion chromatogram (XIC) of *m/z* 253, which is the ion mass of possible monooxygenation products. Accurate mass measurements were in line with the postulated incorporation of one oxygen atom into the CBZ structure, i.e. $C_{15}H_{13}N_2O_2$ for the $[M+H]^+$. Two of the four peaks (at 3.65 and 4.03 min) were abundant enough to provide significant product ion intensities and information on the exact mass of the ions. They showed identical fragmentation patterns in the (+)ESI-MS/MS experiments, and resembled the CBZ spectrum in that protonated molecules underwent loss of ammonia (17 Da) or HNCO (43 Da) to produce the ions at *m/z* 236 and 210, respectively (Figure 4A). The compounds were assigned the structures of 2-hydroxy CBZ and 3-hydroxy CBZ as they matched in retention time and mass spectral fragmentation with the corresponding chemical standards. The two hydroxy derivatives and some analogues have been detected as CBZ-related metabolites in wastewater treatment plant (Miao and Metcalfe, 2003), and upon CBZ photodegradation by Fe(III) (Chiron et al., 2006) and TiO2 (Calza et al, 2012).

TP 250. The molecular ion of the TP 250 showed an accurate mass of *m/z* 251.0819, with a mass error of -0.2 ppm from the theoretical mass for the composition $C_{15}H_{11}N_2O_2$ (Table 1). In the product ion spectrum (Figure 4B), the diagnostic loss of 28 Da to form the fragment ion at m/z 223 suggests the presence of a carbonyl group which can be readily cleaved off under CID conditions. This observation can be rationalized as originating from the cyclic ketone in TP250 (Figure 4B). Ring contraction upon loss of CO leads to a pyridine form. The loss of CO is common for compounds with endocyclic carbonyl group especially if it leads to resonance stabilization of the ionic products (McLafferty and Turecek, 1993). Subsequent loss of HNCO from the m/z 223 gives rise to the fragment ion at m/z 180, that corresponds to the $[M+H]^+$ ion of acridine (Table 1). If the elimination of HNCO precedes the loss of CO, the fragment ion at m/z 208 is formed. The fragmentation pattern observed for TP250, and the proposed chemical structure is in agreement with the one reported by Hu *et al* (2009) for an intermediate formed upon oxidation of CBZ in water by Fe (VI) (Hu et al., 2009). Using LC-(+ESI)-(LTQ Orbitrap)-MS/MS, Calza et al (Calza et al, 2012) proposed an alternative structure for m/z 251 formed after hydroxylation and 7-member ring contraction of CBZ, followed by an intermolecular ring formation between N-aminocarbonyl group and aromatic ring. The same set of fragment ions was observed by Chiron et al (Chiron et al., 2006) in LC-(+ESI)-MS/MS, but they suggested alternative structure of the TP after the analysis in LC-(+)APCI-MS/MS. All the proposed structures were not confirmed by additional ^{1}H-NMR analysis either in the cited studies or in ours, and thus the assignment of the structures has only a speculative character. ***TP 266.*** An intensive and 3 very low-intensity peaks were detected in the XIC of *m/z* 267, indicating presence of possible isomeric forms of TP 266. From the acquired MS/MS spectra, only the intensive signal at 3.4 min provided useful MS/MS spectra. The MS/MS spectra contained fragmentation ions at m/z 239 and 211 that resulted from two sequential losses of C=O (28 Da) from the parent ion. The fragment at m/z 196 is accompanied by a peak at m/z 168, related to the loss of C=O group, indicating a keto-acridine structure for the ion. According to the fragmentation pattern and DBE, the TP has been assigned a structure of diketo-CBZ that resulted from the oxidation of hydroxy-groups present in TP 268 and TP 270 (Figure 4C). Alternative structures for TP266, consistent with fragmentation patterns, have been reported previously (Chiron et al., 2006; Hu et al., 2009).

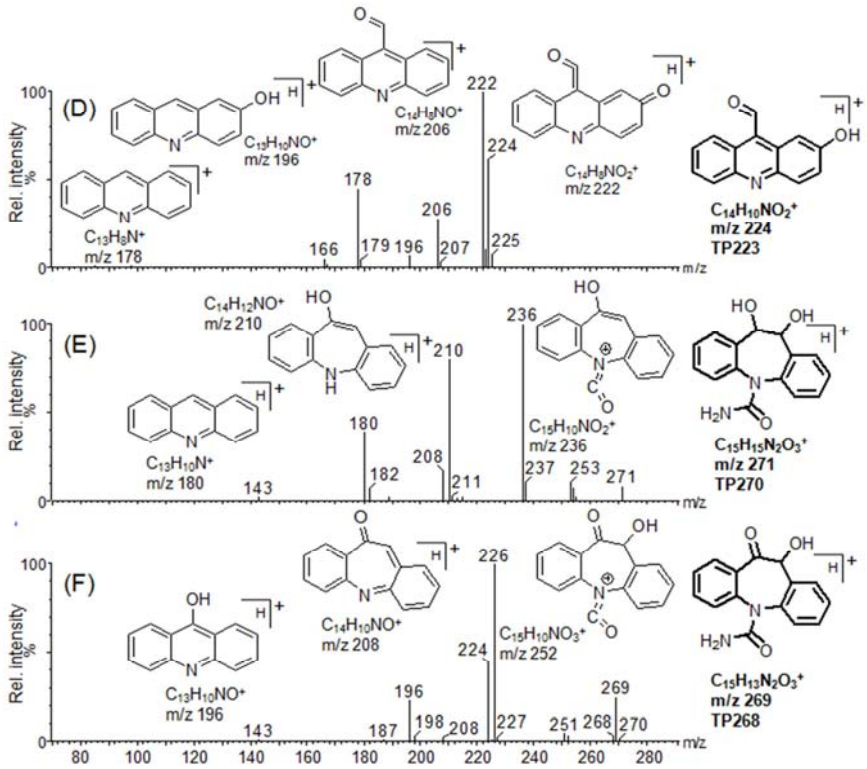


Figure 3. UPLC-(+)ESI-QqToF-MS product ion spectra of (**D)** TP223 (*m/z* 223). (**E**) TP270 (*m/z* 271), and **(F)** TP268 (*m/z* 269); and the putative structures of the TPs and characteristic fragmentation ions.

TP 223. The MS/MS spectrum of TP223 is characterized by sequential loss of H_2O (18 Da) and CO (28 Da) from the protonated molecule at m/z 224 resulting in the fragment ions at m/z 206 and m/z 178, respectively (Figure 4D). An intensive peak at m/z 222 corresponds to dehydrogenation of the ion at m/z 224 to form a resonance-stabilized ion. TP 223 was tentatively identified as a hydroxyacridine-9-carboxaldehyde. The structure is consistent with a previously reported product of heterogeneous photocatalysis (Doll and Frimmel, 2005), direct photolysis (Chiron et al., 2006) and UV/H_2O_2 treatment of CBZ in water (Vogna et al., 2004).

TP 270. Regarding the TP at 3.27 min, the full-scan mass spectrum showed a molecular ion $[M+H]^+$ at *m/z* 271 accompanied by an abundant sodium adduct ion at m/z 293. The measured accurate mass of m/z 271.1077 for the $[M+H]^+$ showed a mass difference of -2.2 ppm from the theoretical mass calculated for $C_{15}H_{15}N_2O_3$ (Table 1), which is a change in the elemental composition of CBZ that corresponds to the addition of two hydrogen and two oxygen atoms (+H_2O_2). The introduction of oxygen-bearing functional groups into the molecule of CBZ is in agreement with the enhanced tendency to form sodium adducts. The MS/MS fragmentation of the protonated molecule of TP270 resulted in a fragment ion at m/z 253, corresponding to dehydration, which is indicative of the presence of an aliphatic hydroxyl group (Figure 4E). This ion can then either cleave off ammonia (m/z 236) or undergo the characteristic neutral loss of HNCO (m/z 210), followed by a loss of HCHO to yield a resonance-stabilized fragment ion at m/z 180. This fragmentation pathway corresponds to a dihydroxylated molecule of CBZ described elsewhere (Chiron et al., 2006; Miao and Metcalfe, 2003), but specific hydroxylation positions were not established as the authentic standards were not available for structural confirmation. Few isomers of dihydroxy-derivative of CBZ have been detected by Chiron et al (Chiron et al., 2006) upon photodegradation by Fe(III) at pH 7.5, and by Calza et al (Calza et al., 2012) upon TiO2 photocatalysis. Hu et al (Hu et al., 2009) detected and identified 10,11-dihydroxy-CBZ by LC-(+ESI)-(ion trap)-MS/MS as a product of CBZ-oxydation by Mn(VII).

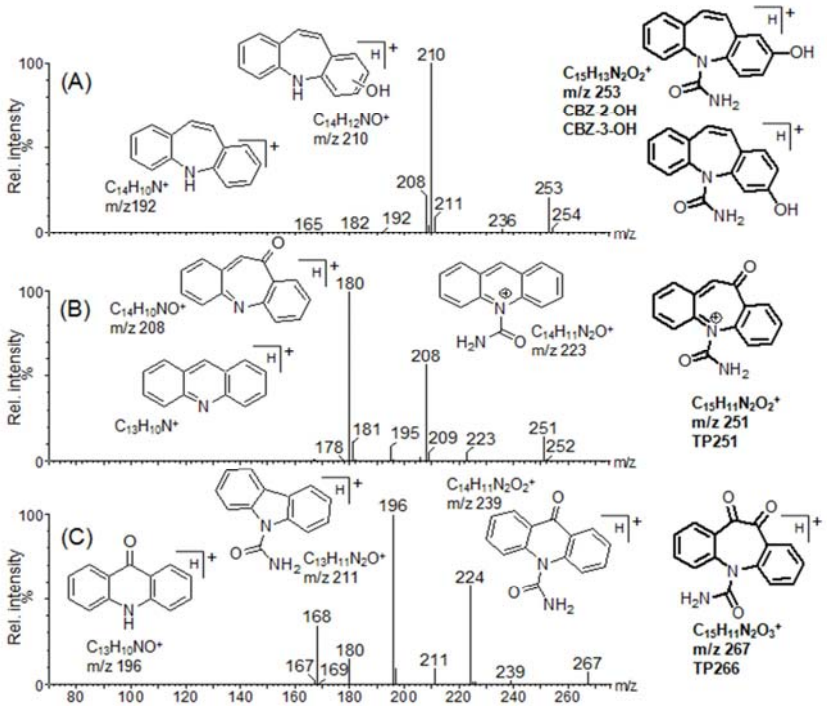


Figure 4. UPLC-(+)ESI-QqToF-MS product ion spectra of (**A**) CBZ-2-OH and CBZ-3-OH (*m/z* 253), (**B**) TP 251 (*m/z* 251) (**C**) TP266 (*m/z* 267); and the putative structures of the TPs and characteristic fragmentation ions.

TP 268. The two peaks observed in the XIC of m/z 269 (TP268-A at 2.60 min and TP268-B at 3.45 min) had identical MS/MS spectra suggesting positional isomers of CBZ-related TPs. The molecular ions of the two TPs showed accurate masses of m/z 269.0927 for TP268-A, and 269.0925 for TP268-B, suggesting an elemental composition of C15H13N2O3 with errors of -0.4 and 0.3 ppm, respectively. The MS/MS spectrum of the protonated TP268 showed losses of 17 Da (m/z 252) and 18 Da (m/z 251) due to losses of NH_3 and H_2O from the parent ion, respectively. The loss of a molecule of H2O from the precursor ion indicated the presence of a hydroxyl group (whose position was not established). On the other hand, two sequential losses of C=O from m/z 252 led to the formation of the fragment m/z 196, i.e. hydroxyl-acridine. According to the accurate mass measurements and the fragmentation pattern, and the similarity to the MS/MS spectrum of oxcarbamazepine reported by Li and colleagues (Li et al., 2011), a hydroxy-ketone structure has been assigned to TP 268 (Figure 4F). Figure 5 shows the decay of CBZ and the evolution of its TPs during the experiments.

3.5 Formation of TPs and proposed transformation pathway

Figure 4 illustrates the decay of CBZ and the evolution of its TPs during the experiments. Since chemical standards were not available for their quantitative analysis, the evolution of the TPs is depicted based on chromatogram area counts, assuming that the parent compound and its TPs have similar ionization efficiencies and response factors during ESI. The formation of the TPs is plotted as a ratio of their chromatogram areas to the area of the spiked CBZ as a function of time of irradiation (right ordinate Figure 4). In all the experiments, the TPs emerged within the first 15 min of irradiation, with different rates of increase, and remained in the media until the end of the experiments.

The initial CBZ concentrations decreased by around 95% after 120 min of SPC and PC treatments. During PC experiments, the highest rate of formation and degradation/transformation was observed for TP270 and TP 251, but 2-OH-CBZ and 3-OH-CBZ had the highest amount at the end of the experiments. During and until the end of SPC experiments, 2-OH-CBZ and 3-OH-CBZ were the most abundant. Two

isomeric forms of TP 223 were detected in PC and SPC, but not in SSI experiments. During solar irradiation, CBZ degraded slowly and to a small extent, *i.e.* <10% (Figure 4). Also TPs were formed at lower rate in comparison to PC and SPC, with an increase in concentrations over the time-course of the experiment.

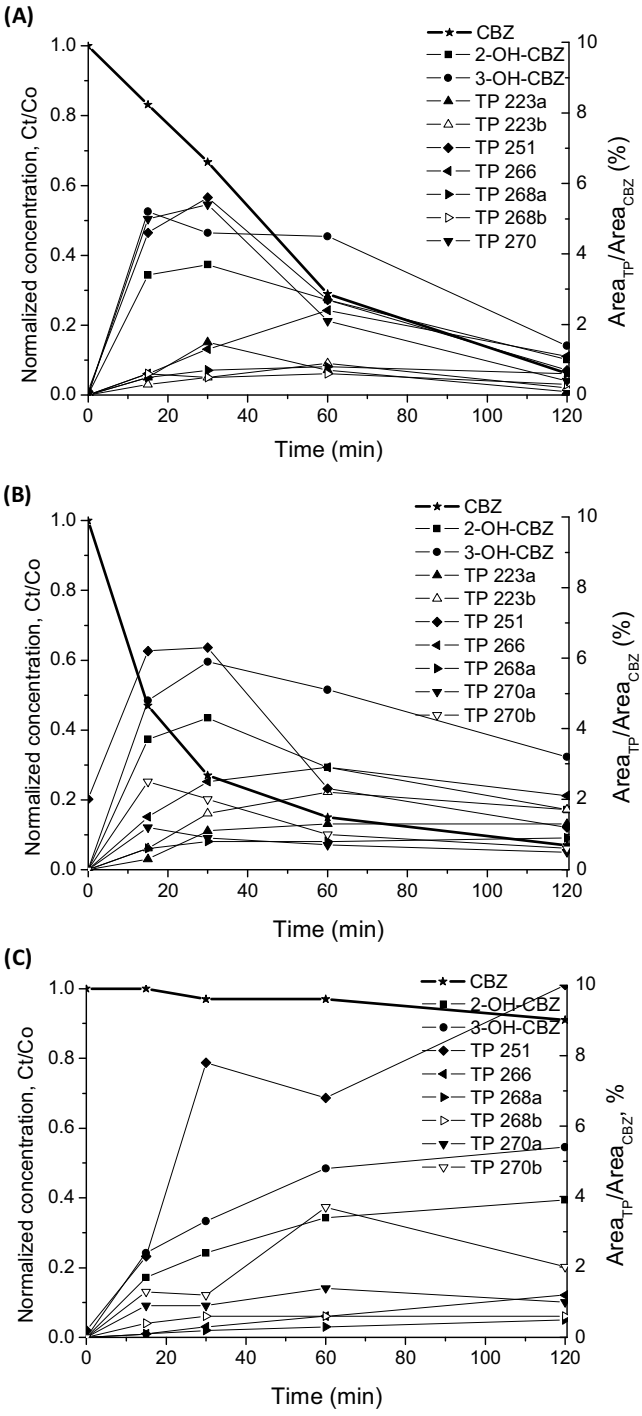
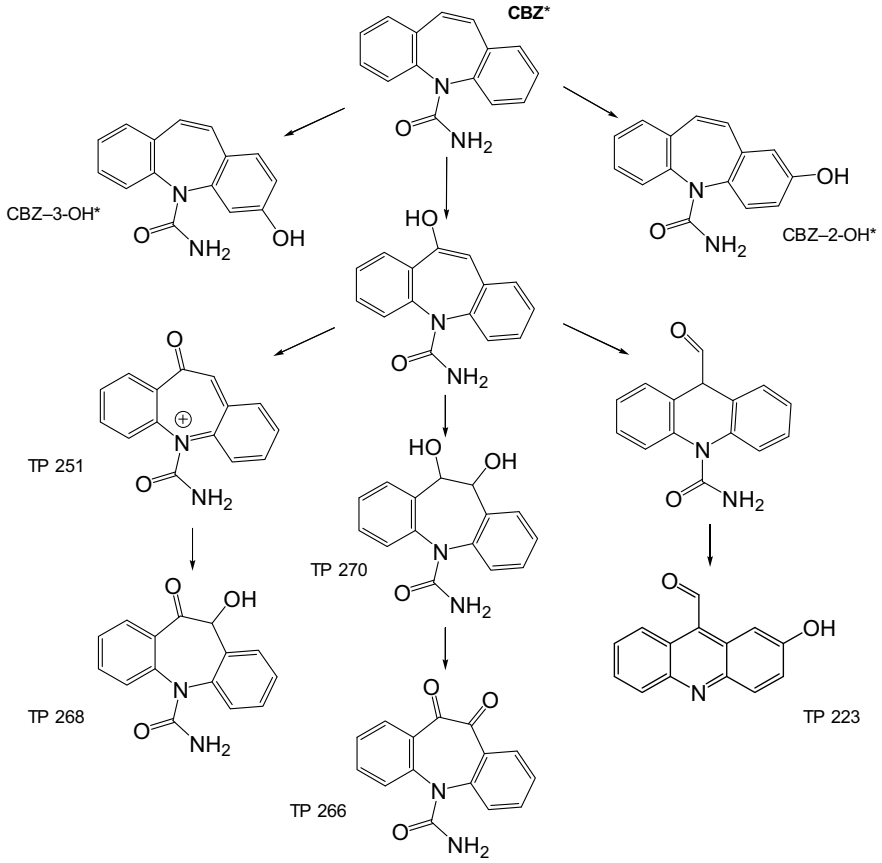


Figure 5. Time course of CBZ degradation and formation of TPs in the (A) PC, (B) SPC and (C) SSI experiments

Proposed CBZ transformation pathway, shown in Schematics 1, indicates formation of products more polar than the parent compound. The major route likely involves mono-hydroxylation of CBZ to TP 253 (*e.g.* 2-OH-CBZ, 3-OH-CBZ), followed by (a) oxidation of the hydroxyl group to give mono-keto intermediate, TP 251, and/or (b) another hydroxylation to give dihydroxy-CBZ, TP 270. Subsequent

hydroxylation of TP 251 likely leads to a formation of a hydroxy-keto-intermediate, TP266. TP 267 could be formed from di-hydroxy-CBZ by oxidation of one hydroxyl- to keto-group. Another pathway involves hydroxylation and central heterocyclic ring contraction with subsequent hydroxylation and the loss of $CONH_2$ to give TP 223 (Schematic 1) in PC and SPC experiments. The iminostilbene core structure remained intact under the mild reaction conditions in SSI experiments.



Schematic 1. Tentative transformation pathway of CBZ degradation by PC, SSI and SPC (the position of hydroxyl and keto-groups was not determined, except in the cases marked with * that were confirmed with authentic chemical standards).

3.7. Ecooxicity

The evolution of acute toxicity of the CBZ treated solutions towards *D. magna* was monitored as a function of the treatment time and exposure time. Primarily, blank aqueous samples containing TiO_2 only, at concentration of 100 mg L^{-1}, (without CBZ) were tested to exclude the possibility that toxicity is owing to catalyst particles; these tests showed no toxicity to *D.magna* after 24 and 48 h exposure. The toxicity tests showed no toxicity of the untreated 10 mg L^{-1} CBZ solution to *D.magna* after 24 h of exposure; however after 48 h of exposure the immobilization of daphnids was dramatically increased to 100%. Under simulated solar light, the acute toxicity of CBZ in *D.magna* was generally very high over the time-course of the SSI experiments. The toxicity of treated samples after 30 min of UV-A irradiation, increased to approximately 90% after 24 h of exposure, after which a decrease in *D. magna* immobilization to 50% was observed at the end of the PC experiments. The same toxicity level was observed after 48h of exposure after 120 min of irradiation. This toxicity trend may be a result of the formation/degradation of TPs of CBZ during the treatment. When CBZ solution was treated by sonophotocatalysis, toxicity increased during first 60 min, but then it dropped to 15% immobilization after 24h of exposure. On the other hand, after 48 h of exposure, toxicity value reached 80%

immobilization of *D. magna* at the end of the SPC treatment. The evolution profile of toxicity does not follow the trend of the degradation of CBZ, indicating that the toxicity might be a result of the formation of toxic TPs during the treatments. Nevertheless, when the toxic samples were diluted to 80%, 40%, 20% and 10%, the toxicity decreased, and at 10% and 20% dilution all samples were non-toxic. It should also be noted that the concentrations of CBZ that were used in the experiments and were toxic for *D. magna* are much higher than the levels detected in the environment, and thus the risks associated with this compound and it TPs in the environment may be limited.

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Supplementary data

The supplementary data includes: the description of the experimental setups and additional information on degradation kinetics.

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Supplementary data for

Photocatalytic and sonophotocatalytic degradation of the anticonvulsant carbamazepine and identification of transformation products

S1. Materials and methods

S1.1. Photochemical reactor

The photochemical reactor used for the photocatalytic experiments (ACE glass) is a three-compartment apparatus and consists of an inner, double-walled, borosilicate glass housing the lamp and an external cylindrical reaction vessel joined together with an internally threaded connection with the aid of a nylon bushing connector and an O-ring (Figure S1A). The reaction mixture was placed in the external cylindrical reaction vessel and the inner double-walled borosilicate glass was immersed inside the reaction mixture. The lamp was placed inside the inner borosilicate glass and was effective cooled by a water circulation stream through the double-walled compartment, acting as a cooling water jacket. During photocatalytic experiments the temperature was maintained constant at 25±2°C. The external reaction vessel was covered with aluminium foil to reflect irradiation exerting the outer wall of the reaction vessel.

Artificial solar irradiation was provided by a Phillips xenon lamp (Xe-OP) of 1000 W nominal power in a Newport (91113) solar simulator (Figure S1B). Reactions were conducted in a glass vessel illuminated from the top (at a distance of 8 cm).

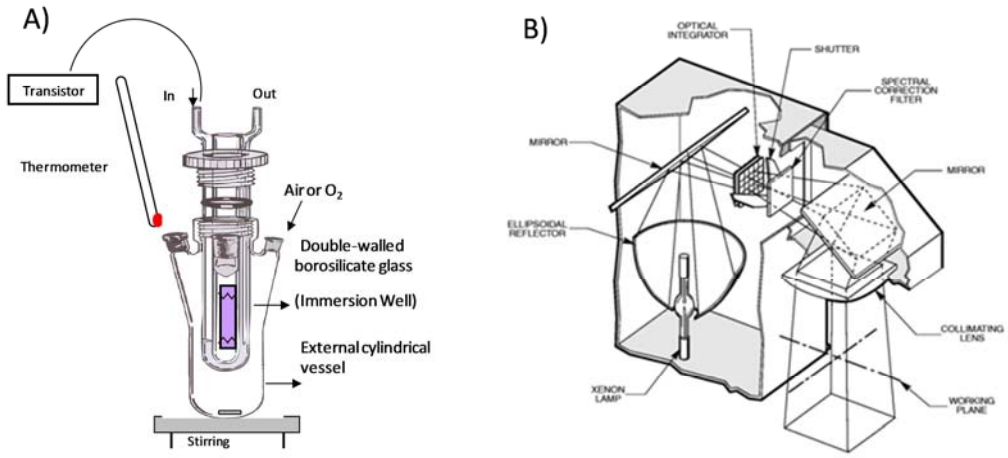

Figure S1. Scheme of the photochemical reactor ACE Glass (A), and of the solar simulator (B)

S1.2. Sonochemical experimental setup

The digital sonifier used in the experiments related to the application of sonolysis (also sonophotocatalysis) is shown in Figure S3.

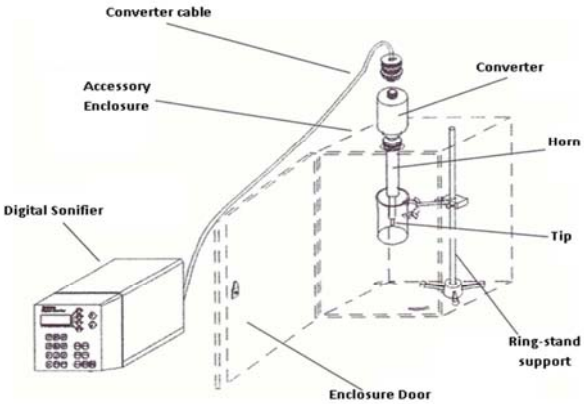

Figure S3. Scheme of the sonochemical experimental setup

The system consists of three core elements: the power supply, the converter and horn. The ultrasonic vibration transmitted through the horn can also be applied directly to a solid workpiece. The sonochemical experiments were carried out using a digital Sonifier 450 Branson device equipped with a titanium horn tip of 1.9 cm in diameter. Its maximum available output power is up to 400 Watts, while the operating frequency was 20 kHz.

S1.3. Sonophotocatalytic experiments

Sonophotocatalytic experiments were carried out in a cylindrical Pyrex glass vessel (Figure S3). A titanium-made probe immersed in the liquid from the open to the atmosphere top of the vessel was used to deliver the ultrasound energy in the reaction mixture. The maximum available output power of the sonifier is up to 400 Watts, while the operating frequency was 20 kHz. The bottom of the vessel was fitted with a glass cylindrical tube housing the light source (9 W/78, UV-A). The vessel was fed with a 350 ml CBZ aqueous solution. This experimental setup was suitable to study the effects of the individual or combined use of ultrasound and light avoiding any modifications in the reactor geometry.

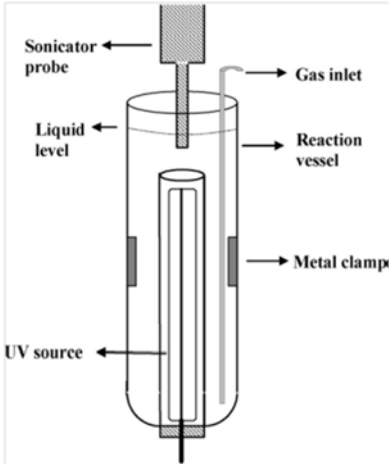

Figure S4: Scheme of the sonophotocatalytic experimental setup

S1.3.Chemical actinometry

The photon flux of the lamp was determined actinometrically using the potassium ferrioxalate method. The experiment was carried out under red safe light. Green crystals of $K_3Fe(C_2O_4)_3.H_2O$ were prepared by mixing 3 volumes 1.5 M $K_2C_2O_4$ (Riedel de Haen, 99%) with 1 volume 1 M $FeCl_3$ (Riedel de Haen, 99%) solution, recrystallized three times from warm water, dried at 45 ºC and kept in the dark. 0.006 M solution (≥405nm) was used for actinometry and was prepared by dissolving 2.947 g of the crystals in 100 ml 1 N H_2SO_4 and dilution with distilled water to 1 L. 350 ml (V_1) solution were irradiated using UV-A irradiation provided by a 9 W lamp (Radium Ralutec, 9W/78, 350-400 nm) under efficient stirring. One ml (V_2) of the irradiated solution was provided into a 10 ml (V_3) volumetric flask containing a mixture of 4 ml 0.1% 1,10- phenanthroline solution (Fluka, 99%, store in dark) and 0.5 ml buffer (stock solution: 82 g NaOAc (Himedia, 99%), 10 ml conc. H_2SO_4, diluted to 1 L with distilled water) which was then diluted to the mark with distilled water. A reference was prepared in the same way except that it had not been irradiated. Both solutions were kept in dark (about an hour) until full colour development was achieved and the absorbance of the first minus that of the second sample was measured at 510 nm. A_{510} was kept within the range 0.4-1.8 as required. The evaluation of the photon flow Φ_p was given below:

$$\Phi_p = \frac{\Delta A.V_1 10^{-3}.V_3}{\Phi_\lambda.\varepsilon_{510}.V_2.t} \text{ (einstein/s)}$$

where: t is in seconds, Φ_λ at room temperature equals 1.20-1.26 (0.006 M) for the wavelength range 254-366 nm, and ε= 11100/$dm^{3.}$mol.cm.

S2. Results

S2.1. Kinetics

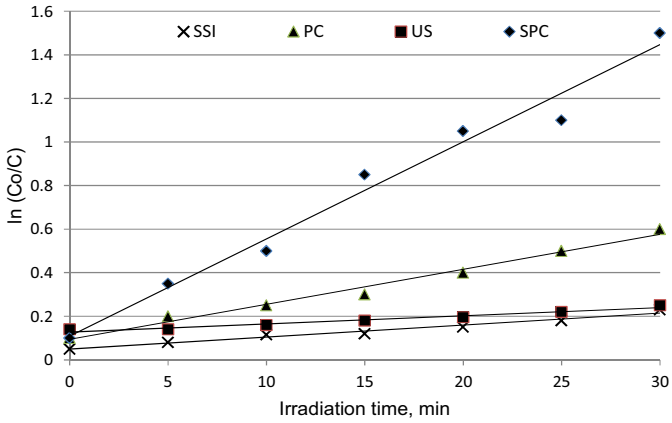

Figure S6. Kinetics of CBZ degradation (10 mg/L) during SSI (100 mg/L TiO_2 + simulated solar irradiation), PC (100 mg/L TiO_2 + 9W UV-A), US (sonocatalysis: US 640 W/L +100 mg/L TiO_2) and SPC (100 mg/L TiO_2, 9W UV-A irradiation + US 640 W/L) treatments

S2.2. Toxicity

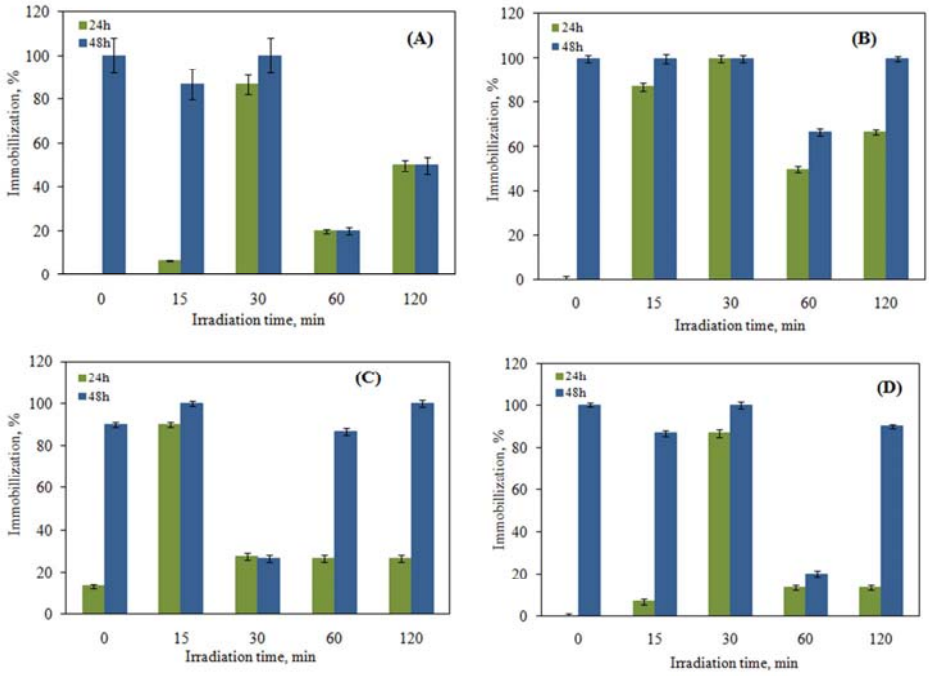

Figure 5. Toxicity of the solutions containing CBZ at 10 mg/L and 100 mg/L of TiO2: (A) PC treatment: 9W UV-A irradiation, (B) SSI treatments: solar irradiation, (C) sonolysis: 640 W/L, (D) SPC treatment: 9W UV-A irradiation, US 640 W/L

3.6. Fungal *versus* photocatalytic treatments for CBZ-degradation

We observed that the white rot fungus *Trametes versiocolor* is capable of degrading CBZ in lab-scale bioreactor operating in batch and continuous mode, with degradation efficiencies of 96 % and 54 %, respectively. It is worth noting that the experiments in our study were performed with pure water. In real wastewater, we can expect that the performance of fungal treatment is altered due to various factors, such as sorption, possible toxicity inhibition, competition with native bacterial population in the system etc. For this reason, a firm conclusion on the treatment performances cannot be made. Anyhow, this technology deserves much attention, because of, at least, two reasons: a) this family of fungi is ubiquitous in nature, and b) fungal treatment, in general, has a cost advantage because it requires relatively cheap inputs (e.g. nutrients), and does not require external sources of energy.

UVA driven photocatalysis and sonophotocatalysis have been shown to reduce the initial concentrations of CBZ for about 95% in 120 min. Sonophotocatalysis offered faster photodegradation of CBZ: after 30 min, 70% was degraded during sonophotocatalysis; and 35% during UV photocatalysis. The use of ultrasound doubled the efficiency of UV-photocatalysis in terms of degradation rates, thus offering the possibility to reduce reaction time in this particular case. Solar photocatalysis achieved only around 10 % of CBZ degradation and yielded higher overall toxicity in comparison with other two processes. As regards the DOC removal during the studied photocatalytic processes, it should be noted that it was similar in all three cases (around 40 %), and always lower than CBZ degradation. This indicates that a considerable organic load remains in the treated solutions that can be attributed to the presence of persistent oxidation products. And thus, the identification and quantification of transformation products generated during the processes is a crucial step in order to evaluate the technologies. We should have in mind that solar photocatalytic systems offer cheaper and very abundant energy source, in comparison with UV-driven photocatalysis. This is the reason why much focus has been given to sunlight as the initiator of the photochemical reactions with TiO2 as the catalyst. Solar photocatalysis strongly depends on the configuration of reaction system that has to permit collection of the light from a broad area, and this might be the main reason why our sunlight-driven experiments did not yield better results regarding the CBZ-degradation.

Finally, I believe that the choice of technology will depend on the particular problem to be solved, as well as on the economic evaluation of the technology implementation. Preliminary research, i.e. lab-scale studies, is necessary in order to assess potential treatment options, and optimize the conditions for any specific problem, but, without the use of real wastewater matrices and further scale-up research, decisions cannot be made.

3.7. Conclusions

In this chapter two alternative approaches for degradation of carbamazepine in aqueous media were discussed: a) biodegradation using white rot fungus *Tremates versiolor* in an air pulsed fluidized bioreactor operated in batch and continuous modes, and b) AOP using TiO_2-heterogeneous photocatalysis in three different conditions: under visible and UVA irradiation, and in combination with ultrasound treatment.

a) The results indicated that *T. versicolor* is capable of degrading CBZ in aqueous medium in both operational modes of bioreactor. Epoxy-CBZ, diol-CBZ, acridone and acridine were identified as the major TPs of CBZ. Around 96% of CBZ was removed/transformed after 2 days when the bioreactor was operated in batch mode, and epoxy-CBZ was found as unique TP. In bioreactor operated in continuous mode with a hydraulic retention time of 3 d, 54% of the inflow concentration (about 200 μg L^{-1}) was reduced at the steady state (25 d) with a CBZ degradation rate of 11.9 μg CBZ g^{-1} dry weight d^{-1}. No metabolite was detected in the final culture broth. Acute toxicity tests showed that the final culture broths in both experiments were nontoxic, with 15 min EC50 values of 24% in batch, and 77% in continuous mode. And thus, degradation using white-rot fungi may be a good strategy for the degradation of CBZ. Nevertheless, additional experiments should be carried out in reactors fed with real wastewater in order to draw firm conclusions on the process. Only afterwards, a possibility of scaling-up could be evaluated.

b) During UVA-photocatalytic and sonophotocatalytic experiments, about 95% of the initially present CBZ was transformed after 120 min of the experiments. Similar degradation efficiencies, in terms of DOC removal as well, were observed for SPC and PC treatments under the operating conditions. Nevertheless, the use of ultrasound with UVA radiation resulted in an enhanced CBZ-transformation rate. During photocatalytic experiments under SSI only 10% of the initially present CBZ was transformed/removed. The acute toxicity of the treated solutions was generally high over the time-course of the conducted photocatalytic experiments, mainly because of the high CBZ concentrations used for the studies. The mineralization of the substrates was found to be lower than CBZ conversion in all three experiments, and it accounted for about 40% in all three cases, indicating the presence of persistent transformation products. Nine TPs of CBZ were tentatively identified in SPC and PC experiments, and eight in SSI experiment. The TPs are hydroxy- and keto-derivatives of CBZ formed as a result of oxidative transformation reactions involving hydroxyl radicals. The fact that the same TPs were detected in the different experiments indicated similar reaction mechanisms during the treatments. The TPs were detected, although at low concentration, at the end of the experiments. Additional experiments applying a longer process time would be convinient, in order to determine the moment when CBZ and its TPs are degraded to become less toxic and better biodegradable.

Chapter IV

Resumen en castellano

4.1. Introducción

Los fármacos son sustancias orgánicas destinadas a prevenir, aliviar y/o currar enfermedades en humanos y animales. Comprenden un amplio y diverso grupo de compuestos diseñados para ser muy activos y interaccionar con receptores específicos en los humanos y animales, o bien para ser tóxicos para organismos infecciosos (e.g. hongos, bacterias etc.). Anualmente, cientos de toneladas de fármacos se administran y consumen en todo el mundo, y su uso seguirá creciendo constantemente debido a los descubrimientos de nuevos fármacos, el crecimiento y envejecimiento de la población, así como a causa de la caducidad de las patentes y también debido a una mayor disponibilidad de los medicamentos genéricos menos costosos (1).

Una vez ingeridos, los fármacos intervienen en diversos procesos metabólicos y son excretados principalmente por vía renal y/o por vía biliar-entérica. La vía de ingestión – metabolización – excreción de fármacos y su excreción después de la administración tópica, seguidas por el tratamiento de las aguas residuales, se consideran las vías principales de entrada de fármacos (y de sus correspondientes metabolitos activos e inactivos) al medio ambiente. La mayoría del agua tratada en las estaciones depuradoras de aguas residuales (EDAR) (el efluente) se vierte al medio receptor local (e.g. ríos, mar etc.), o bien es reutilizada para el riego de áreas verdes o de cultivos. Los fangos que se forman en el proceso de depuración se utilizan en agricultura como abono orgánico, para estabilizar o rellenar terrenos, o bien se retiran a vertederos controlados. Otra fuente de entrada de estos contaminantes al medio ambiente se debe a la inapropiada eliminación de los medicamentos sobrantes por el inodoro y/o por la basura. La importancia relativa de esta fuente de entrada, con respecto a los niveles globales de fármacos en el medio ambiente, es todavía desconocida (2).

Como consecuencia del desarrollo de nuevas técnicas analíticas y con la aparición de nuevos equipamientos instrumentales cada vez más sensibles, se han detectado niveles de concentración de fármacos muy bajos (e.g. hasta ng/L) en diversas matrices medioambientales (3). Se han encontrado principios activos, y sus metabolitos y/o productos de transformación, en aguas superficiales, sedimentos de río (4-6), en suelos regados con agua tratada (7-9) y/o con lodos de EDARs (10-11). Esos estudios han indicado que las tecnologías aplicadas en la depuración de aguas residuales no son suficientemente eficaces como para eliminar los fármacos y, por consiguiente estos micro-contaminantes van a parar al medio ambiente.

Una vez los fármacos han entrado en el medio ambiente, aunque estos se encuentren en bajas concentraciones, pueden bioacumularse en organismos acuáticos y terrestres, especialmente en aquellos organismos acuáticos que están expuestos continuamente y durante largos periodos de tiempo a efluentes de aguas residuales

(12-14). Aunque los efectos y mecanismos de acción de los fármacos han sido ampliamente evaluados en estudios de seguridad y toxicidad en la práctica clínica, sus potenciales impactos sobre el medioambiente son parcialmente desconocidos (15). Algunos estudios revelan algunos de sus efectos negativos sobre la biodiversidad. El diclofenaco (antiinflamatorio) ha causado mortalidad en poblaciones de buitres G*yps bengalensis* en el subcontinente indio, después de que estos hubieran ingerido restos de animales tratados con este fármaco (16). Efectos similares se han observado en la trucha arcoíris (Oncorhynchus mykiss) después de una exposición de 28 días a diclofenaco presente en bajas concentraciones (17). Algunos estudios señalan efectos en el crecimiento, sexualidad, fertilidad y comportamiento reproductivo de los organismos acuáticos expuestos continuamente a los esteroides sexuales naturales y sintéticos (15). Sin embargo, la mayoría de los estudios se han realizado con compuestos individuales, y raramente con mezclas de estas sustancias que son las que realmente se encuentran en el medio ambiente. Los científicos todavía no han encontrado evidencias de que la presencia, a bajas concentraciones, de compuestos farmacológicamente activos en el medio ambiente (e.g. vía agua potable) pueda provocar efectos adversos para la salud humana, sin embargo, sí que se ha observado que las mezclas complejas pueden provocar efectos nocivos a largo plazo en el medio ambiente acuático, por lo que estos escenarios deben de ser investigados en profundidad.

La presencia de fármacos en el medioambiente ha suscitado un especial interés científico en los últimos años, y también se ha dedicado un importante esfuerzo en el estudio de la eliminación de estos compuestos durante el proceso de depuración de aguas. Se trata de un tema relativamente reciente, por lo que la presencia de estos compuestos en el medioambiente no está incluida en la normativa actual. A pesar de este vacío en la legislación, diferentes agencias gubernamentales y no-gubernamentales (agencias reguladoras, suministradoras de agua, así como el público en general) son conscientes del riesgo potencial que supone para el medioambiente la presencia de fármacos. Una vez identificado este peligro potencial, nuestros esfuerzos han de dirigirse hacia minimizar este problema y encontrar unas soluciones idóneas via mejor tratamiento en las EDARs y/o buenas prácticas en la utilización de fármacos por parte de la población.

4.2. Objetivos

Los objetivos generales de esta tesis han sido:

I) evaluar la presencia, comportamiento y eliminación de 43 fármacos en plantas convencionales de tratamiento de aguas residuales (EDAR);

II) estudiar el grado de degradación y los productos de transformación del fármaco carbamazepina generados durante los experimentos a) usando el hongo *Trametes versicolor*, y b) mediante procesos de oxidación avanzada, i.e. fotocatálisis y sono-fotocatálisis.

4.3. Presencia, comportamiento y eliminación de fármacos en sistemas convencionales de tratamiento de aguas residuales y lodos

4.3.1. Fármacos de interés

Los estudios presentados en esta tesis se han realizado con 43 compuestos pertenecientes a diferentes grupos terapéuticos seleccionados en base a la información sobre el uso de fármacos y su presencia en las muestras ambientales, asi como con a la posibilidad de analizarlos conjuntamente. Los compuestos analizados pertenecen a los siguientes grupos terapéuticos: *antiinflamatorios no esteroideos* (AINE) (reducen los síntomas de la inflamación, el dolor y la fiebre), *los agentes que reducen los lípidos séricos* (para el tratamiento de la hipertrigliceridemia), *ansiolíticos y antiepilépticos* (en el tratamiento de la epilepsia, el trastorno bipolar etc.), *los agentes bloqueadores beta-adrenérgicos* (en el tratamiento de los trastornos del ritmo cardíaco y en la cardioprotección posterior a un infarto de miocardio), *agonistas β2 adrenérgico* (para el alivio del asma y la enfermedad pulmonar obstructiva crónica), *antagonistas* H_2 (para la disminución en la producción de ácido del jugo gástrico), *antibióticos* (en el tratamiento de las infecciones provocadas por bacterias), *inhibidores de la enzima convertidora de angiotensina (IECA)* (el tratamiento de la hipertensión y insuficiencia cardíaca crónica), *diuréticos* (para reducir la hipertensión arterial) y *antidiabéticos* (en el tratamiento de la diabetes mellitus). Los compuestos seleccionados se muestran en la Tabla 2.1 de esta tesis.

4.3.2. Metodologías

Las muestras de aguas residuales se han procesado y analizado por el método desarrollado y validado por Gros et al (199). El método se basa en la extracción en fase

sólida con cartuchos Oasis® HLB (Waters, Milford, MA, USA) y análisis mediante cromatografía líquida acoplada a espectrometría de masas en tándem, empleando un sistema híbrido triple quadrupolo/trampa de iones lineal (4000 QTRAP - *Applied Biosystems*/MDS SCIEX, USA) (SPE-HPLC-QqLIT-MS/MS). En resumen, justo después del muestreo, el agua residual se filtró con filtros de 1 µm de fibra de vidrio y 0.45 µm de nailon, y se conservó a − 20 °C hasta su análisis. Se tomó una alícuota de muestra (i.e. 100 mL de agua de afluente, y 200 mL del efluente), y se procesó mediante extracción con cartuchos Oasis® HLB. Posterior elución se realizó con 8 mL de metanol. Después de la evaporación de los extractos, las muestras se reconstituyeron con 1 mL de agua/metanol, 3/1, v/v. Antes de analizarlas por HPLC-MS/MS, las muestras se enriquecieron con una mezcla de estándares internos hasta una concentración de 20 ng/mL.

La cromatografía líquida ha sido realizada con el sistema Symbiosis™ Pico (SP104.002, Spark, Holland), con autosampler, acoplado en serie con el espectrómetro de masas 4000 QTRAP equipado con fuente Turbo Ion Spray (Applied Biosystems-Sciex, Foster City, CA, USA). La separación cromatográfica se ha llevado a cabo a través de una columna C-18 - Purospher Star RP-18 (125 mm × 2.0 mm, 5 µm) *precedida por* una *columna* de guarda C_{18} (4 mm× 4 mm, 5 µm) de Merck (Darmstadt, Germany), usando un gradiente de agua HPLC (A) y acetonitrilo/metanol (1/1, v/v) (B) con un caudal de 0.2 mL/min en modo negativo, y usando el gradiente de 0.1% acido fórmico en agua HPLC (A) y acetonitrilo (B) con un caudal de 0.3 mL/min en modo positivo.

El método de Gros et al (199), para análisis de fármacos en aguas, ha sido el punto de partida para el desarrollo del método de análisis de dichos fármacos en las muestras de lodos, ya que incluía los compuestos de interés y ofrecía elevada recuperación. El método para la determinación de fármacos en las muestras de lodo, basado en extracción con fluidos presurizados, purificación de los extractos mediante SPE, y análisis por HPLC-QqLIT-MS/MS (199), está explicado en detalle en la publicación**:** *Multi-residue method for trace level determination of pharmaceuticals in solid samples using pressurized liquid extraction followed by liquid chromatography/quadrupole-linear ion trap mass spectrometry* (Talanta (2009) 80 (1) 363-371).

La extracción de las muestras sólidas ha sido realizada mediante extracción con líquidos presurizados (PLE) utilizando el sistema ASE200 (Dionex, Sunnyvale, CA). Una vez liofilizado y molido el lodo, se tomó 1 g de muestra y se dopó con mecoprop-d_3 y sulfathiazole-d_3 y se extrajo bajo las siguientes condiciones: metanol/agua, 1/2, v/v, como disolvente de extracción; 100 ºC de temperatura, y 1500 psi de presión; 3 ciclos de extracción estática de 5 min; paso del disolvente al 100 % del volumen de la celda; y la purga de nitrógeno de 60 s. Los extractos PLE (~22 mL) han sido diluidos con agua

HPLC hasta un volumen final de 500 mL, a continuación se procesaron y analizaron como las muestras acuosas, por SPE-HPLC-QqLIT-MS/MS (199).

4.3.3. Análisis de datos

Los datos adquiridos en el modo de monitorización de reacciones seleccionadas - *selected reaction monitoring* (SRM) - se han procesado con el *software* Analyst 4.2 (Applied Biosystems – MDS SCIEX, USA). La integración automática de los picos se ha revisado manualmente. Se han monitorizado dos transiciones SRM entre el ión precursor y dos iones fragmento para cada compuesto excepto para el ibuprofeno, la hidroclorotiazida y la pravastatina. En el caso de los patrones internos – isotópicamente marcados - se ha registrado sólo una transición ya que es poco probable encontrarlos en el medio ambiente. Aunque la detección en SRM ofrece una elevada selectividad y especificad, en algunos casos pueden darse falsos positivos. Por tanto, con el fin de asegurar una confirmación más precisa de la presencia de los compuestos de interés, la identificación y cuantificación de los analitos se han hecho siguiendo las normativas de la Unión Europea (Decisión de la UE 2002/657/EC implementando la Directiva 96/23/EC):

1) El ratio de las intensidades relativas de las dos transiciones SRM del analito de interés en la matriz real debe ser comparable al ratio de dos transiciones del patrón estándar de calibración en disolvente o bien de la misma muestra enriquecida, medidas en las mismas condiciones, y con un margen de tolerancia ±20-50% dependiendo al valor del ratio de la intensidades.
2) El tiempo de retención del analito en la muestra real (i.e. matriz) puede variar hasta un 2.5 % con respecto al tiempo de retención de su patrón estándar correspondiente y el patrón interno en la misma matriz.

Para la cuantificación de los fármacos de interés, en todos los análisis realizados durante esta tesis, se ha utilizado el método de calibración por patrones internos como estrategia para corregir las pérdidas de señal por los efectos matriz, y, de esta manera, evitar errores en el cálculo de las concentraciones de los compuestos detectados. Los patrones internos marcados isotópicamente usados en los análisis han sido compuestos de estructura química y de tiempo de retención cromatográfico parecidos o/y iguales a los compuestos estudiados. Las rectas de calibración correspondientes a cada patrón se han establecido mediante análisis de regresión lineal por mínimos cuadrados, obteniendo coeficientes de correlación de R^2>99% para la mayoría de fármacos analizados en el rango de concentraciones de 0.5-100 ng/mL. La precisión, expresada como la desviación estándar relativa (RSD, %), de los métodos aplicados ha sido determinada mediante estudios de repetitividad (*intra-day)* y de reproducibilidad *(inter-day)*: 5 inyecciones de patrón estándar de 50 ng/mL en un día y durante varios

días consecutivos de análisis (dependiendo en la duración del análisis), respectivamente. En general, la precisión de análisis se ha considerado aceptable para las RSD<15% para las muestras acuosas y <20% para las muestras de lodo. Los límites de detección del método (MDL) y los límites de cuantificación del método (MQL) se han determinado a partir de muestras enriquecidas con la cantidad mínima del analito detectable y cuantificable con una relación señal-ruido *signal-to-noise* (S/N) de 3 y 10, respectivamente. Debido a la excelente sensibilidad del QqLIT-MS, se han obtenido unos MQL muy bajos, i.e. del orden de ng/L para aguas, y de menos de 1 ng/g (peso seco) para la mayoría de fármacos analizados en lodos. Para la determinación de los porcentajes de recuperación, tres muestras de cada matriz analizada se han enriquecido con una mezcla de patrones a concentraciones finales de 50 ng/g en el caso de lodos, y a 100 ng/L en el caso de aguas residuales, y se analizaron junto a los blancos de cada matriz por las metodologías descritas (199, 226). Para la mayoría de los fármacos analizados se han obtenido unas recuperaciones mayores del 50% en todas las matrices. En algunos casos, la recuperación ha sido inferior al 40%, pero no se ha considerado un inconveniente, ya que estos compuestos presentan unos límites de detección bajos y con unos RSD% inferiores al 15%.

La principal ventaja de los métodos multiresiduo es que permiten analizar en un sólo análisis un importante número de compuestos con propiedades fisicoquímicas parecidas, reduciendo de esta manera el número de análisis, la cantidad de muestra necesaria, el tiempo de preparación de muestra y por lo tanto el coste del análisis. La principal desventaja de estos métodos es que el rendimiento analítico no puede ser el óptimo para cada compuesto individual, por lo que para desarrollar estos métodos se debe de alcanzar un compromiso a la hora de seleccionar las condiciones experimentales de manera que sean las más adecuadas para la mayoría de compuestos.

4.3.4. Muestreo

El primer objetivo de la presente tesis consiste en ampliar el conocimiento sobre la presencia de fármacos en afluentes y efluentes de estaciones depuradoras de aguas residuales (EDARs) urbanas, y estudiar en profundidad su destino y eliminación durante los tratamientos aplicados. Para ello, se hicieron dos estudios explicados a continuación:

El primer trabajo consta de 8 campañas realizadas entre Julio de 2007 y Marzo de 2009, en el marco del proyecto CENIT SOSTAQUA ("Desarrollos Tecnológicos hacia un ciclo urbano del agua autosostenible"). Se analizaron muestras de la entrada y de la salida de 3 estaciones depuradoras convencionales ubicadas en la región de Cataluña. Aunque en las plantas seleccionadas no se realizan todos los posibles

tratamientos de aguas residuales urbanas, se puede considerar que estas EDARs presentan configuraciones típicas de las EDARs de esta región. Las 3 EDARs constan de un proceso de sedimentación primaria, seguido de un tratamiento secundario (biológico) de lodos activos. La EDAR#3 además dispone de un sistema de depuración terciario mediante filtración por arena y desinfección. En la EDAR#1, la mezcla de lodo primario y secundario se deshidrata mecánicamente mediante centrifugación y se destina para ser usado como compostaje. En caso de las EDAR#2 y EDAR#3 el lodo está estabilizado mediante digestión anaerobia mesófila y deshidratado mediante centrifugación (EDAR#2) o mediante filtros de presa (EDAR#3).

Las muestras de agua, integradas de 24h cada una, se recogieron tanto en afluentes como en efluentes de las EDARs. Las muestras se tomaron mediante muestreadores automáticos teniendo en cuenta los tiempos de retención hidráulica (HRT) para el muestreo de efluentes. Las muestras de lodos se recogieron como muestras puntuales.

En el segundo estudio se evaluó el rendimiento de una estación de depuración de aguas residuales urbanas situada en la región del Veneto (Italia). El agua de entrada fue tratada mediante un proceso de eliminación biológica de nutrientes (BNR) con fangos activos. En esta EDAR, debido a una infiltración de agua subterránea en una tubería del sistema de aguas residuales (lo que origina una dilución del afluente), no se realiza la sedimentación primaria con tal de conservar la demanda química de oxigeno (COD) y mejorar así el proceso de BNR. El exceso de lodo (WAS) se mezcla con la fracción orgánica de los residuos sólidos urbanos (OFMSW), se espesa y se somete a una co-digestión anaerobia mesófila. El sobrenadante de la co-digestión anaerobia se trata en un proceso de *short-cut* nitrificación/desnitrificación en un reactor “batch” en secuencia (Sequencing Batch Reactor - SBR), y finalmente se devuelve a la entrada de la EDAR.

Para este estudio se realizaron dos campañas de muestreo, una en Diciembre de 2010 y otra en Febrero de 2011. Las muestras de afluente y efluente proporcionales al volumen de agua de afluente y las muestras integrales (compuestas por 5 muestras puntuales por día) de los lodos provenientes del espesador de lodos, digestor anaerobio y filtro-prensa se recogieron durante tres días consecutivos.

4.3.5. Discusión de resultados

Para la realización de estos estudios se siguieron los procedimientos resumidos anteriormente (199, 226) y los resultados fueron discutidos en las publicaciones científicas:

- Nº2: *Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment* (Water Research (45) 3: 1165–1176);
- Nº3: *Tracing pharmaceuticals in a municipal plant for integrated wastewater and organic solid waste treatment* (Science of The Total Environment (433) 352–361).
- Nº4: *New indexes for compound prioritization and complexity quantification on environmental monitoring inventories* (Environmental Science and Pollution Research (2012) 19 (4): 958-970);

Los resultados obtenidos indican que los tratamientos de depuración realizados en las EDARs estudiadas son sólo moderadamente eficaces en la eliminación de los fármacos. La eficacia en el proceso de eliminación de fármacos depende de multitud factores, entre ellos las propiedades físico–químicas de los fármacos, las características de las aguas residuales, la tecnología empleada para la depuración, las condiciones de funcionamiento, etc. Por lo que se observaron diferencias significativas entre las tres EDARs estudiadas.

Las EDARs actuales fueron diseñadas principalmente para su eficaz eliminación de materia orgánica y en algunos casos de nutrientes. Sin embargo, esto no es extensible a la eliminación de algunos fármacos, que pueden pasar por las diferentes etapas de depuración sin prácticamente sufrir cambios, en parte debido a su baja biodegradabilidad. Por tanto, no se puede esperar que los tratamientos de depuración convencionales sean el unico mecanismo para reducir la entrada de fármacos en el medio ambiente.

Además de desarrollar nuevas estrategias para mejorar los sistemas de depuración actuales, también se deberían gestionar las aguas residuales antes de entrar en las EDARs (e.g. la separación de corrientes para tratarlas en plantas más pequeñas). También sería necesario implementar unas medidas concretas de prevención y control de las fuentes de emisión de fármacos para poder reducir su impacto medioambiental.

A partir de los dos estudios realizados se obtuvieron las siguientes conclusiones más específicas:

- No se observaron diferencias estadísticamente significativas entre las eficiencias de eliminación de fármacos de las aguas residuales crudas (Nº2) o diluidas (Nº4). No obstante, cabe señalar que las eficiencias de eliminación, calculadas como una diferencia entre la masa de los principios activos (fármaco padre) en la entrada y salida, pueden ser sub- o sobre-estimadas por varias razones: a) no se determinó la presencia de los metabolitos / productos de transformación y b) los muestreos empleados no fueron los más adecuados para los análisis realizados.

  Los fármacos detectados en los efluentes de las EDARs cubren un amplio rango de propiedades físico – químicas y actividades biológicas y farmacológicas. En ambos estudios, los coeficientes de riesgo (calculados a partir de la concentración máxima

de fármaco y de los valores de toxicidad aguda en peces, algas y *Daphnia magna* obtenidos en la literatura) indicaron que el agua de efluente de las EDAR no presenta ningún riesgo para los organismos acuáticos estudiados. La toxicidad crónica de las mezcla de fármacos detectadas en ambos casos se desconoce, por tanto el riesgo real de estos compuestos no se puede evaluar. Por tanto, se requieren nuevas interpretaciones para poder evaluar el riego medioambiental de cada fármaco.

- Aunque los compuestos analizados son polares, algunos de ellos se detectaron también en lodos. Esto indica que, además de la hidrofobicidad, existen otros factores que pueden afectar y contribuir a su sorción en lodos/sólidos suspendidos. Se ha observado que estas interacciones no son lo suficientemente fuertes como para que la adsorción sea el mecanismo más importante en la eliminación de los fármacos estudiados en la fase acuosa. Para obtener más información acerca de los mecanismos que intervienen en la eliminación de fármacos durante la depuración, se calcularon los balances de masa para cada fármaco cuantificado en las muestras de agua y lodo y se calcularon (experimentalmente) los correspondientes coeficientes de distribución (K_d). Ambos experimentos indicaron que la biotransformación es el mecanismo principal para la eliminación de estos fármacos en el tratamiento biológico aplicado.

4.4. Degradación del anticonvulsivante carbamazepina

La desaparición de un compuesto padre (i.e. principio activo) en el tratamiento de aguas residuales no significa que el compuesto haya sido eliminado ni que el tratamiento empleado haya sido realmente eficiente. En cambio, sí que puede indicar que el compuesto padre se ha transformado en unos (sub)productos, pudiendo estos ser bioactivos y/o tóxicos. Por tanto, es muy importante identificar y cuantificar los productos de transformación generados durante la degradación/transformación de los compuestos de interés en los tratamientos de aguas residuales, así como estimar su toxicidad con tal de evaluar el riesgo medioambiental de los fármacos.

En la presente tesis se realizaron dos series de experimentos con el anticonvulsivante carbamazepina (CBZ):

I) degradación biológica mediante un tratamiento ecológico y suave con el hongo *Trametes Versicolor* en un reactor *batch* a escala de laboratorio.

II) degradación mediante procesos químicos avanzados: fotocatálisis (con radiación solar y UVA) y sonofotocatálisis.

Se seleccionó la CBZ ya que en estudios previos se observó que presenta un comportamiento recalcitrante durante los procesos biológicos de las estaciones de tratamiento de aguas residuales.

4.4.1. Carbamazepina

La carbamazepina (CBZ, 5H-dibenzo[b,f]azepine-5-carboxamide) es un fármaco derivado del imidoestilbeno de estructura similar a la imipramina con propiedades anticonvulsivantes. Está indicado en el tratamiento de las crisis epilépticas parciales simples, parciales complejas con o sin generalización secundaria, crisis generalizadas tónicas, clónicas o tónico-clónicas, neuralgia del trigémino, neuralgia del glosofaríngeo y neuropatía diabética. La CBZ es una molécula neutra y lipófila, prácticamente insoluble en agua pero bastante soluble en acetona y alcohol.

Según la Agencia Española de Medicamentos y Productos Sanitarios, tiene un consumo de 1.3 DDD/1000 habitantes por día en el año 2006 en España. El consumo global está estimado en 1000 toneladas por año. Este fármaco parece ser de los más resistentes a la biodegradación, y prácticamente no se encuentra ningún porcentaje de eliminación superior al 10-20 % en EDARs. Muchos estudios indicaron que la CBZ es un compuesto ubicuo en aguas residuales y en diferentes matrices ambientales (suelo, ríos y aguas subterráneas). Incluso, se ha propuesto usar la CBZ como marcador molecular de la contaminación humana por aguas residuales, por su alta resistencia a la degradación.

Recientemente, varios estudios has considerado a los hongos como una interesante alternativa para la eliminacion de los compuestos recalcitrantes y tóxicos, debido a su acción enzimática no-especifica de oxidasas y peroxidasas. Aunque la CBZ no es un sustrato para las enzimas de los hongos, se han obtenido unas eliminaciones superiores al 50 % utilizando mediadores redox (e.g. quinonas) durante los tratamientos con hongos.

Los procesos de oxidación química (AOP), e.g. ozonización, fotocatálisis heterogénea (TiO2), fotolisis directa y catalizada (UV/H_20), ofrecen una elevada eliminación (>90%), pero el principal inconveniente que presentan es que, si no se consigue la mineralización completa del medio tratado, se pueden generar productos de transformación y subproductos de degradación, posiblemente tóxicos. Se necesitaría, por tanto, profundizar en el conocimiento de los productos/subproductos (i.e. identificación) y de la toxicidad del medio generado para evaluar su impacto ambiental y tomar decisiones adecuadas sobre la aplicación de AOPs en el tratamiento de aguas residuales. Aunque en últimos años se publicaron diversos estudios en ese tema, el conocimiento es todavía escaso.

4.4.2. Análisis cualitativo de los productos de transformación de CBZ

Los análisis cualitativos se llevaron a cabo empleando un sistema UPLC Waters ACQUITY UPLC™ (Micromass, Manchester, RU) acoplado con un espectrómetro de masas Waters/Micromass QqToF-Micro™, utilizando una columna Waters ACQUITY BEH C18 (10 x 2.1 mm, 1.7 µm). El primer paso en el estudio de identificación de los productos de transformación de CBZ generados durante los experimentos realizados fue el análisis en modo full-scan en el QqToF-MS, con el objetivo de detectar posibles derivados de la CBZ formados durante los experimentos. Después, para cada pico obtenido en el cromatograma de iones totales, se realizó una serie de experimentos MS/MS de masa exacta, aplicando diferentes energías de colisión, con tal de obtener información sobre la fragmentación de los productos de interés. El software MassLynx v4.1 fue una herramienta importante a la hora de elucidar los intermediarios. Este software permite asignar las formula empíricas (composiciones moleculares) más probables para la masa medida con una tolerancia de +/- 5 ppm de error (entre la masa medida y calculada). Los espectros de masas adquiridos se compararon con los patrones correspondientes cuando se disponía de ellos, en caso contrario, las estructuras fueron asignadas a partir de la fragmentación MS/MS y composición molecular que mejor se correspondía.

4.4.3. Degradación biológica mediante hongo *Trametes Versiolor*

En colaboración con un grupo del Departamento de Ingeniera Química de la Universidad Autónoma de Barcelona, estudiamos el potencial del hongo *Trametes versiolor* para degradar la CBZ en un biorreactor, a escala de laboratorio, operando en los modos *batch* y continuo. Los resultados de este estudio se describen en detalle en la publicación (Nº5): *Degradation of carbamazepine by Trametes versicolor in an air pulsed fluidized bed bioreactor and identification of intermediates* (Water Research (2012) 46 (4) 955-964).

En este estudio se llegó a las siguientes conclusiones:

El hongo *Trametes Versicolor* es capaz de degradar la CBZ en medio líquido en los dos modos de operación (batch y continuo). En ensayos preliminares, con la CBZ a una concentración de 9 mg/L, se determinaron cuatro productos de transformación: 10,11-epoxicarbamazepina, 10,11-dihidro-10,11-dihidroxicarbamazepina, acridina y 9,10-dihidro-9-oxoacridina. En los experimentos en modo batch, la CBZ (a 200 µg/L) fue eliminada/transformada con un porcentaje de 96 % después de 2 días. El único producto de transformación identificado fue la 10, 11-epoxicarbamazepina. Trabajando en modo continuo, con un HRT de 3 días, se logró alcanzar un porcentaje de degradación de 54% en el estado estacionario (25 días), pero no se detectó ningún

producto de transformación. Ensayos de toxicidad aguda demostraron que el medio de cultivo final de ambos experimentos no fue tóxico, con unos valores de EC50 (15 min) de 24 % en modo batch, y 77 % en modo continuo. Por lo tanto, el tratamiento mediante hongos de podredumbre blanca puede considerarse una potente estrategia para mejorar la degradación de la CBZ. No obstante, se necesitan pruebas adicionales con agua residual para poder evaluar la efectividad y posibilidad de scale-up del proceso en condiciones reales.

4.4.4. Degradación *vía* AOPs: fotocatálisis heterogénea TiO2/UV y TiO2/solar, y sonofotocatálisis

En colaboración con un grupo de Ingeniera Civil de la Universidad de Chipre, evaluamos la posibilidad de utilizar procesos avanzados de oxidación como fotocatálisis heterogénea TiO_2/UV y TiO_2/solar, y sonofotocatálisis, para eliminar la CBZ del medio líquido. Los estudios se llevaron a cabo a escala de laboratorio en un foto-reactor cilindro-parabólico compuesto en operación *batch*. Los resultados se discuten en detalle en la publicación (Nº6): *Photocatalytic and sonophotocatalytic degradation of the anticonvulsant carbamazine and identification of transformation products.*

Tanto con la fotocatálisis heterogénea vía TiO_2/UV como con la sonofotocatálisis se obtuvieron elevados porcentajes de eliminación para la CBZ (95 %). Al comparar los resultados obtenidos en ambos procesos, transcurridos los 120 min de los experimentos, las diferencias encontradas fueron mínimas. Por lo que es difícil concluir cuál de los dos procesos fotocatalíticos es más eficiente para la eliminación de la CBZ. En los dos procesos se generaron unos productos que presentaban mayor toxicidad que el compuesto padre, y los cuales no desaparecieron a lo largo de los experimentos. El porcentaje de degradación de la CBZ para la fotocatálisis heterogénea solar con TiO_2 fue del 10 %. El ensayo de toxicidad, basado en la inmovilización de *Daphnia magna*, también señaló un ligero aumento de la toxicidad para este tratamiento. Sería conveniente realizar experimentos prolongados (para los tres procesos) con tal de determinar el momento a partir del cual la composición del medio tratado sea constante y su toxicidad sea la mínima, i.e. hasta su completa mineralización. Se identificaron nueve productos de transformación de la CBZ durante la sonofotocatálisis y fotocatálisis heterogénea mediante TiO_2/UV, y ocho durante la fotocatálisis heterogénea solar. En general, se observó la formación de los derivativos oxigenados de CBZ (e.g. hidroxi- y oxo- compuestos), probablemente generados a través mecanismos similares en los tres experimentos. Estos compuestos parecen ser más persistentes que la CBZ porque estaban presentes, en concentraciones bajas, hasta el final del experimento (120 min).

Chapter V

General conclusions

5.1. Conclusiones generales

Desde hace más de 40 años se ha detectado la presencia de fármacos en el ciclo de aguas, debido a los avances en la química analítica que han permitido el desarrollo de nuevas metodologías analíticas para la determinación de estos compuestos de modo fiable y a bajas concentraciones. A pesar de que los niveles de concentración de cada fármaco considerándolos individualmente en aguas superficiales y en aguas de consumo humano no son críticos para la salud humana y el medio ambiente, las consecuencias a nivel de mezclas de estos compuestos no son claras. Las estaciones depuradoras de aguas residuales (EDARs) han sido identificadas como la ruta principal de entrada de fármacos de origen humano en el medioambiente.

El principal objetivo de esta tesis ha sido el estudio de la presencia, destino y eliminación, de 43 fármacos seleccionados, durante el tratamiento convencional realizado en las depuradoras de aguas residuales. Los compuestos estudiados fueron seleccionados en base a los índices de consumo en España, a la frecuencia de detección en aguas residuales y además en base a la posibilidad de ser analizados bajo las mismas condiciones experimentales. Estos compuestos pertenecen a diferentes clases terapéuticas, i.e. antiinflamatorios no esteroideos , los agentes que reducen los lípidos séricos, ansiolíticos y antiepilépticos, los agentes bloqueadores beta-adrenérgicos, agonistas β2 adrenérgico, antagonistas H_2 , antibióticos, inhibidores de la enzima convertidora de angiotensina, diuréticos y antidiabéticos.

En esta tesis se observaron grandes diferencias en las eficiencias de eliminación de los fármacos seleccionados, independientemente de la clase terapéutica a la que pertenecen y de los procesos llevados a cabo durante el tratamiento en las depuradoras. Por lo que no se aprecia ninguna tendencia clara en la eliminación de alguno de estos compuesto en particular. De acuerdo con los balances de materia calculados y con los coeficientes de partición estimados, la eliminación de los fármacos estudiados, durante el tratamiento biológico de las depuradoras, puede ser atribuida completamente a los procesos de biodegradación/biotransformacion. Ninguno de los compuestos estudiados fue eliminado totalmente (i.e. biodegradado y/o transformado) durante el tratamiento biológico realizado en las depuradoras, pero las concentraciones detectadas estuvieron por debajo de los niveles considerados peligrosos, de acuerdo con los datos de toxicidad disponibles actualmente.

Las EDARs convencionales se consideran como el único mecanismo para controlar la entrada de fármacos en el medioambiente, pero no fueron diseñadas para este propósito y no pueden garantizar la total eliminación de estos compuestos. Por lo tanto, los esfuerzos deben dirigirse en buscar soluciones económicas y efectivas para

prevenir que los fármacos alcancen las aguas naturales. Por lo tanto, se deberían considerar aspectos como mejorar la gestión de las aguas residuales antes de su entrada en las plantas depuradoras, realizar controles más estrictos de los efluentes, así como también invertir esfuerzos en desarrollar nuevos diseños y estrategias para mejorar las estaciones depuradoras existentes.

Ante estos hechos, en esta tesis como segundo objetivo hemos considerado evaluar tratamientos avanzados alternativos al tratamiento convencional para la eliminación de un fármaco antiepiléptico, la carbamazepina, uno de los compuestos más recalcitrantes al tratamiento biológico convencional. Se procedió a estudiar su degradación en medio acuoso mediante dos procedimientos a escala laboratorio: a) biodegradación utilizando el hongo ligninolitico *T.Versicolor* en un reactor fluidizado por pulsos de aire operando en modo "batch" y continuo, y b) oxidación avanzada mediante un tratamiento fotocatalitico en presencia de TiO_2 bajo irradiación UV-A y solar, y aplicando la radiación UV en combinación con ultrasonidos (sonofotocatálisis). Ambos métodos, a pesar de ser de naturaleza muy diferente, demostraron ser muy efectivos para la degradación de carbamazepina en medio acuoso. El tratamiento con hongos presentó unas medias de eliminación del 54 y 96% para reactores *batch* y en continuo, respectivamente. También se evaluó la toxicidad con el ensayo *Vibrio Fischeri* de las muestras tratadas observándose una disminución de la toxicidad en ambos reactores. En el otro estudio, se alcanzó prácticamente la una fotodegradación total de la carbamazepina (~95%) durante los 120 min que duraron los experimentos de UVA-fotocatalisis y sonofotocatalisis; mientras que solo el 10% de la carbamazepina se degradó al realizar los experimentos fotocatalíticos con luz solar. En los ensayos de toxicidad que se realizaron con *Daphnia magna* de las muestras fotodegradadas, se observó un leve aumento en la toxicidad respecto al tiempo "zero" de degradación. Este hecho se puede asociar a la formación de los productos de transformación de la carbamazepina.

Para la evaluación de los tratamientos alternativos, se identificaron los productos de transformación de la carbamazepina. Se utilizó la cromatografía de líquidos de alta eficacia acoplada a un espectrómetro de masas con analizador de tipo cuadrupolo-tiempo de vuelo. La mayoría de los intermedios que fueron identificados tentativamente, presentaron sólo pequeñas modificaciones en sus estructuras moleculares. Tanto el proceso mediante hongos como el fotocatalítico originaron productos de transformación oxigenados. En el tratamiento por hongos, se formaron productos por epoxidación enzimática e hidroxilación del anillo heterocíclico central de la estructura carbamazepina. Y en el tratamiento fotocatalítico, se generaron productos por hidroxilación y oxidación en diferentes partes de la molécula. Los productos de transformación formados parecen ser más persistentes que la carbamazepina, ya que, se detectaron, aunque a bajas concentraciones, hasta el final de los experimentos.

Los resultados presentados en esta tesis contribuyen a un mayor conocimiento sobre a) la presencia y la cantidad de los 43 fármacos estudiados en el medioambiente una vez realizado el tratamiento de aguas residuales, b) la eficiencia que presentan las depuradoras convencionales en la eliminación de estos fármacos en aguas residuales y c) posibles alternativas y estrategias a tener en cuenta para mejorar la eliminación de estos compuestos.

5.2. General conclusions

Pharmaceuticals have been detected in natural waters for more than forty years, but with improvements in sample preparation procedures and analytical instrumentation, the number of scientific publications on the issue has increased significantly. Even though the concentration of pharmaceutical residues in surface and drinking water is not critical for human health according to the present level of knowledge, the consequences for the environment are not clear. Wastewater treatment plants have been identified as the primary route of pharmaceuticals to the environments, with households as the major source point for most of the over-the-counter and prescription drugs.

In this thesis, the first aim was to study the occurrence, fate and removal of 43 pharmaceuticals during conventional wastewater treatment. The target compounds were selected on the basis of their high consumption in Spain or/and frequently reported detection in wastewaters and the possibility to be analyzed under the same experimental conditions. They belong to different therapeutic classes, i.e. nonsteroidal anti-inflammatory agents and analgesics, lipid modifying agents, psycholeptic and antiepileptic drugs, beta-blocking agents, beta-2-adrenoreceptor agonists, H2-receptor antagonists, antibiotics, angiotensin converting enzyme agents, diuretics and antidiabetic drugs.

A wide variation in removal efficiencies was observed even for individual compounds, and across therapeutic classes and treatment processes, without clear conclusion on the removal of any particular compound. According to mass balance calculations and estimated partition coefficients, the loss of the selected pharmaceuticals during biological wastewater treatment can be fully attributed to biodegradation/biotransformation. None of the studied compounds was entirely removed (i.e. biodegraded and/or transformed) during biological wastewater treatment, but the measured concentrations were below the levels of concern according to available toxicity data.

Conventional wastewater treatment plants cannot be expected to be the only mechanism for controlling the entry of pharmaceuticals into the environment because they were neither designed nor can provide their complete removal. Therefore, the challenge is to look for solutions that would be the most economical and effective means of preventing further pollution of natural waters by pharmaceuticals. More appropriate management of sewage waters before they enter treatment plants as well as a stricter control of effluent discharges, along with an in-depth investigation on the development of new designs and strategies for the improvement of existing wastewater treatments should be considered.

In light of this, as the second objective of this thesis, alternative approaches for the removal of the antiepileptic carbamazepine were studied in aqueous media in two laboratory scale experiments: a) biodegradation using white rot fungus *T. versicolor* in an air-pulsed fluidized bioreactor operated in batch and continuous modes, and b) advanced oxidation using TiO_2-heterogeneous photocatalysis under simulated solar and UV-A irradiation, and under the combined use of ultrasound and UV-A irradiation (sonophotocatalysis). We selected carbamazepine as a representative example of compounds that are found to be refractory to biological treatment and ubiquitous in various environmental matrices. Both, the fungal and UVA-driven TiO_2-photocatalytic treatments, very different in their nature, have been shown to be very effective in degrading carbamazepine in aqueous media. The fungal treatment resulted in average removals of 54 and 96% in batch and continuous reactor, respectively. Acute toxicity test using the bioluminescent marine bacterium *Vibrio fischeri* showed a decrease in toxicity during the treatment in both types of bioreactor. In the other study, almost complete photodegradation of carbamazepine (~95%) was achieved during 120 min of the UV-driven photocatalytic experiment and sonophotocatalysis, while only 10 % of carbamazepine was photodegraded during photocatalytic experiments under solar irradiation. A slight increase in toxicity in *Daphnia magna* acute toxicity testing was observed over the time-course of the photocatalytic experiments, which can be associated with the formation of transformation products of carbamazepine.

Ultraperformance liquid chromatography coupled to a quadrupole-time-of-flight mass spectrometry was used for a tentative identification of the transformation products of carbamazepine formed during the performed experiments. Most of the tentatively identified intermediates exhibited only slight modifications of the CBZ molecular structure. The fungal as well as the photocatalytic processes yielded oxygenated transformation products. In the biological treatment using *T. versicolor*, transformation products were formed by enzymatic epoxidation and hydroxylation of seven-membered heterocyclic ring of the carbamazepine molecule. During the photocatalytic experiments, CBZ-related transformation products emerged from hydroxylation and further oxidation of different parts of the molecule of carbamazepine. The generated transformation products appeared to be more persistent than their parent compound, as they were present, although at low concentration, until the end of the experiments.

The results of the thesis contribute to a better understanding of a) the magnitude of the selected pharmaceuticals that reach the environment through the wastewater and sludge discharge, b) the efficiency of typical conventional wastewater treatment plants regarding the removal of these compounds from raw wastewater, and c) possible developments of alternative technologies for their enhanced elimination.

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Annexes

ANNEX I – List of Abbreviations

ACE – Angiotensin Converting Enzyme (agents)
ACN – Acetonitrile
AcoD –Anaerobic-co-Digestion
AOP –Advanced Oxidation Processes
ASE – Accelerated Solvent Extraction
ATC system - Anatomical Therapeutic Chemical Classification system
BNR – Biological Nutrient Removal
BOD - Biochemical Oxygen Demand
CBZ - Carbamazepine
COD - Chemical Oxygen demand
DDD - Defined Daily Dose
EDAR - Estación Sepuradora de Aguas Residuales
ERA – Environmental Risk Assessment
ESI- ElectroSpray Ionization
GC – Gas Chromatography
GWD - Ground Water Directive
HF-LPME - Hollow Fiber Liquid Phase MicroExtraction
HILIC - Hydrophilic Interaction LIquid Chromatography
HLB - Hydrophilic-lipophilic-balanced (reversed-phase sorbent)
HPLC – High-Performance Liquid Chromatography
HRT – Hydraulic Retention Time
IDA - Information Dependent Acquisition
LiP - Lignin Peroxidase
LLE - Liquid Liquid Extraction
LOD – Limit Of Detection
LOQ – Limit Of Quantification
LPME - Liquid-Phase MicroExtraction
MAX - Mixed-mode Anion-eXchange (sorbent)
MBR – Membrane BioReactor
MeOH -Methanol
MIP - Molecularly Imprinted Polymer
MnP - Manganese Peroxidase
MS – Mass Spectrometry
NI – Negative Ion (mode)
NOM - Natural Organic Matter
NSAID – NonSteroidal Anti-Inflammatory Drugs
OECD - Organisation for Economic Co-operation and Development

OFMSW – Organic Fraction of Municipal Solid Waste
·OH – Hydroxyl radical
OSPAR - Oslo/Paris convention (for the Protection of the Marine Environment of the North-East Atlantic)
PAH - Polycyclic Aromatic Hydrocarbons
PC – TiO_2-PhotoCatalysis under UV-A irradiation
PCB - Polychlorinated Biphenyls
PI – Positive Ion (mode)
PLE – Pressurized Liquid Extraction
QqLIT-Quadrupole-Linear Ion Trap
QqQ – Triple-Quadrupole
QqTOF –Quadrupole-Time-Of-Flight
SBR – Sequencing (Sequential) Batch Reactor
SDME - Single-Drop MicroExtraction
SPC - SonoPhotoCatalysis
SPE - Solid Phase Extraction
SRM – Selected Reaction Monitoring
SRT – Solid Retention Time
SSI – TiO_2-photocatalysis under Simulated Solar Irradiation
TiO_2 – Titanium dioxide
TPs – Transformation Products
U.S. EPA – United States Environmental Protection Agency
U.S. FDA – United States Food and Drug Administration
USE – UltraSonic Extraction
UV – Ultraviolet light
WAS - Waste Activated Sludge
WAX - Mixed-mode Weak Anion-eXchange (sorbent)
WFD - Water Framework Directive
WHO – World Health Organization
WWTP – WasteWater Treatment Plant

ANNEX II – List of Tables and Figures

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ANNEX III – List of publications and participation in conferences

List of Publications

SCI Journals

- **Jelic, A.,** Petrovic, M., & Barceló, D. (2009). Multi-residue method for trace level determination of pharmaceuticals in solid samples using pressurized liquid extraction followed by liquid chromatography/quadrupole-linear ion trap mass spectrometry. Talanta, 80(1), 363-371.

- **Jelic, A.,** Gros, M., Ginebreda, A., Cespedes-Sánchez, R., Ventura, F., Petrovic, M., & Barcelo, D. (2011). Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment. Water Research, 45(3), 1165-1176.

- **Jelic, A.,** Cruz-Morató, C., Marco-Urrea, E., Sarrà, M., Perez, S., Vicent, T., Petrović, M., & Barcelo, D. (2012). Degradation of carbamazepine by Trametes versicolor in an air pulsed fluidized bed bioreactor and identification of intermediates. Water Research, 46(4), 955-964.

- **Jelic, A.,** Fatone, F., Di Fabio, S., Petrovic, M., Cecchi, F., & Barcelo, D. (2012). Tracing pharmaceuticals in a municipal plant for integrated wastewater and organic solid waste treatment. Science of the Total Environment, 433 (1) 352-361

- **Jelic, A.,** Michael, I., Achilleos, A., Lambropoulou, D., Perez, S., Petrovic, M., Fatta-Kassinos, D., Barcelo, D. Photocatalytic and sonophotocatalytic degradation of the anticonvulsant carbamazepine and identification of transformation products (submitted)

- Díaz-Cruz, M.S., García-Galán, M.J., Guerra, P., **Jelic, A.,** Postigo, C., Eljarrat, E., Farré, M., López de Alda, M.J., Petrovic, M., Barceló, D. (2009) Analysis and occurrence of selected emerging contaminants in sewage sludge, Trends in Analytical Chemistry TRAC, 28 (11) 1263-1275

- Radjenovic, J., **Jelic, A.,** Petrovic, M., Barceló, D. (2009) Determination of pharmaceuticals in sewage sludge by pressurized liquid extraction (PLE) coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS), Analytical and bioanalytical chemistry 393 (6-7) 1685-1695

- Ferreira da Silva, B., **Jelic, A.,** Lopez Serna, R., Mozeto, A. A., Petrovic, M., Barceló D. (2011) Loads and distribution of pharmaceutical discharges into the aquatic environment of the Ebro river basin,Chemosphere 85 (8) 1331-1339

- Rodríguez-Rodríguez, C.E., **Jelic, A.,** Llorca, M., Farré, M., Caminal, G., Petrović, M., Barceló, D., Vicent, T. (2011) Solid-phase treatment with the fungus *Trametes*

versicolor substantially reduces pharmaceutical concentrations and toxicity from sewage sludge, Bioresource Technology, Volume 102 (10)5602-5608

- Al Aukidy, M., Verlicchi, P., **Jelic, A.,**Petrovic, M., Barcelò, D. (2012) Monitoring release of pharmaceutical compounds: Occurrence and environmental risk assessment of two WWTP effluents and their receiving bodies in the Po Valley, Italy, Science of the Total Environment, 438, 15-25

- Rodríguez-Rodríguez, C.E., Barón, E., Gago-Ferrero, P., **Jelić, A**., Llorca, M., Farré, M., Díaz-Cruz, M.S., Eljarrat, E., Petrović, M., Caminal, G. , Barceló, D., Vicent, T. (2012) Removal of pharmaceuticals, polybrominated flame retardants and UV-filters from sludge by the fungus Trametes versicolor in bioslurry reactor, Journal of Hazardous Materials,Vol. 233-234, 235-243

- Ginebreda, A., **Jelic, A.,** Petrovic, M., López de Alda, M, Barceló, D. (2012) New indexes for compound prioritization and complexity quantification on environmental monitoring inventories, Environmental Science and Pollution Research, 19 (4) 958-970

- Rodríguez-Rodríguez, C. E., **Jelic, A.** , Alcina Pereira, M., Sousa, D. Z., Petrović, M., Alves, M.M., Barceló, D., Caminal, G., Vicent T. (2012) Bioaugmentation of Sewage Sludge with *Trametes versicolor* in Solid-Phase Biopiles Produces Degradation of Pharmaceuticals and Affects Microbial Communities, Environemntal Science and Technology, DOI: 10.1021/es301788n

- Cruz-Morató, C., **Jelic, A**., Perez, S., Petrovic, M., Barceló, D., Marco-Urrea, E., Sarra, M., Vicent, T.Continuous treatment of clofibric acid by Trametes versicolor in a fluidized bed bioreactor: identification of transformation products and toxicity assessment (Submitted)

Book chapters

- **Jelic, A.**, Gros, M., Petrović, M., Ginebreda, A., & Barceló, D. (2012) Occurrence and Elimination of Pharmaceuticals During Conventional Wastewater Treatment. In H. Guasch, A. Ginebreda & A. Geiszinger (Eds.), Emerging and Priority Pollutants in Rivers, The Handbook of Environmental Chemistry, Volume 19 (pp. 1-23): Springer
- **Jelic, A.,** Petrović, M. and Barceló, D. (2012) Pharmaceuticals in Drinking Water, In D. Barcelo, A. G. Kostianoy (Eds.) Emerging organic contaminants and human health, The Handbook of Environmental Chemistry, Volume 20 (p.p. 47-70): Springer

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Attendance at conferences - Platform presentations:

Event: SIDISA 2012, June 2012, Milan, Italy
Title: Exposure assessment of pharmaceuticals in wastewaters and surface water - the case study of the catchment area of Ferrara
Authors: P. Verlicchi, **A. Jelic**, M. Al Aukidy, M. Petrovic, D.Barcelo

Event: SIDISA 2012, June 2012, Milan, Italy
Title: Fate of pharmaceuticals during the integrated treatment of municipal wastewater and organic fraction of municipal solid waste
Authors: **A. Jelic**, F. Fatone, S. Di Fabio, M. Petrovic, F. Cecchi, D.Barcelo

Event: Environmental Microbiology and Biotechnology - EMB2012, April 2012, Bologna, Italy
Title: Bioremediation of emerging pollutants from sewage sludge by fungal bioaugmentation
Authors: C. E. Rodríguez-Rodríguez, **A. Jelic**, M.A. Pereira, D.Z. Sousa, P. Blánquez, M. Sarrà, E. Marco-Urrea, M. Petrović, M.M. Alves, D. Barceló, G. Caminal, T. Vicent

Event: 5th European Bioremediation Conference, July 2011, Chania, Greece
Title: Biodegradation of pharmaceuticals by fungi and identification of metabolites: study from bench-scale to pilot bioreactor
Authors: M. Sarrà, C. Cruz-Morato, **A. Jelic**, E. Marco-Urrea, S. Perez, M. Petrovic, D. Barcelo, G. Caminal, T. Vicent

Event: Symposium on Emerging Pollutants, Water Treatment and Remediation, March 2011, Barcelona, Spain
Title: Pharmaceuticals in the Ebro river basin: occurrence, distribution and elimination in wastewater treatment plants
Authors: M. Petrovic, **A. Jelic**, D. Barcelo,

Event: Symposium on Emerging Pollutants, Water Treatment and Remediation, March 2011, Barcelona, Spain
Title: New indexes to characterize risk assessment of environmental multichemical exposure: tentative relationships with ecotoxicity and ecosystem variables
Authors: A. Ginebreda, **A. Jelic**, M. Köck, M. Petrovic D. Barcelo, I. Muñoz, M. Ricart, S. Sabater

Event: SETAC North America 31st Annual Meeting, November 2010
Title: Occurrence and distribution of pharmaceuticals in the Ebro river basin
Authors: D. Barcelo, M. Petrovic, **A. Jelic**, B. Ferreira da Silva, R. Lopez

Event: SETAC 2010
Title: Occurrence and distribution of pharmaceuticals in the Ebro river basin
Authors: D. Barcelo, M. Petrovic, B. Ferreira da Silva, **A. Jelic**, R. Lopez

Event: 28th International Symposium on Chromatography (ISC) Valencia (Spain) 2010.
Title: LC-MS/MS study of distribution of pharmaceuticals in water, suspended solids and sediments of the Ebro river
Authors: B. Ferreira da Silva, **A. Jelic**, R. Lopez Serna, A. A. Mozeto, M. Petrovic, D. Barceló

Event: 20Th SETAC Europe, Sevilla (Spain) 2010.
Title: Fate and occurrence of pharmaceuticals during wastewater treatment
Authors: **A.Jelic**, M. Gros, M. Petovic, D. Barcelo)

Event: 1st CEFSER Training course: Capabilities of UPLC-MS/MS in Analysis of Contaminants and Pharmaceutical Compounds in Food and the Environment, Novi Sad (Serbia) 2010.
Title: Application of UPLC-MS/MS in the environmental studies and emerging risks
Authors: **A. Jelic**, M. Petrovic

Event: INNOVA-MED Water Conference Ramallah (Palestine) 2009,
Title: Fate and behavior of emerging contaminants in wastewater sludge
Authors **A. Jelić**, M. Petrović , D.Barceló

Event: 30th SETAC North America, New Orleans (USA) 2009.
Title: Fate and analysis of multi-class pharmaceuticals in sewage sludge
Authors: D. Barcelo, M. Petrovic, **A. Jelic**

Event: 1st REPHAD Workshop: Pharmaceuticals and their degradation products in the environment, Varazdin (Croatia) 2009.
Title: Identification and determination of pharmaceuticals and their degradation products in the environment by LC-MS systems
Authors: M. Gros, J. Radjenović, **A. Jelić**, M. Petrović, D. Barceló

Attendance at conferences - Poster presentations:

Event: 8th Annual LC/MS/MS Workshop on environmental applications and food safety, Barcelona, Spain, July 2012
Title: *Trametes versicolor* degrades the antiepileptic carbamazepine: Identification of intermediates formed in an air pulsed fluidized bed bioreactor
Authors: **A. Jelic**, C. Cruz-Morató, E. Marco-Urrea, M. Sarrà, S. Perez ,T. Vicent ,M. Petrovic,D. Barcelo

Event: ICCE 2011, Zurich, Switzerland
Title : Tracing pharmaceuticals in an integrated municipal plant for wastewater and organic waste treatment
Authors: **A. Jelić**, S. di Fabio, C. Cavinato, F.Fatone, M. Petrović , D.Barceló

Event: SedNet Conference, Venezia, Italy, April 2011
Title : Pharmaceuticals in the Ebor river basin: Occurrence and distribution between aqueous and solid phase
Authors: D.Barcelo, B. Ferreira de Silva, **A. Jelić**, R. Lopez, M. Petrović

Event: INNOVA MED Conference Girona (Spain) 2009. Procesos y prácticas innovadoras para el tratamiento de aguas residuales y su reutilización en la zona mediterránea

Title : Multi-residue method for trace level determination of pharmaceuticals in solid samples using pressurized liquid extraction followed by liquid chromatography/quadrupole linear ion trap mass spectrometry
Authors: **A. Jelić**, M. Petrović , D.Barceló

Event: 6th Annual LC/MS/MS workshop on environmental application and food safety, Barcelona, Spain, 2010.
Title: Fate and occurrence of pharmaceuticals during wastewater treatment
Authors: **A. Jelić**, M. Petrović , D.Barceló