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THE REMOVAL OF XENOBIOTIC COMPOUNDS FROM WASTEWATER THROUGH THE USE OF BIOLOGICAL PROCESSES AND ADVANCED OXIDATION TECHNOLOGIES

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SUMÁRIO

Xenobióticos são compostos estranhos aos organismos e de difícil degradação no ambiente. Entre estes compostos incluem-se os princípios activos de compostos farmacêuticos (PACF) e fragrâncias. A ocorrência e comportamento destes compostos nas estações de tratamento de águas residuais (ETARs) em Portugal era pouco conhecida, quer ao nível da detecção à entrada, quer na eficiência de remoção da própria estação. Este trabalho teve como objectivos: 1) seleccionar princípios activos de compostos farmacêuticos e fragrâncias e optimizar a sua detecção em matrizes reais; 2) estudar a sua ocorrência e comportamento em ETARs; 3) estudar em ambiente controlado à escala de laboratório, os processos de remoção biológica e de oxidação avançada, usando a radiação UV.

Numa primeira fase foram desenvolvidas e validadas metodologias analíticas para a detecção dos compostos farmacêuticos e fragrâncias nas amostras de águas residuais e nas lamas secundárias de 5 ETARs, durante diferentes períodos do ano. Com este estudo, foi possível priorizar as famílias mais importantes de PACFs e concluir que a ocorrência era independente da estação do ano. O PACF mais abundante foi o ibuprofeno, embora o enalapril, cafeína e o ácido clofibrico também estivessem presentes em concentrações relativamente elevadas no afluente e efluente. Com base na informação recolhida, foi efectuado um estudo detalhado da estação de tratamento de Fernão Ferro (Seixal), onde foram colhidas amostras de água residual e de lamas com o objectivo de estudar a variabilidade e reprodutibilidade de ocorrência dos compostos farmacêuticos e fragrâncias, bem como os mecanismos de remoção. As concentrações dos PACFs no afluente apresentaram maior variabilidade do que as das fragrâncias, que foram mais reprodutíveis. O mecanismo de remoção mais importante na ETAR para os PACFs foi principalmente a biodegradação e enquanto para as fragrâncias foi a adsorção. A radiação UV teve um efeito importante sobre a remoção de alguns PACFs (por exemplo, o diclofenaco), devido à baixa remoção biológica encontrada para este composto e elevada concentração detectada nas lamas.

Numa segunda fase, foi estudada a biodegradação e biotransformação de ácido clofibrico em reactor descontínuo sequencial com um consórcio de microrganismos adaptados à biodegradação do propanil. Neste estudo, observou-se a remoção de cerca de 51% de ácido clofibrico após 3 dias. Foi provado que as bactérias heterotróficas tiveram um papel mais importante na biodegradação do que as bactérias autotróficas nitrificantes. Os principais metabolitos gerados (ácido α-hidroxiisobutirico, 4-clorofenol e ácido láctico) foram identificados e quantificados. Com base nesta identificação, foi proposto um mecanismo de remoção biológica do ácido clofibrico.

Finalmente, foram realizados estudos de cinética de oxidação do atenolol, diclofenaco e cetoprofeno através de lâmpadas UV de baixa e média pressão. O efeito da matriz, água pura e efluentes (filtrada e não filtrada) também foi estudado. O coeficiente de absorção molar e os valores de rendimento quântico de fotodegradação foram mais elevados para o cetoprofeno, seguido pelo diclofenaco. O

efeito da fotólise na oxidação do atenolol provou-se ser baixo. A presença de matéria orgânica particulada no efluente teve um impacto negativo na fotodegradação de PACFs. Os produtos de transformação mais persistentes da MP/fotólise UV do cetoprofeno, diclofenaco e atenolol foram identificados e monitorizados ao longo do tempo de irradiação.

Palavras – chave: Princípios activos de compostos farmacêuticos, fragrâncias, estações tratamento águas residuais, reactores biológicos, radiação UV

SUMMARY

Xenobiotics are compounds foreign to living organisms and difficult to biodegrade in the environment. This group of compounds includes the pharmaceutical active compounds (PhACs) and musk fragrances. Prior to this work, the occurrence and fate of these compounds in Portuguese wastewater treatment plants (WWTP) was still unknown, not only regarding the influent to WWTPs, but the removal efficiencies of the different biological, physical and chemical processes occurring in the plants had never been studied together. This thesis aimed at the identification of the most relevant PhACs and musks for studying their occurrence and fate in WWTPs, as well as the implementation of biological removal and photolysis processes for a select group of compounds at the laboratory scale. In the first phase, analytical methodologies were developed and validated for the detection of these PhACs and musks in wastewater and sludge samples in five WWTPs during different periods of the year. With this study, it was possible to prioritize the most important families of PhACs independently of the season. The most abundant PhACs were the NSAIDs (e.g. ibuprofen), while enalapril, caffeine, and clofibric acid were also present in relatively high concentrations in the influent and effluent. Based on this information, an intensive survey was carried out in the WWTP of Fernão Ferro, where samples of wastewater and sludge were collected to study the variability and repeatability of the occurrence in the influent and the removal mechanisms in the WWTP. PhAC concentrations in the influent were subject to a higher variability than the musks, which were more repeatable. The most important removal mechanism in the WWTP for PhAC was mainly biodegradation, while adsorption was most important for the musks and the UV radiation had an important effect on some PhAC (e.g diclofenac) due to the low biological removal found for these compounds. In the second phase, the biodegradation of the recalcitrant clofibric acid was studied in a sequential batch reactor with a consortium of microorganisms adapted to transform propanil, a structurally similar compound. In this study, we observed the removal of about 51% of clofibric acid after 3 days. It was proved that mainly the heterotrophic bacteria were responsible for the biodegradation when compared to autotrophic nitrifier bacteria. The main metabolites (e.g. α-hydroxyisobutiric acid, 4-chlorophenol and lactic acid) were also identified and quantified, forming the basis for a proposed metabolic pathway for clofibric acid biodegradation. Finally, photolysis studies of the kinetics of atenolol, diclofenac and ketoprofen degradation were performed through low and medium pressure UV lamps. The matrix effects in pure water and wastewater effluent (filtered and unfiltered) was also studied. The decadic molar absorption coefficient and quantum yield values indicated high photodegradation for ketoprofen, followed by diclofenac, and low photolysis for atenolol. The presence of particulate organic matter in the wastewater had a negative impact on the photodegradation of PhACs. The most persistent transformation products from MP/UV photolysis of ketoprofen, diclofenac and atenolol were identified and monitored throughout irradiation time.

Key words: Pharmaceutical active compounds, musks, wastewater treatment plants, biological reactors, UV radiation

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Abs(λ) - compound absorbance at wavelength λ

ADBI - 4 4-Acetyl-6-tert-butyl-1,1-dimethylindane, celestolide

AHIBA - α-hydroxyisobutyric acid

AHMI - 6-Acetyl-1,1,2,3,3,5-hexamethylindane, phantolide

AHTN - 6-Acetyl-1,1,2,4,4,7-hexamethyltetralin, tonalide

AITI - 5-acetyl-3-isopropyl-1,1,2,6-tetramethylindane, traseolide

AOB - ammonia-oxidizing bacteria

AMO - ammonium monooxygenase

amu - atomic mass unit

AOP - advanced oxidation processes

AOX - adsorbable organic halogenated compound or adsorbable organic halides

APCI - atmospheric pressure chemical ionization

ATU - allylthiourea

BBD - biocatalysis and biodegradation database

BDO_5 - biochemical oxygen demand

BSTFA - *N*,*O*-bis(trimethylsilyl)-trifluoracetamide

C - concentration

C_{Ads} - concentration of substance adsorb in the biomass

CAR-PDMS - carboxen/polydimethylsiloxane fiber

CAR-PDMS-DVB - carboxen/ polydimethylsiloxane /divinylbenbenzene fiber

CAS - conventional activated sludge system

CI - chemical ionization

CID - collision-induced dissociation

CLF - clofibric acid

C_{Liq} - concentration of substance in the liquid

CNS - central nervous system

COD - chemical oxygen demand

4-CP - 4-chlorophenol

C_s - sorbed concentration per amount of suspended solids

C_w - soluble concentration

CW-DVB - carbowax/divinylbenbenzene fiber

DAD - diode array detector

DCA - 3,4-dichloroaniline

DEHP - di(2-ethylhexyl)phthalate

DI - direct infusion

DPMI - 6,7-dihydro-1,1,2,3,3-pentamethyl-4(5*H*)-lindone, cashmeran

DVB - divinylbenbenzene fiber

ε - decadic molar absorption coefficient

$\varepsilon_{atr,\lambda}$ - molar absorption coefficient of atrazine at wavelength λ

ECD - electron-capture detector

EDC - endocrine disrupting compounds

EE2 - 17β-ethinylestradiol

E2 - β−estradiol

E1 - estrone

EI - electronic impact ionization

E_p^0(200 – 450 nm) - photon fluence rate determine through atrazine actinometry in the wavelength interval of 200 to 450 nm

ESI - electrospray ionization

eV - electron Volt

FISH - fluorescence in situ hybridisation

f_{atr} - quantum yield of atrazine depletion

$f_{p,\lambda}$ - emission spectrum of the lamp based on the photon flux and normalized to the chosen wavelength interval

GC - gas chromatography

GC-MS - gas chromatography associated with mass spectrometry

GC-MS/MS - gas chromatography tandem mass spectrometry

GPC - gel permeation chromatography

HHCB - 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-(g)-2-benzopyran, galaxolide

GC- CIRMS - gas chromatography with combustion isotope ratio mass spectrometry

HLB - hydrophilic lipophilic balance cartridges

HPLC - high performance liquid chromatography

HPLC-DAD-MS - high pressure gas chromatography coupled diode array detector and to mass spectrometry

HR - high resolution

HRT - hydraulic retention time

HS - head space

ICM - iodinated X-ray contrast media

INE - instituto nacional de estatística

INFARMED - autoridade nacional do medicamento e produtos de saúde

IR - infra red spectrometry

IT - ion trap

k_d - sorption constant

k_{atr} - pseudo-first-order rate constant of atrazine depletion

$k_{E_p^o}$ - photon fluence-based rate constants

k - time-based pseudo-first-order rate constants

$k_s(\lambda)$ - specific rate of light absorption

K_{OW} - partition coefficient octanol/water

λ - wavelength

LA - lactic acid

LAS - linear alkyl benzenesulfonates

LC-MS - liquid chromatography

LC-MS - liquid chromatography coupled with mass spectrometry

LC-MS/MS - liquid chromatography tandem mass spectrometry

LOD - limits of detection

LOQ - limits of quantification

LP - low-pressure mercury UV lamp

L_{sld} - total load of the PPCP in the excess sludge

m - slope of the calibration curve

MBR - membrane bioreactors

MDMA - 3,4-methylenedioxymethamphetamine, ecstasi

MP - medium-pressure mercury UV lamp

MS - mass spectrometry

MS^2- MS/MS system

MSTFA - *N*-methyl-*N*-trimethylsilyltrifluoroacetamide

MTBSTFA - *N*-*tert*-Butyldimethylsilyl-*N*-methyltrifluoracetamide

m/z - mass/charge ratio

M_W- molecular weight mass

NHS – national health system

NMR - nuclear magnetic resonance

NPE - nonylphenols and ethoxylated nonylphenols

NOB - nitrite oxidizing bacteria

NSAID - non-steroidal anti-inflammatory drug

PA - polyacrylate fiber

PAHs - polycyclic aromatic hydrocarbons

PCB - polychlorinated biphenyl compounds

PCDD/F - polychlorinated dibenzodioxins/furans

PCP - personal care products

PDMS - polydimethylsiloxane fiber

PDMS/DVB - polydimethylsiloxane/divinylbenbenzene fiber

PET - plastic of polyethylene terephthalate

PhAC - pharmaceutical active compound

pKa - acid dissociation constant at logarithmic scale

PLE - pressurized liquid extraction

PMF - polycyclic musk fragrances

POP - persistent organic pollutant

PPCP - persistent personal care products

PPCP - pharmaceutical and personal care products

PPS - pathway prediction system

Q - quadrupole

Q_{in} - influent wastewater flow rate

Q_w - purged sludge flow rate

QTOF - quadrupole-time-of-flight

Qtrap - hybrid triple Quadrupole with ion trap mass spectrometer

RP C18 - reverse phase Sep-Pak C18 cartridge

SAID - steroidal anti-inflammatory drug

SBR - sequencing batch reactor

SD - standard deviation

SIMARSUL - sistema integrado multimunicipal de águas residuais da península de Setúbal

SMAS - serviços municipalizados de águas e saneamento

SPE - solid phase extraction

SPME - solid phase microextraction

SRT - sludge or solid retention time

SS - suspended solids concentration

STP - sewage treatment plant

SS_{sld} - suspended solid concentration in the sludge

TMS - trimethylsulfonium hydroxide solution

TMSi - Trimethylsilyl group

TOF - time-of-flight

t_r - retention time

TSS - total suspended solids

U.K. - United Kingdom

UM - University of Minnesota

UPLC - ultra performance liquid chromatography

U.S. - United States

USE - ultrasonic solvent extraction

UV - ultraviolet radiation

WW - wastewater

WWTP - wastewater treatment plant

VSS - volatile suspended solids

X_{SS} - suspended solid concentration

z - solution depth

CHAPTER 1

MOTIVATION AND THESIS OUTLINE

1. Motivation and Thesis Outline

1.1 Background

Over the last decade, xenobiotic compounds have become a big concern to the environment. The development of more sensitive analytical techniques was responsible for the possibility of detection of a very large number of substances (organic and inorganic) in very small concentrations in the ranges of ng L^{-1} and µg L^{-1}. Since that time, many compounds have been identified as xenobiotics. These chemicals are often called micro pollutants (because their concentrations are very low) or emerging contaminants (since the concern about them is only recent) or xenobiotics (since most of them are synthetic, i.e. xenon to bios which means foreign to life). Xenobiotic compound can be defined as a chemical which is found in an organism, but is not normally produced or expected to be present. It can also cover substances which are present in higher concentrations than are usual (Fatta-Kassinos *et al.*, 2010).

For a long time the production of chemicals and pharmaceuticals, their usage and application was connected with the heavy pollution of the environment and serious health effects. At the end of the last century, it was realized that the products of chemical and pharmaceutical industries are presenting a new type of environmental pollution that may cause a health risk to the consumer. Most chemicals are used in excessive amounts e.g. for personal care, hygiene, health care products and pharmaceutical active compounds. Some other compounds are of natural biological origin such as mycotoxins, aflatoxins, some hormones and others. They can also be included in the group of xenobiotics, since they are foreign to the organisms in which they are found, due to uptake and bioconcentration processes, after their release in the environment.

The xenobiotics often spread within the water cycle, however, the knowledge on their fate and effects and on opportunities for their removal and input prevention is scarce. In particular in Portugal there was no information available about their presence in the wastewater until 2005. The presence of xenobiotics in the aquatic environment seems to be a big challenge for sustainable water in future when the reuse of the wastewater treated from the wastewater treatment plants will became important. In countries with no water reuse, the wastewater discharges in the environment can be responsible for groundwater infiltration and possible contamination of the drinking water supply aquifers. Hazardous chemicals, like many of the xenobiotic organic compounds, are of rising concern in urban water management since water supply, urban drainage and wastewater treatment systems

were originally designed only to solve other problems (supply of potable water, flooding prevention and sanitation). There is need to understand, in an integrated manner, the sources, flow paths, fate and effects of hazardous chemicals on both humans and ecosystems.

Most of them are released into the environment according to their use, like for example, personal care products and pharmaceuticals. These types of chemicals enter the environment continuously via domestic and industrial sewage systems and via wet weather or raining run-off (e.g. from animal farms). Many of the chemicals of emerging interest, including pharmaceuticals, have not been fully examined yet for their negative environmental and health effects. The pharmaceuticals, for example, are designed to modulate immune and endocrine systems and cellular signal transduction and have a potential to interfere with organisms in the environment. Many of these chemicals are designed to have profound physiological effects, so it would not be surprising if they were found to affect fish, insects and other forms of life. Relatively short-living chemicals can cause chronic exposures because they are continuously infused into the environment.

Even if the individual concentrations of such chemicals are low, the combined concentrations from those sharing a common mechanism of action could have a substantial effect on the living organisms. The transformation of the parent compounds may be incomplete in the environment and in treated effluents. These recalcitrant or persistent chemical entities may also add to the already huge number of potentially toxic, suspicious, unsafe chemicals present in the environment. Less is known about these transformed chemicals compared to their parent compounds, in particular in relation to their possible effects on environmental organisms and humans (easily reachable e.g. via drinking water).

Illicit drugs constitute a new class of chemicals with potent psychoactive properties and unknown effects to the aquatic environment. The occurrence of such drugs in water resources in various countries such as the U.S. (Bartelt-Hunt *et al.*, 2009), Italy (Zuccato *et al.*, 2006), Germany (Wick *et al.*, 2009) and Croatia (Terzic *et al.*, 2010) is an example of the importance of studying micro pollutants and PPCP in wastewater treatment system and combined with the effect with the urban water cycle.

Some of the studies reported the occurrence and fate, effects and risks associated with the presence of xenobiotics in urban waters such as:

- Occurrence and fate in water and wastewater by the application of different technologies
- Co-metabolism removal of different organic compounds in pilot and laboratory studies

- Photochemical transformation of pharmaceutical active compounds when present in the aquatic environment and/or during the application of photo induced treatment processes
- Analytical identification and quantification of the transformation products of selected pollutants

Storm water is also another major pathway for the introduction of xenobiotics into the water cycle. The treatment of storm water should of course constitute an integral part of precipitation water management. This could also contribute to the current demands concerning water quality including this group of compounds called xenobiotics. In this respect, the requirements of the Water Framework Directive (2000/60/EU, WFD) continue to play a central role and provide a basis for discussion leading to further innovative solutions for the persistent compounds not quite well studied. In order to assess these requirements, major investments are necessary in water and wastewater treatment in future, to extent and nature of the xenobiotics released from these different sources that will certainly differ and each can have different potential and cause different environmental pollution effects, and consequently will became a potential challenge for environmental management.

The inherent properties (e.g. solubility, volatility, biodegradability, etc.) of xenobiotics are varied, as well as their potential sources and uses, and the behavior of different xenobiotic substances released to the environment is not equal between the different classes of compounds. The ecotoxicity and environmental persistence of a substance have a large range of variability to the environmental hazard, and it is clear that an emission level for one compound may be orders of magnitude higher or lower than that specified for another. Some sources and uses of xenobiotics may release a single harmful substance, and others release a mixture which difficult how to deal with this problem. The spatial distribution and scale of that source and use in the area of interest (e.g. a city, country, etc.) and the type of regulatory and/or voluntary controls imposed, play a major role in determining its importance in relation to the overall emission of hazardous substances into the environment and to the overall risk posed by the particular substance emitted. This knowledge also forms an important basis for pollution monitoring, exposure assessment, and source control strategies for emission prevention and/or reduction. It is important to know which sources are the most important in terms of the quantity released, which sources are relatively easy to control using on-site treatment technologies, which sources are mobile, which sources are continually emitting. This knowledge is vital for building a picture of the patterns and pathways of substance flow throughout the environment and for supporting the calculation of mass balances and the evaluation of different pollution control strategies. The pharmaceutical active compounds (PhACs) and

musks are included and have been detected in many sources, such as urban, industrial and hospital wastewaters, sludges and even in the surface and ground water, sources for drinking water system. Marine sediments are also characterized as to have memory of the pollution constituting a sink source for the more hydrophobic compounds that can cause risks to aquatic biota, in which these compounds can, bioaccumulate, and affect the human health through the ingestion of contaminated fish and shellfish. Due to the importance of the detection of these pharmaceutical active compounds and musks in many parts of the urban water cycle, many studies are being carried out to understand the mobility of these compounds in the water cycle and the potential toxicity effect on wildlife, human beings, animals and plants.

The changes and interferences in endocrine function may lead to altered growth, development, or reproduction in exposed animals and these changes may be expressed later in the life cycle or even in future generations. The presence of estrogenic substances in municipal effluents has been linked to a number of biological responses, such as induction of plasma vitellogenin and intersex in fish exposed in the environment immediately adjacent to the outfalls (Servos *et al.*, 2005). The exposure during critical life stages may result in a variety of biological impacts mediated through endocrine systems affecting the female and male ratio, mainly on fish. The impact of 17α-ethinylestradiol (EE2) on fish reproduction under laboratory conditions have been performed by Liebig *et al.*, (2006) and PhACs by Grung *et al.*, (2008) and Sanderson *et al.*, (2009).

Numerous potential endocrine disrupting compounds (EDC) have been reported in municipal effluents including natural and synthetic estrogens (Liniert *et al.*, 2007). Many of these compounds can be detected in surface waters and sediment that receive wastewater discharges (Kolpin *et al.*, 2002, Servos *et al.*, 2005). The major contribution to the estrogenicity in effluents has been shown in previous studies to be related to the presence of natural and synthetic estrogens in several municipal effluents. The presence of industrial chemicals, such as alkylphenols and natural estrogens such as β-estradiol (E2) and estrone (E1) (Servos *et al.*, 2005) as well as the active ingredient of most birth control drugs, 17α-ethinylestradiol (EE2) (Ternes *et al.*, 1999, Andersen *et al.*, 2003), has also been shown to contribute to the estrogenicity of effluents, depending if there are important contributions of the industrial or other sources to the effluent. This potential effect of the other similar micro pollutants such as PhACs and musks in the environmental organisms was also a source of motivation to proceed with further studies on this area and also because of a lack in information about the most important compounds that should be prioritized.

It is important to prioritize the most harmful and the less harmful compounds to the organisms and environment. This is the main reason why so much research is carried out nowadays on the different sources of PhACs and musks, especially in wastewater treatment plants, one of the main receptors of these compounds. Biodegradation, adsorption and oxidation studies with different technologies are being tested for evaluation of the efficiency of removal, and to suggest potential changes in the design of the treatment plants in order to remove this kind of compounds.

1.2 Research Objectives

This work aims at contributing to the development of methods for measuring xenobiotic compounds in real wastewater systems, and to assess their abundance in various wastewater treatment plants (WWTPs) in the south region of Lisbon. The same analytical procedures are employed throughout the study to monitor the oxidation and/or biotransformation extent of the studied compounds. The xenobiotic screening in the selected WWTP indicates which compounds are present in higher amounts in this socio-economic context (an urban, densely populated region of Portugal). The pharmaceutical and personal care product (PCP) selection is done according to those that are known to be more toxic to human health and aquatic life or more resistant to biodegradation for the subsequent treatment studies.

The level of xenobiotic removal currently achieved by WWTPs is assessed through analysis of influent and effluent samples and sludge samples. Single and composite liquid samples reveal those compounds that appear in high peak concentrations (e.g. in the morning), and those present in significant levels on a 24 h basis. The xenobiotic removal efficiency in the existing treatment systems are assessed by comparing the concentrations in the influent and effluent of the WWTP. While some biological degradation of the xenobiotics is expected, removal may also take place by adsorption to biomass cells, which must be accounted for through analysis of the sludge. This process is particularly important for hydrophobic compounds, such as the musks.

The metabolic transformations that the target xenobiotic compounds undergo in conventional biological wastewater treatment plants (WWTPs) was assessed through laboratory bioreactor tests. The biodegradation/adsorption of each compound was studied and compared with the screening of real WWTP carried out, and the metabolic by-products that are generated were monitored by the analytical procedures.

Accurate assessment of the by-products produced by the sludge can only be done when each xenobiotic was fed individually to a bioreactor. This assessment is critical since

some of the by-products are potentially more harmful than their parent compounds. Most previous studies focusing on xenobiotic removal from wastewater have not investigated this potential problem, thus this work aims to provide a more comprehensive outlook on the capacity of biological treatment systems to prevent toxic xenobiotics from entering into waterways. The chemical structure of each compound provide an indication of the operational conditions (aerobic/anaerobic, reactor pH, sludge age, etc.) that are likely to be more efficient for the biodegradation of each compound. The extent of biodegradation was followed by kinetic reaction rates and stoichiometric yields that calculated for each set of reactor operational conditions.

After the identification and quantification of the target xenobiotics, bench scale reactors were operated under specific conditions to study their removal. One important feature of this work is the identification of metabolic products formed by the microbial cultures as well as the intermediate oxidation step. The isolation and identification of these by-products were conducted by chromatographic separation techniques: gas chromatography coupled to mass spectrometry (GC-MS); high pressure gas chromatography coupled diode array detector and to mass spectrometry (HPLC-DAD-MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS).

Additional degradation steps are provided by advanced oxidation processes (AOP) (e.g. ultraviolet (UV) radiation). AOP are expected to be more efficient in degrading the selected compounds than direct photolysis due to the production of the highly reactive and unselective $^{\bullet}OH$ radicals. The efficiency of direct photolysis depends on the selected compound's absorbance, since for a compound to be photo liable it needs to have the capacity to absorb light. Therefore, the absorbance of each target xenobiotic has to be measured to be able to predict how efficient UV radiation degrades it. The presence of suspended matter can also interfere with the efficiency of the UV treatment and should be monitored by using real wastewater supernatant as matrix. The oxidation processes are expected to produce more by-products than the photolysis processes and the compounds formed more toxic since they will be more oxidized than the parent compound.

The chemical structures of the by-products give information about the possible (bio-) chemical transformations of the parent compound, suggesting potential (metabolic) degradation pathways. Based on this information, we also aim at developing metabolic pathways for the biodegradation of some of the studied xenobiotic compounds and/or their by-products. Of special interest was whether or not the compounds are completely mineralized, or if other, potentially harmful, by-products are formed.

This work is an important contribution for:

i) the study of the occurrence and fate of the PhACs and musks in the WWTPs in Portugal as well as the analytical methodological techniques applied
ii) assessing the variability of the PhACs and musks in the influent of a WWTP and how repeatable are the samples obtained
iii) assessing the removal mechanisms by the sequence of treatment in a WWTP by establishing mass balances for the PhACs and musks, including the effects of the secondary biological treatment as well as UV radiation
iv) the study of the biological removal of a recalcitrant PhAC such as clofibric acid with a specific consortium and identification of the metabolites produced
v) the study of the kinetic oxidation and sub-product identification of the reaction in UV irradiation with low and medium pressure lamps of 3 PhACs (atenolol, diclofenac and ketoprofen) in different matrices.

1.3 Research Strategy

The starting point for the project was the lack of information about the presence or absence of PhACs and musks in our WWTP in Portugal. One of the main objective of this work was to identify and quantify pharmaceutical active compounds (PhACs) and musk fragrances present in wastewater treatment plants (WWTP) in the south region of Lisbon (Almada, Seixal and Setúbal). The analytical techniques for the detection of xenobiotics and their by-products have been developed. A preliminary screening, based on consumption statistics for Portugal (INFARMED, INE) was carried out. Among the PhACs selected, it was included antidepressives (e.g. carbamazepine, hydroxyzine); anti-inflammatory drugs: non-steroidal (e.g. diclofenac) or steroidal (e.g. tramadol); drugs for asthma and allergic diseases (e.g. salbutamol); antibiotics (e.g. amoxicillin); contraceptives – steroid hormones (e.g. progesterone, 17β-estradiol, 17α-ethinylestradiol) and musks (e.g. galaxolide, cashmeran), among others.

The concentration in the influent, effluent and sludge was measured in order to determine the removal efficiency of the target compounds by different existing treatment processes (activated sludge (WWTP of Valdeão, Setúbal and Parque Industrial do Seixal) and trickling filters (Quinta da Bomba and Fernão Ferro)). These WWTP are managed by Águas do Sado, SMAS Almada and SIMARSUL. A partnership agreement was signed with these companies in order to ensure the continuous transfer of materials and knowledge throughout the study. Single and composite liquid samples (influent and effluent) and grab sludge samples were collected. The sludge samples were treated by

solid phase microextraction (SPME) for the musks and/or solid phase extraction (SPE) methods for PhACs. Different techniques were used to extract the PhACs, depending on their nature: the acidic compounds were extracted using OASIS HLB as extracting medium and the neutral compounds, with RP-C18. The analysis of the liquid samples was also depending on the compound's properties. The collected samples were treated with headspace extraction techniques using SPME fibers, PDMS/DVB (polydimethylsiloxane/divinylbenzene) to selectively isolate the musks. The analysis of the non-polar compounds (e.g. musks) was done by gas-liquid chromatography (GC) and/or gas-liquid - mass spectrometry (GC-MS) and the polar compounds (i.e. most PhACs), by liquid chromatography-diode array detector-mass spectrometry (LC-DAD-MS). With the results of detection of the PhACs and musks in the influent, effluent (before and after UV) and sludge samples, it was possible to study not only the influent variability of these compounds but also to establish mass balances to the plant to get an understanding of the removal mechanisms of biodegradation and/or biotransformation, adsorption and the effect of the UV radiation of low pressure lamps on the degradation of the PhACs and musks in full scale WWTP.

The second phase of the study was to investigate the biological removal and effect of UV of specific xenobiotics found to be relevant in the first phase. Bioreactors were inoculated with sludge collected at WWTPs known to receive the xenobiotic. The fate of selected organic compounds was assessed in a bench scale reactor operated under similar conditions as the WWTP. The aim was to investigate the adsorption and biotransformations occurring for each target compound as well as to monitor the resulting by-products associated with each compound, using the optimized analytical methods. The bioreactor was operated under aerobic conditions for the degradation of clofibric acid. The microbial diversity and activity of these consortia was also investigated in this thesis. Initially, the xenobiotic were the sole carbon and energy source, to determine the sludge capacity for direct metabolism. Since the biotransformation of the xenobiotic compound was not observed, an easily biodegradable carbon source (i.e. propanil) was supplied in combination with the xenobiotic to test the potential for co-metabolism and enhanced degradation.

The efficiency in degradation of some target xenobiotics (atenolol, diclofenac, ketoprofen) using ultraviolet (UV) radiation was also tested. Batch experiments were conducted to evaluate the xenobiotic degradation kinetics and to determine the main parameters that influence their degradation (such as decadic molar extinction coefficient, quantum yield and source water quality) and also identifying the by-products formed. The photodegradation experiments were performed in a bench scale collimated low pressure

lamp and medium pressure UV reactor. The selected oxidation technique was finally tested with real wastewater to verify its efficiency in the presence of suspended particles and of the other components of the effluent.

1.4 Thesis Structure

This thesis is composed of eight chapters, including the current introductory chapter describing the motivation and outline of the work developed (Chapter 1). The remaining chapters include an overview of the state of art in xenobiotics, in particular the pharmaceutical active compounds and musks (Chapter 2), five chapters (Chapters 3 to 7) describing the work developed according with the specific objectives laid out above, and a final chapter summarizing the conclusions draw from this study (Chapter 8).

Chapter 1, *Motivation and thesis outline:* This chapter contains a short description of the motivation to get involved in the research of the effect of PhACs and musks in wastewater treatment plants. The aim is to describe the main activities developed during the PhD and a brief description of the project. This chapter also contains a short description of the main objectives of this thesis.

Chapter 2 *State of art and introduction:* This chapter contains a short description of the xenobiotic problem in wastewater treatment plants and the environment. The chapter also contains an introduction to the specific pharmaceutical active compounds and musks studied in the wastewater treatment processes and their removal processes in the WWTP, which is extensively discussed in the thesis. An overview of the literature on the subjects of occurrence and fate of PhACs and musks in WWTPs, the analytical techniques for the detection of these kind of compounds and lab scale reactors for biological and oxidation studies is presented.

Chapter 3 *Analysis of 65 Pharmaceuticals and Personal Care Products in 5 Wastewater Treatment Plants in Portugal Using a Simplified Analytical Methodology:* This chapter consists of a survey of PhACs and musk fragrances in different WWTPs. In this study, the abundance of 65 PPCPs was analysed in 5 Portuguese WWTPs during the spring and autumn. The analytical methods were described in detail for the wastewater and sludge samples for the determination of PhACs and musks and also the validation of the method was performed using influent wastewater matrices, showing comparable limits of detection and quantification as literature values for most PPCPs.

Chapter 4 *Assessing the Diurnal Variability of Pharmaceutical and Personal Care Products in a Full-Scale Activated Sludge Plant:* In this chapter, an intensive sampling campaign has been carried out in a municipal wastewater treatment plant (WWTP) to

assess the dynamics of the influent pharmaceutical active compounds (PhACs) and musks. The mass loadings of these compounds in wastewater influents displayed contrasting diurnal variations depending on the compound. The range of PhACs loadings in the influent to WWTPs can vary several orders of magnitude from one day or week to the next, representing a challenge in obtaining data for steady-state modelling purposes.

Chapter 5 *Assessing the Removal of Pharmaceutical and Personal Care Products in a Full-Scale Activated Sludge Plant:* In this chapter, was investigated the different removal mechanisms of PhACs and musks in a WWTP. Biological removal and adsorption in the activated sludge tank was studied as well as the effect of UV radiation used for disinfection purposes. By establishing a mass balance for the WWTP, the main removal mechanism (biological, adsorption and UV radiation) of PhACs and musks in the WWTP was possible to obtain, as well as its variability over different sampling days.

Chapter 6 *Biodegradation of clofibric acid and identification of its metabolites:* In this chapter, the environmentally persistent and refractory clofibric acid, the pharmacologically active metabolite of the lipid regulator family of pharmaceuticals, was subjected to biological transformation by a microbial consortium. The goal of this study was to evaluate the biotransformation of this compound and the production of metabolites. Kinetic studies of the carbon source consumption and also ammonium, nitrite and nitrate have been performed in order to investigate the effect that nitrifiers and heterotrophic bacteria have in the biotransformation of clofibric acid. For the detection and identification of clofibric acid metabolites, GC-MS combined with HPLC-DAD-MS was employed.

Chapter 7 *Photodegradation kinetics and intermediates of ketoprofen, diclofenac and atenolol in pure water and treated wastewater:* In this chapter, kinetic studies were performed with UV radiation using low and medium pressure lamps with atenolol, diclofenac and ketoprofen as PhACs. The matrix effect is compared in pure water as well as filtered and unfiltered treated wastewater, where pseudo-first order UV kinetic constants are determined. Metabolites of the transformations are also identified using GC-MS and UPLC-MS/MS.

Chapter 8 *Conclusion:* This chapter provides an aggregated discussion of the results obtained in the thesis as well as a summary of the main conclusions. Main achievements, contributions to the advancement of PPCP monitoring and removal studies in WWTPs and future work are also listed in this chapter.

CHAPTER 2

STATE OF THE ART

2.1 Xenobiotic definition and occurrence in the environment

2.2 Analytical methods

2.3 Variability and removal of PhACs and musks in full scale WWTP

2.4 Sampling of PhACs and musks in full scale WWTP

2.5 Biodegradation and oxidation studies of PhACs

2. State of art

2.1 Xenobiotic definition and occurrence in the environment

The term xenobiotic is derived from the Greek words (*xenos*) = foreigner, stranger and (*bios*) = life. Specifically, drugs such as pharmaceutical active compounds (PhACs) and their metabolites are xenobiotics in humans because the human body does not produce them itself, nor are they part of a normal diet (Fatta-Kassinos *et al.*, 2010). The pharmaceutical active compounds (PhACs) are considered xenobiotic, man-made chemical compounds, foreign to living organisms that are discharged in municipal wastewater treatment plants (WWTP). The term xenobiotics is often used in the context of pollutants such as musk fragrances, pharmaceutical active compounds and other persistent personal care products (PPCP) and their effect on the biota.

Natural compounds can also become xenobiotics if they are taken up by another organism, such as the uptake of natural human hormones by fish found downstream of sewage treatment plants (Fatta-Kassinos *et al.*, 2010). The exposure to these xenobiotic compounds can alter the oxidative state of cells and thereby increase oxidative stress. The induction of oxidative metabolism by the hemoprotein family of cytochrome P450s is well known to release oxygen radicals that can lead to oxidative stress. Aquatic biota exposed to these pharmaceutical compounds can be adversely affected, as they are not possibly as efficient as mammals in eliminating lipophilic drugs and oxygen radicals. The persistent personal care products (PPCP) act at various tissues and most of them are metabolized in the liver (hepatocytes). These metabolize drugs differently attempt to eliminate them and prevent toxic accumulation in tissues. This is of particular importance because municipal wastewaters from major urban areas are considered to emit these substances into the aquatic environment on a continuous basis, where they can persist for days (Gagné *et al.*, 2006).

These substances are problematic for the sewage treatment systems, since they are relatively new substances without defined regulations and are very difficult to categorize. There is a lack in the European Community regulation for these compounds (PhACs and musks) in sewage wastewater and sludges, only other groups of organic compounds are considered as for example polycyclic aromatic hydrocarbons (PAHs) and polychorobiphenyls (PCBs).

Many tons of drugs are produced every year, because of its application in human and veterinary medicine. The exact production figures are difficult to obtain in INFARMED statistics. The INFARMED is national authority of medicines and health products, a

governmental agency accountable to the health ministry with the objective monitoring, assessing and regulate all activities related with human medicines and health products for the protection of public health. The amount of pharmaceuticals prescribed by physicians can be evaluated by multiplying the amount of daily dose with the amount of prescribed daily doses per year. This amount can increase dramatically when drugs that can be purchased without prescription in the pharmacy are considered. These concentrations are dependent of the pharmacokinetical behavior (half life, urinary and fecal excretion, metabolism, etc.) or the application mode as injection, pill, where the human body metabolism is needed to process the drug, or by direct application in cream or gel form on the skin (Ternes *et al.*, 1998). Due to this high application level, detectable concentrations of drugs and their metabolites may be expected in sewage. According to INFARMED, the most prescribed and consumed PhACs in Portugal from 2003 to 2010 are shown Table 2.1.

The PhACs selected in many studies are those most sold or prescribed compounds in the market and also those showing human or animal excretion rates, where the compound is unchanged by the organism. According to the INFARMED data, almost all of the selected PhACs are still widely produced and even in some cases the consumption is increasing. The occurrence of PhACs in wastewater is not a temporary problem and studies are needed in the different countries to understand the removal behavior in the WWTP.

Table 2.1 - 65 target compounds, human effects statistics of the Top ranking position of 100 active substances with highest number of packages in NHS (INFARMED, 2008, 2009, 2010)**.

Compound	Ranking 2003	Number package 2003	Ranking 2007	Number package 2007	Ranking 2008	Number package 2008	Ranking 2009	Number package 2009	Number package 2010	Daily Dose of PhAC (max)* (mg d^{-1})	Excretion rates unchanged (%)
Acidic PhAcs											
Acetylsalicylic acid	31	714545	18	1107377	12	1428978	11	1778370	3130809	500-1000 (4000)	
Ampicillin										1000-4000	30-60[a]
Amoxicillin	5	1880150	4	2091167	4	2180559	5	2239124	2150900	750-1500	80-90[a]
Captopril	28	739000	58	532863	62	500663	83	440138		12.5-150	
Ciprofloxacin	38	628122	40	673604	43	655694	47	640648	529272	500-1500	45[c], 20[d]
Clofibrate ethyl											
Clofibric acid											6[b]
Diclofenac	4	1986824	7	1947175	7	1945838	10	1817520	2669287	50-150	15[b]
Enalapril maleate	65	449869	96	384646	65	369296				10-40	30[d]
Fentiazac										100-400	
Flurbiprofen										50-200	
Furosemide	24	826682	16	1146044	14	1227322	16	1312465		20-80	90[d]
Ibuprofen	12	1276608	11	1633842	9	1794553	8	2016009	3225265	900-2400	1-8[b]; 10[d]
Indomethacin	68	440176								50-150	10-20[b]
Ketoprofen	90	364532	86	404076	78	428862	82	441070		160-320	
Naproxen					-	40488	-	61500		250-1000	1-2[c]
Telmitarsen					73	450465	57	576054			
Tiaprofenic acid											

Table 2.1 - **(cont.)** 65 target compounds, human effects statistics of the Top ranking position of 100 active substances with highest number of packages in NHS (INFARMED, 2008, 2009, 2010)**.

Compound	Ranking 2003	Number package 2003	Ranking 2007	Number package 2007	Ranking 2008	Number package 2008	Ranking 2009	Number package 2009	Number package 2010	Daily Dose of PhAC (max)* (mg d^{-1})	Excretion rates unchanged (%)
Neutral PhACs											
Allopurinol	56	497021	43	650226	37	702030	38	771022		100-300	
Alprazolam	2	2502476	2	2208290	5	2179887	4	2280686	3363097	0.75-1.5	
Atenolol	82	380196	-	106988	-	110683	-	144844		25-100	> 50[c]; 90[d]
Azithromycin	23	856205	27	892528	26	906615	30	912055	632481	500-1000	
Betamethasone	60	474978	98	383654						4-20	
Bromazepam	9	1457640	14	1207555						3-18	
Budesonide	87	369407	80	417512	84	417202	85	426855		0.2-0.8	
Caffeine										20-40(++)	
Carbamazepine	74	410837	93	391173	-	37908	-	323872		200-1200 (1600)	1-2[b]
Clorazepate										45-80	
Codeine										10-120	
Diazepam	11	1338429	15	1146901	16	1115754	21	1130220	494745	6-30	
Digoxin	40	621657	59	529235	61	503423	75	478958		0.125-1.25	
Diltiazem	55	504038	76	437312	83	418277				120-360	3[c]
Dimethylamino-phenazone											
Domperidone	35	660360	38	734096	36	749950	37	775022		30-60	
Escitalopram			49	605352	56	531752	64	532923			

Table 2.1 - **(cont.)** 65 target compounds, human effects statistics of the Top ranking position of 100 active substances with highest number of packages in NHS (INFARMED, 2008, 2009, 2010)**.

Compound	Ranking 2003	Number package 2003	Ranking 2007	Number package 2007	Ranking 2008	Number package 2008	Ranking 2009	Number package 2009	Number package 2010	Daily Dose of PhAC (max)* (mg d^{-1})	Excretion rates unchanged (%)
Neutral PhACs											
β-Estradiol										1-2	
Estrone										10-20	
17α-Ethinylestradiol	14	1222358	17	1125664	25	938287	34	828145	2893722	0.02-0.03	
Etofenamate	48	557973	68	494069	60	503712	68	504178		100-1000	
Fluoxetine	30	721617	41	671204	41	678481	41	708266	505527	20-60	
Fluticasone	67	445511	84	409010	94	392352				0.2-0.5 (1)	
Glibenclamide	15	1181286	50	601419	63	491854	100	393940		5-15	
Hydroxyzine	88	367047	90	398482	89	398428	89	418354		50-100 (600)	
Indapamide	10	1450582	9	1775043	10	1794144	12	1743762		1.25-2.5	
Latanoprost	85	373446	75	440808	72	451624	78	466279		0.05-0.125	
Lorazepam	7	1682991	8	1888586	8	1869183	9	1909169	2107513	2-6 (10)	
Nifedipine	26	763697	32	783840	35	781599	39	769754		30-120	
Nimesulide	3	2254935	12	1622152	11	1487913	13	1510441	2790311	100-400	
Omeprazol	13	1260328	10	1650237			7	2029495	1345138	20-40 (120)	20[d]

NSAID – Non-steroidal anti-inflammatory drug; [a]Hirsch *et al.* (1999); [b]Ternes T. (1998); [c]Sedlak *et al.*(2001); [d]Zucatto *et al.* (2005); *Carmona, M., Carneiro, C., Esteves, A.P., Filipe, H, Gonçalves, J., Macedo, T., Osswald, W., Pinheiro, R.L., Rodrigues, A., Sampaio, C., Teixeira, A.A, Prontuário terapêutico, INFARMED, Ministério da Saúde (2008); **Medicines statistics, Infarmed (Instituto Nacional da Farmácia e do Medicamento, 2003-2010; (++) Associated with paracetamol.

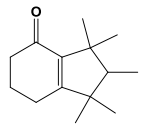
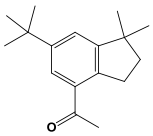
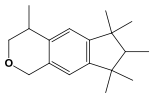
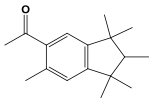
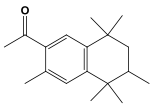
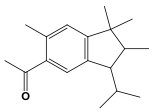
Table 2.1 - **(cont.)** 65 target compounds, human effects statistics of the Top ranking position of 100 active substances with highest number of packages in NHS (INFARMED, 2008, 2009, 2010)**.

Compound	Ranking 2003	Number package 2003	Ranking 2007	Number package 2007	Ranking 2008	Number package 2008	Ranking 2009	Number package 2009	Number package 2010	Daily Dose of PhAC (max)* (mg d^{-1})	Excretion rates unchanged (%)
Neutral PhACs											
Oxazepam										15-30 (50)	
Paracetamol	1	3505698	1	3520490	1	3465215	1	3642302	10345595	500-1000 (4000)	
Paroxetine	39	624423	95	385901	98	384420	98	396592		20-50	
Phenazone (antipyrine)											
Piroxicam	43	582015	91	397681	-	6995	-	6286		10-20	
Progesterone										5-10	
Propranolol			94	388750	76	430745	81	451256		40-320	< 1[b]
Ramipril	75	409403	55	557552	49	583716	49	623243		1.25-10	
Ranitidine	96	356211	-	104281	-	95247	-	100947		300-600 (1200)	40[d]
Reserpine											
Salbutamol	34	682926	54	580501	55	548259	62	538312		0.2-1.6 (5)	
Salicylic acid											
Sertraline	66	446561	56	551892	51	573605	48	630785		50-150 (200)	13[c]
Tramadol	71	422418	89	400974	88	399709	53	615053		50-400	25-50[c]
Warfarin											
Zolpidem	22	862477	21	980308	23	974065	23	1078175			

NSAID – Non-steroidal anti-inflammatory drug; [a]Hirsch *et al.* (1999); [b]Ternes T. (1998); [c]Sedlak *et al.*(2001); [d]Zucatto *et al.* (2005); *Carmona, M., Carneiro, C., Esteves, A.P., Filipe, H, Gonçalves, J., Macedo, T., Osswald, W., Pinheiro, R.L., Rodrigues, A., Sampaio, C., Teixeira, A.A, Prontuário terapêutico, INFARMED, Ministério da Saúde (2008); **Medicines statistics, Infarmed (Instituto Nacional da Farmácia e do Medicamento, 2003-2010; (++) Associated with paracetamol.

The personal care products (PCPs) are produced and used in large amounts around the world. Some examples are soaps, household detergents, perfumes, etc. Fragrances are common ingredients of PCPs and in contrast with other chemicals, almost no knowledge exists on their long-term environmental effects (Llompart *et al.*, 2003). The musks are ubiquitous, persistent, bioaccumulative pollutants, and some generate toxicologically active compounds. Musks are continuously introduced into the environment mainly via urban wastewater. The musk fragrances can include e.g. galaxolide, tonalide, cashmeran, traseolide, celestolide and phantolide as polycyclic musks (Table 2.2) and musk xylene, musk moskene, musk tibetene and musk ketone as nitro musks.

Table 2.2 - Uses, chemical structure, chemical proprieties of the musks studied.

Compound	**Uses**	**CAS**	**Chemical structure**	**M_w**	**logK_{ow}**
Cashmeran (DPMI)	Health care product	33704-61-9		206	4.84[b]
Celestolide (ADBI)	Health care product	13171-00-1		244	4.37[b]
Galaxolide (HHCB)	Health care product	1222-05-5		258	5.9[a] 4.6[b]
Phantolide (AHMI)	Health care product	15323-35-0		244	5.85[g]
Tonalide (AHTN)	Health care product	1506-02-1		258	5.7[a;f] 4.84[b]
Traseolide (ATII)	Health care product	68140-48-7		258	6.3[g]

[a](Ternes *et al.*, 2004); [b](Carballa *et al.*, 2008); [c](Carrara *et al.*, 2008); [d](Comeau *et al.*, 2008); [e](Lin *et al.*, 2009); [f](Grung *et al.*, 2007); [g](Xia *et al.*, 2005)

The polycyclic musks account for nearly 85% of the worldwide production of musks, while nitro musks account for the rest (Llompart *et al.*, 2003). The manufacturing

industries have introduced some limitations on the kind and proportion of musks in PCP formulations, mainly the nitro musk.

Since 1999 in Europe, these compounds are included in the list of Persistent Organic Pollutants (POP) and special attention and many studies have been conducted since that time (Ternes *et al.*, 1999). The sewage treatment plant have been designed to combat problems of organic matter removal and microbial contamination, or more advanced processes to treat nitrogen and phosphorus. However, there are many natural and synthetic compounds, such as PhACs and musks that are not being removed by these systems, leading to their discharge in the natural environment. Studies of occurrence of PhAC and musk in the wastewater influent (Joss *et al.*, 2006, Santos *et al.*, 2009) and effluent of WWTP, surface water and sediments (Hernando *et al.*, 2006), and rivers (Moldovan, 2006, Ort *et al.*, 2009), have also been done to select the most harmful compounds to the environment.

In potable water and ground water some drugs like diazepam, and antibiotics have also been detected (Ternes in 1998). Clofibric acid for example, a metabolite of three lipid regulating agents, has been identified in the river and ground water in Germany and even in drinking water with concentration levels ranging up to 165 ng L^{-1} (Ternes, 1998). The behavior of drugs in their passage through the WWTP and about the treated wastewater contamination of the aquatic environment by PPCPs is due to the diversity of the drugs applied in medicine and is still not completely known nowadays.

WWTP sludge may contain many xenobiotic (anthropogenic) organic chemicals which have the potential to be taken up by plants and animals and accumulate in the terrestrial food chain, as well as to leach into the groundwater (Kreuzinger *et al.*, 2004). The sludge can also be a problem in WWTP management because many PPCP can adsorb to the sludge and in some countries, the main application for the sludges is the agriculture. The unknown behaviors of the PPCP desorption from the sludge and the possible contamination of surface and ground water used for drinking water can be a problem for human health. The management of sludge from WWTPs represents one of the major challenges in wastewater treatment today, with costs amounting to more than the treatment cost of the liquid in many cases (Carballa *et al.*, 2007). Some micro pollutants adsorb onto the sludge solids during wastewater treatment. Depending on the efficiency of treatment technology on their degradation, these compounds will be mostly present in the supernatant of the sludge and thus recycled from the dewatering system or thickener to the entrance of the WWTP or disposed with the sludge. The inorganic compounds, such as heavy metals, are analyzed on a routine basis, the characterization and long-term observation of organic contaminants in sludge has received little attention so far, probably

due to the inherent difficulties associated with the analysis of sludge samples (Carballa *et al.*, 2007). Some of these substances (e.g. PhACs and musks) have the potential to cause adverse effects on plants, soil microbes, invertebrates and other microorganisms above certain concentrations (Bundschuh *et al.*, 2011). The problem is that not only those substances with high partitioning to sludge are recycled with it, but also a significant part of the non-degraded compounds which tend to remain in the aqueous phase are recycled (Carballa *et al.*, 2007).

The compounds monitored in effluent treated wastewater of the WWTPs was first regulated in nineties, with the most important parameters to measure being the organic concentration as biochemical oxygen demand (BDO_5) and chemical oxygen demand (COD), total suspended solids (TSS), nutrients (nitrogen and phosphorus) and some heavy metals (*Decreto-Lei nº 236/98 de 1 de Agosto* and *Decreto-Lei nº 152/97 de 17 de Junho*). Between the organic compounds, only lauryl sodium sulphate, pesticides and some of the organic chlorinated compounds are listed in the European Union regulation of Water Framework Directive (2000/60/EU, WFD). The application of the WWTP in agriculture was regulated in 1991 but only in 1996 was it established maximum concentration values for heavy metals (e.g. Cd, Ni, Cu, Pb, Zn, Hg and Cr) in the sludge. In 2005, the legislation starts to include in the monitoring plan not only heavy metals but also some organic compounds such as AOX (adsorbable organic halogenated compound or adsorbable organic halides), LAS (linear alkyl benzenesulfonates), DEHP (di(2-ethylhexyl)phthalate), NPE (nonylphenols and ethoxylated nonylphenols), PAHs (polycyclic aromatic hydrocarbons), PCB (polychlorinated biphenyl compounds), dioxin and PCDD/F (polychlorinated dibenzodioxins/furans). (UE Directive nº 86/278/EC, June 12 transposed to the Portuguese legislation by the *Decreto-Lei n.º 118/2006 de 21 de Junho* and *Decreto-Lei n.º 276/2009 de 2 de Outubro*).

Other unknown organic compounds (e.g. xenobiotic compounds) present in the wastewater need to be studied in the water, wastewater and in the sludges in order to extend the list of compounds that should be monitored in the future and to include in future legislation.

2.2 Analytical methods

2.2.1 Pharmaceutical active compounds

Solid-phase extraction (SPE) is the most used clean-up technique for pre-concentration of wastewater samples prior to analysis of the pharmaceutical active compound. Different sorbents can be used for clean-up wastewater samples: Oasis HLB (hydrophilic lipophilic

balance) cartridges and reverse phase Sep-Pak C18 cartridges, which assures good recovery of compounds in a wide range of polarities. For most of the PhACs, Oasis HLB and reverse phase (RP C18) as SPE cartridges are the most used in literature due to the polar nature of the compounds and the acidic and neutral characteristics of most of them (Ternes *et al.*, 2004; Ternes *et al.*, 2005). The selection of the SPE media is directly related with the proprieties of the compound (e.g. acidic or neutral characteristics, (see Table 2.3). RP C18 is most appropriate for the neutral and Oasis HLB general it can be more appropriate for the acidic compounds.

For the extraction of the PhAC adsorbed to the sludges, the procedure consists of ultrasonic solvent extraction (USE) using solvents (e.g. methanol/acetone) or pressurized liquid extraction (PLE) using 100% methanol. After this extraction step, non-selective, an additional clean-up can be performed with SPE (Ternes *et al.*, 2005).

Table 2.3 - Human effect, chemical structure, and chemical proprieties of the pharmaceuticals studied.

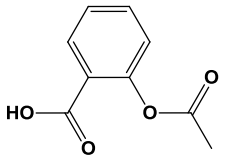
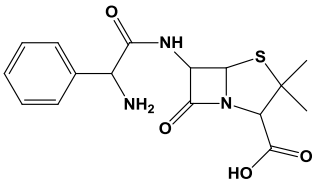
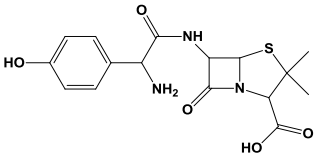
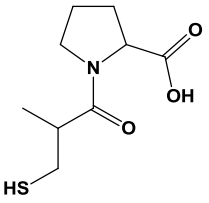
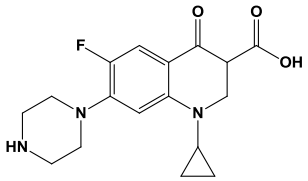
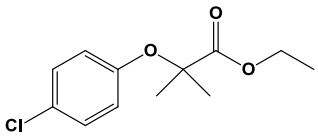
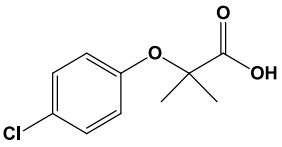
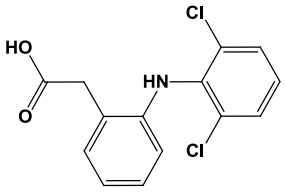
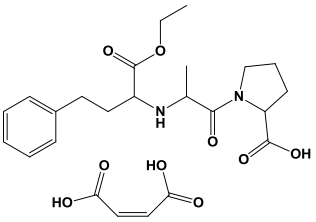
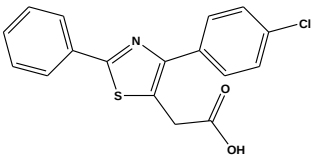
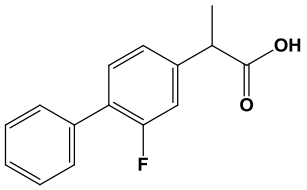
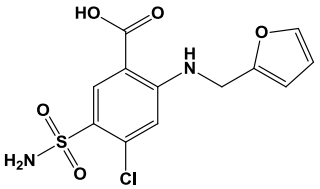
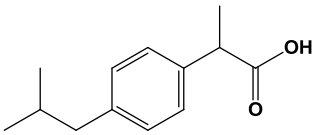
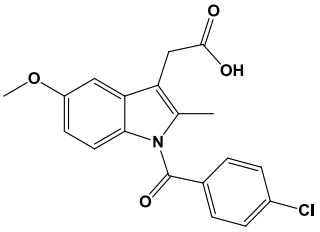
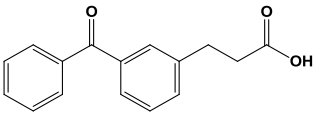
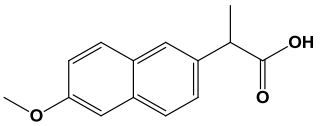
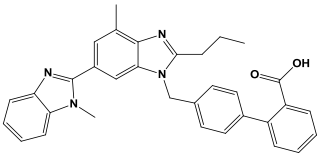
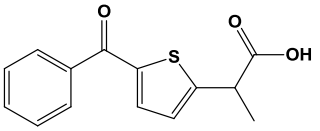
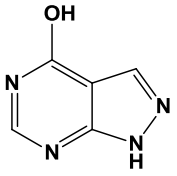
N.	Compound	Human effect	CAS	Formula	Chemical structure	M_W	pK_a (at 25ºC)
	Acidic PhACs						
1	Acetylsalicylic acid	Analgesic	50-78-2	$C_9H_8O_4$		180.2	3.5
2	Ampicillin	β-lactam antibiotic	69-53-4	$C_{16}H_{18}N_3O_4S$		349	2.5 (-COOH) 7.3 (-NH_2)
3	Amoxicillin	β-lactam antibiotic	26787-78-0	$C_{16}H_{19}N_3O_5S$		365	2.8 and 7.2
4	Captopril	Antihypertensive	62571-86-2	$C_9H_{15}NO_3S$		217	3.7 (carboxyl group) 9.8 (thiol group

Table 2.3 - (cont.) Human effect, chemical structure, and chemical proprieties of the pharmaceuticals studied.

N.	Compound	Human effect	CAS	Formula	Chemical structure	M_W	pK_a (at 25ºC)
5	Ciprofloxacin	Antibiotic	85721-33-1	$C_{17}H_{18}FN_3O_3$		331	8.6 and 6.2
6	Clofibrate ethyl	Lipid modifying agent	637-07-0	$C_{12}H_{15}ClO_3$		243	
7	Clofibric acid	Lipid modifying agent metabolite	882-09-7	$C_{10}H_{11}ClO_3$		243	3.6-4.9
8	Diclofenac	NSAID	15307-86-5	$C_{14}H_{11}Cl_2NO_2$		318	4.2
9	Enalapril maleate	Antihypertensive	77549-59-8	$C_{24}H_{32}N_2O_9$		376	3.0-5.5
10	Fentiazac	NSAID	18046-21-4	$C_{17}H_{12}ClNO_2S$		329.8	
11	Flurbiprofen	NSAID	5104-49-4	$C_{15}H_{13}FO_2$		244	4.2

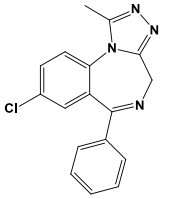
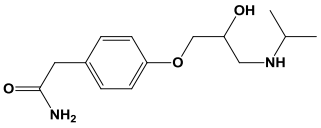
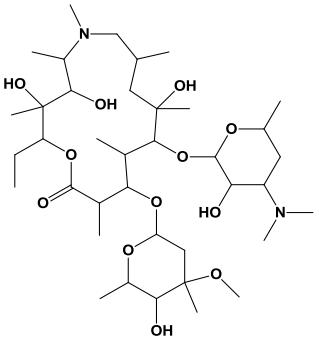
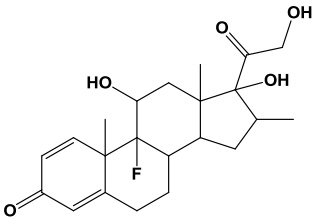
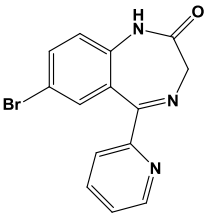
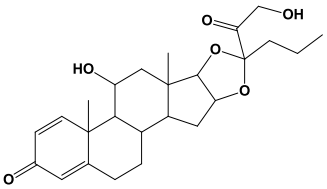
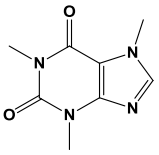
NSAID - Non-steroidal anti-inflammatory drug;

Table 2.3 - (cont.) Human effect, chemical structure, and chemical proprieties of the pharmaceuticals studied.

N.	Compound	Human effect	CAS	Formula	Chemical structure	M_W	pK_a (at 25ºC)
12	Furosemide	Loop diuretic	54-31-9	$C_{12}H_{11}ClN_2O_5S$		330.7	6.0
13	Ibuprofen	NSAID	15687-27-1	$C_{13}H_{18}O_2$		206	4.5-5.2
14	Indomethacin	NSAID	53-86-1	$C_{19}H_{16}ClNO_4$		444	4.5
15	Ketoprofen	NSAID	22071-15-4	$C_{16}H_{14}O_3$		254	3.9-4.2
16	Naproxen	NSAID	22204-53-1	$C_{14}H_{14}O_3$		230	4.2
17	Telmitarsen	Angiotensin	144701-48-4	$C_{33}H_{30}N_4O_2$		514.6	
18	Tiaprofenic acid	NSAID	33005-95-7	$C_{14}H_{12}O_3S$		260	
	Neutral PhACs						
19	Allopurinol	Gout treatment	315-30-0	$C_5H_4N_4O$		136	9.4

NSAID - Non-steroidal anti-inflammatory drug;

Table 2.3 - (cont.) Human effect, chemical structure, and chemical proprieties of the pharmaceuticals studied.

N.	Compound	Human effect	CAS	Formula	Chemical structure	M_W	pK_a (at 25ºC)
20	Alprazolam	Anxiolytic; Tranquilizer	28981-97-7	$C_{17}H_{13}ClN_4$		308.8	2.4
21	Atenolol	β-Blocker; Anti-hypertensive	29122-68-7	$C_{14}H_{22}N_2O_3$		266	9.5
22	Azithromycin	Macrolide antibiotic	83905-01-5	$C_{38}H_{72}N_2O_{12}$		749	
23	Betame-thasone	Corticosteroid (SAID)	378-44-9	$C_{29}H_{33}FO_6$		392	
24	Bromazepam	Anxiolytic; Tranquilizer	1812-30-2	$C_{14}H_{10}BrN_3O$		316	2.9
25	Budesonide	Corticosteroid (asthma)	51333-22-3	$C_{25}H_{34}O_6$		430	7.6
26	Caffeine	CNS Stimulant	58-08-2	$C_8H_{10}N_4O_2$		194	10.4

SAID - Steroidal anti-inflammatory drug

Table 2.3 - (cont.) Human effect, chemical structure, and chemical proprieties of the pharmaceuticals studied.

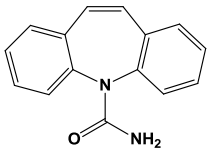
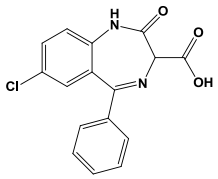
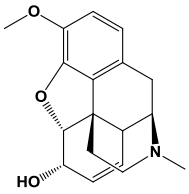
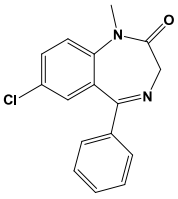
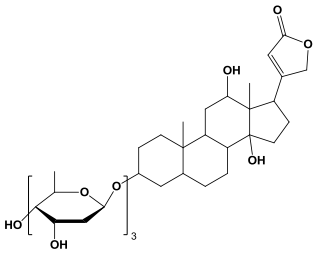
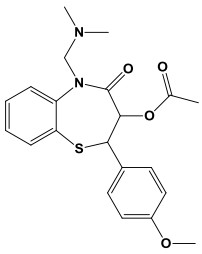
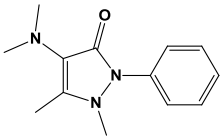
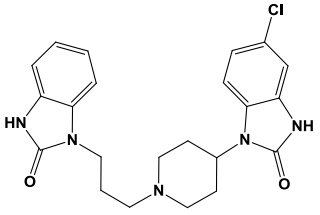
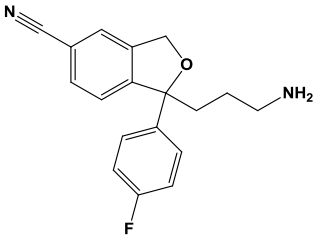
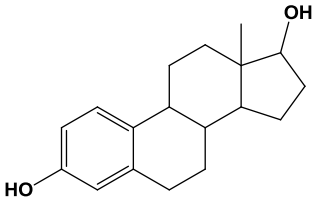
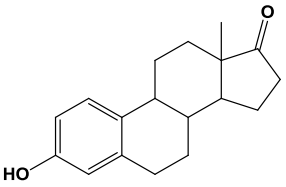
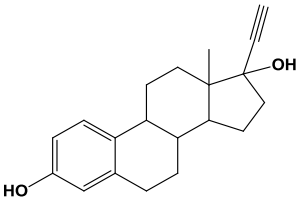
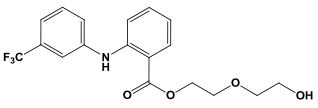
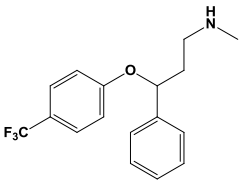
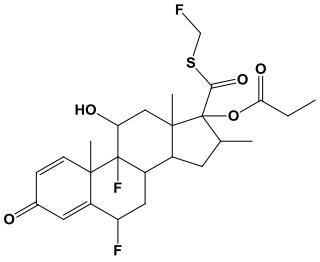
N.	Compound	Human effect	CAS	Formula	Chemical structure	M_W	pK_a (at 25°C)
27	Carbamazepine	Antiepileptic	298-46-4	$C_{15}H_{12}N_2O$		236	
28	Clorazepate	Anxiolytic; Tranquilizer	57109-90-7	$C_{16}H_{11}ClH_2N_2O_4$		409	1.5 (nitro groups) 10.5
29	Codeine	Analgesic	76-57-3	$C_{18}H_{21}NO_3$		299.4	8.2
30	Diazepam	Anxiolytic; Tranquilizer	439-14-5	$C_{16}H_{13}ClN_2O$		284.7	3.4
31	Digoxin	Cardiac glycoside	20830-75-5	$C_{41}H_{64}O_{14}$		781	9.5
32	Diltiazem	Hypertension, calcium blocker	42399-41-7	$C_{22}H_{26}N_2O_4S$		451	7.7
33	Dimethylamino-phenazone	Analgesic, Anti-inflammatory, Antipyretic	58-15-1	$C_{13}H_{17}N_3O$		231	
34	Domperidone	Antidopaminergic	57808-66-9	$C_{22}H_{24}ClN_5O_2$		425.9	7.9

Table 2.3 - (cont.) Human effect, chemical structure, and chemical proprieties of the pharmaceuticals studied.

N.	Compound	Human effect	CAS	Formula	Chemical structure	M_W	pK_a (at 25°C)
35	Escitalopram	Antidepressant drug	128196-01-0	$C_{20}H_{21}FN_2O$		324	
36	β-Estradiol	Sex hormone	50-28-2	$C_{18}H_{20}O_2$		272	10.5
37	Estrone	Estrogenic hormone	53-16-7	$C_{18}H_{22}O_2$		270	10.8
38	17α-Ethinyl-estradiol	Oral contraceptive	57-63-6	$C_{18}H_{24}O_2$		296	4.0
39	Etofenamate	NSAID	30544-47-9	$C_{18}H_{18}F_3NO_4$		369	–
40	Fluoxetine	Antidepressant drug	54910-89-3	$C_{17}H_{18}F_3NO$		345	8.7-9.87
41	Fluticasone	Glucocorticosteroid (asthma)	90566-53-3	$C_{25}H_{31}F_3O_5S$		444.5	

NSAID - Non-steroidal anti-inflammatory drug;

Table 2.3 - (cont.) Human effect, chemical structure, and chemical proprieties of the pharmaceuticals studied.

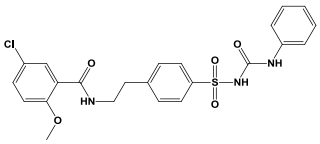
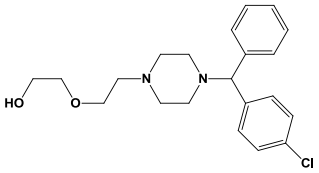
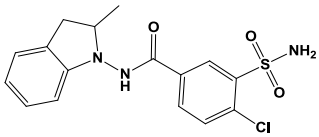
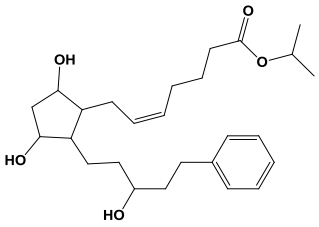
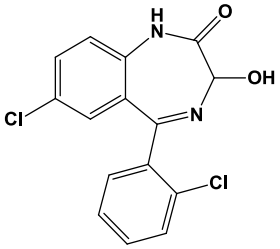
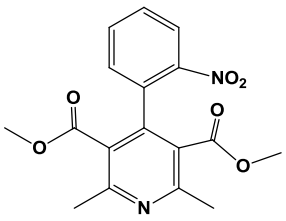
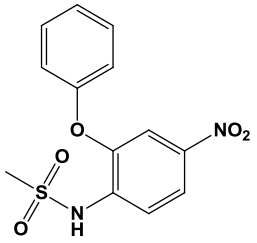
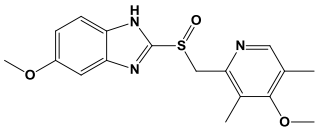
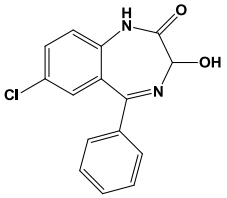
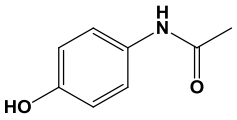
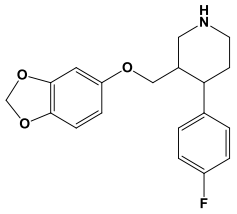
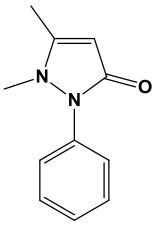
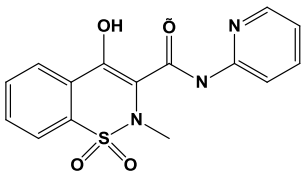
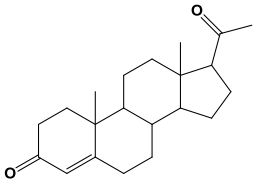
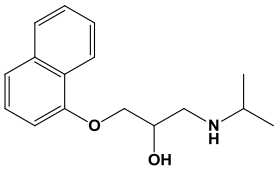
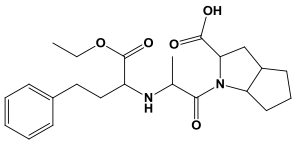
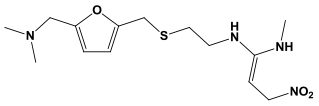
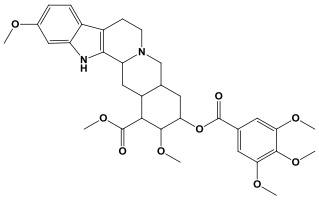
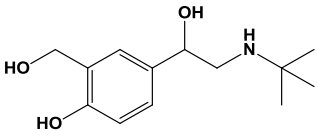
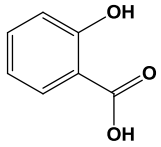
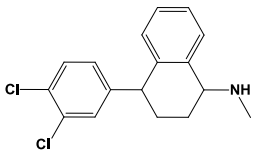
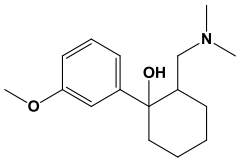
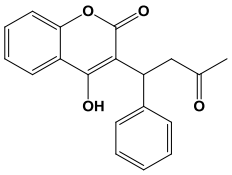
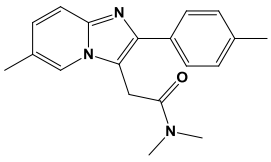
N.	Compound	Human effect	CAS	Formula	Chemical structure	M_W	pK_a (at 25ºC)
42	Glibenclamide	Diabetes type II treatment	10238-21-8	$C_{23}H_{28}ClN_3O_5S$		494	
43	Hydroxyzine	Antihistamine, Antiemetic, Anxiolytic	68-88-2	$C_{21}H_{27}ClN_2O_2$		448	2.1 and 7.1
44	Indapamide	Diuretic (hypertension)	26807-65-8	$C_{16}H_{16}ClN_3O_3S$		365	8.8
45	Latanoprost	Ocular hypertension, Glaucoma	130209-82-4	$C_{26}H_{40}O_5$		434	
46	Lorazepam	Anxiolytic	846-49-1	$C_{15}H_{10}Cl_2N_2O_2$		321	1.3
47	Nifedipine	Calcium blocker	21829-25-4	$C_{13}H_{12}N_2O_5S$		346	
48	Nimesulide	NSAID, Analgesic, Antipyretic	51803-78-2	$C_{13}H_{12}N_2O_5S$		308	6.5

Table 2.3 - (cont.) Human effect, chemical structure, and chemical proprieties of the pharmaceuticals studied.

N.	Compound	Human effect	CAS	Formula	Chemical structure	M_W	pK_a (at 25ºC)
49	Omeprazol	Proton pump inhibitor - ulcer	73590-58-6	$C_{17}H_{19}N_3O_3S$		345	8.8
50	Oxazepam	Anxiolytic	604-75-1	$C_{15}H_{11}ClN_2O_2$		286.7	1.7 and 11.6
51	Paracetamol	Analgesic	103-90-2	$C_8H_9NO_2$		151	9.7
52	Paroxetine	Antidepressant, anxiety, compulsive disorders	61869-08-7	$C_{19}H_{20}FNO_3$		375	9.9-10.3
53	Phenazone (antipyrine)	NSAID, Analgesic, Antipyretic	60-80-0	$C_{11}H_{12}N_2O$		188	5.0
54	Piroxicam	NSAID, Analgesic, Arthritis, Neoplasias	36322-90-4	$C_{15}H_{13}N_3O_4S$		331	5.1 and 2.3
55	Progesterone	Contraceptive, C-21 steroid hormone	57-83-0	$C_{21}H_{30}O_2$		314	
56	Propranolol	β-Blocker	525-66-6	$C_{16}H_{21}NO_2$		259	9.4

NSAID - Non-steroidal anti-inflammatory drug;

Table 2.3 - (cont.) Human effect, chemical structure, and chemical proprieties of the pharmaceuticals studied.

N.	Compound	Human effect	CAS	Formula	Chemical structure	M_W	pK_a (at 25ºC)
57	Ramipril	Congestive heart failure	87333-19-5	$C_{23}H_{32}N_2O_5$		416.5	
58	Ranitidine	Histamine H2 receptor antagonist, peptic ulcer disease	66357-35-5	$C_{13}H_{22}N_4O_3S$		350	8.2 and 2.7
59	Reserpine	Antiadrenergic agent	50-55-5	$C_{33}H_{40}N_2O_9$		609	6.6
60	Salbutamol	β2-Adrenergic receptor antagonist (asthma)	35763-26-9	$C_{13}H_{21}NO_3$		239	9.3 and 10.0
61	Salicylic acid	Antiseptic	69-72-7	$C_7H_6O_3$		138	3.3
62	Sertraline	Antidepressant	79617-96-2	$C_{17}H_{17}Cl_2N$		343	
63	Tramadol	Opiod-centrally action	27203-92-5	$C_{16}H_{25}NO_2$		300	9.4
64	Warfarin	Anticoagulant	81-81-2	$C_{19}H_{18}O_4$		308	5.1
65	Zolpidem	Insomnia treatment	82626-48-0	$C_{19}H_{21}N_3O$		307.4	

The liquid chromatography coupled with mass spectrometry (LC-MS), liquid chromatography tandem mass spectrometry (LC-MS/MS) and liquid chromatography with diode array detector and coupled with mass spectrometry (LC-DAD-MS) with MS in electrospray (ESI) or atmospheric pressure chemical ionization (APCI) in positive or negative mode are the analytical techniques most adequate for the identification and quantification of PhACs in wastewater since most of the compounds present neutral and acidic polar characteristics (Ternes *et al.*, 2004). The spectroscopic proprieties such as the most characteristic absorbance wavelength obtained in the Diode Array Detector (DAD) and mass spectrum (MS) proprieties obtained by direct injection on the MS system in ESI+ is described in Table 2.4.

Table 2.4 - Pharmaceutical active compounds analysed by LC-DAD-MS: analytical data.

Compound	Human effect	CAS	t_r (min)	Wavelength (nm)[1]	M_W	*m/z* (ESI+)
Acetylsalicylic acid	Analgesic	50-78-2	14.14	236, 302	180	136
Ampicillin	β-Lactamic antibiotic	69-53-4	16.34	242, 352	349	350, 106, 191, 160, 175
Amoxicillin	β-Lactamic antibiotic	26787-78-0	3.07	273	365	106, 366, 367, 245, 142
Captopril	Antihypertensive	62571-86-2	20.87	220, 261	217	218, 115
Ciprofloxacin	Antibiotic	85721-33-1	8.65	220, 280	331	244, 115, 136, 227, 301, 332
Clofibric acid	Lipid modifying agent	882-09-7	19.60	197, 224, 277	243	244, 123, 197
Diclofenac	NSAID	15307-86-5	18.78	220, 277	318	111, 91, 130,319, 232, 250, 269
Enalapril maleate	Antihypertensive	77549-59-8	13.68	220, 258	376	175, 181, 376, 377, 339
Fentiazac	NSAID	18046-21-4	21.95	210, 320, 245	330	331
Flurbiprofen	NSAID	5104-49-4	18.12	245	244	240, 245, 152, 193
Furosemide	Loop diuretic	54-31-9	14.64	274, 344	331	91, 329, 332, 137, 209, 285
Ibuprofen	NSAID	15687-27-1	21.91	234, 264, 272	206	140, 184, 53, 192, 207, 89
Indomethacin	NSAID	53-86-1	18.81	221, 230, 318	444	75, 349, 125, 355, 445
Ketoprofen	NSAID, analgesic and antipyretic	22071-15-4	16.31	254, 244	254	255, 240, 231
Naproxen	NSAID	22204-53-1	16.70	272, 281	230	231, 109, 137, 169

NSAID - Non-steroidal anti-inflammatory drug; SAID - Steroidal anti-inflammatory drug [1] Underlined wavelength used for quantification

Table 2.4 - (cont.) Pharmaceutical active compounds analysed by LC-DAD-MS: analytical data.

Compound	Human effect	CAS	t_r (min)	Wavelength (nm)[1]	M_W	*m/z* (ESI+)
Meclofenamic acid	Internal Standard	644-62-2	20.84	242, 276, 336	296	126, 106, 193, 250, 259, 214
Allopurinol	Gout treatment	315-30-0	2.71	249	136	170, 137, 113
Alprazolam	Anxiolytic; Tranquilizer	28981-97-7	17.40	233	309	310
Atenolol	Antihypertensive	29122-68-7	3.07	240, 274	266	267, 121, 104, 178, 186
Azithromycin	Macrolide antibiotic	83905-01-5	25.84	279, 367	749	750, 420, 570, 270
Betamethasone	Corticosteroid (SAID)	378-44-9	14.07	286	392	113, 157, 178, 393
Bromazepam	Anxiolytic; Tranquilizer	1812-30-2	17.37	237,310, 370	316	216, 231, 303
Budesonide	Corticosteroid (asthma)	51333-22-3	16.77	245	430	330, 431, 266
Caffeine	CSN Stimulant	58-08-2	6.07	220, 267, 273	194	126, 143, 212, 218, 195
Carbamazepine	Antiepileptic	298-46-4	13.95	220, 285	236	137, 109, 209, 237
Clorazepate	Anxiolytic; tranquilizer	57109-90-7	14.47	238, 320	409	365, 299, 316
Codeine	Analgesic	76-57-3	14.42	210, 270	299	300
Diazepam	Anxiolytic; tranquilizer	439-14-5	19.07	243, 312	285	286
Digoxin	Cardiac glycoside	20830-75-5	12.18	240	781	360, 116, 190, 397, 316, 779, 782
Diltiazem	Hypertension, calcium blocker	42399-41-7	12.32	240	451	452, 204, 248, 325, 420, 443
Dimethylamino-phenazone	Analgesic, anti-inflammatory, antipyretic	58-15-1	3.67	237, 259	231	232
Domperidone	Antidopaminergic	57808-66-9	11.25	285	425	104, 106, 145, 426
β-Etradiol (E2)	Sex hormone	50-28-2	15.47	224, 281	272	272
Estrone (E1)	Estrogenic hormone	53-16-7	17.53	240, 281	270	158, 262, 263, 177, 271
17α-Ethinyl-estradiol (EE2)	Oral contraceptive	57-63-6	17.01	281	296	106, 145, 297
Etofenamate	NSAID	30544-47-9	21.47	238, 297	369	216, 231
Fluoxetine	Antidepressant drug	54910-89-3	13.21	276, 264	345	137, 91, 281,
Fluticasone	Glucocorticosteroid (asthma)	80474-14-2	19.88	240, 254	501	104, 129, 147, 193, 197, 478, 500, 502

NSAID - Non-steroidal anti-inflammatory drug; SAID - Steroidal anti-inflammatory drug; [1] Underlined wavelength used for quantification

Table 2.4 - (cont.) Pharmaceutical active compounds analysed by LC-DAD-MS: analytical data.

Compound	Human effect	CAS	t_r (min)	Wavelength (nm)[1]	M_W	*m/z* (ESI+)
Glibenclamide	Diabetes type II treatment	10238-21-8	18.47	233, 301	494	495, 369
Hydroxyzine	Antihistamine, antiemetic, anxiolytic	68-88-2	12.61	233, 240	448	356, 256, 449
Indapamide	Diuretic (hypertension)	26807-65-8	14.98	242	365	167, 126, 235, 270, 317, 366
Latanoprost	Ocular hypertension, glaucoma	130209-82-4	3.84	366, 383	434	435, 437, 220, 263, 267, 294, 382, 351
Lorazepam	Anxiolytic	846-49-1	17.08	232, 317	321	217, 231, 261
Nifedipine	Calcium blocker	21829-25-4	16.87	281, 314	346	329, 104, 145, 186, 188, 284, 315, 347
Nimesulide	NSAID, analgesic, antipyretic	51803-78-2	17.97	301	308	307, 309, 229
Omeprazol	Proton pump inhibitor (Peptic ulcer disease)	73590-58-6	11.12	302	345	125, 108, 128, 346, 335, 271
Oxazepam	Anxiolytic	604-75-1	16.82	241, 330, 230	287	269, 288
Paracetamol	Analgesic	103-90-2	4.77	243	151	137, 152
Paroxetine	Antidepressant, anxiety, compulsive disorders	61869-08-7	12.66	243, 265, 297	375	161, 376, 249
Phenazone (antipyrine)	NSAID, analgesic, antipyretic	60-80-0	9.64	220, 242	188	96, 90, 159, 168, 171, 136, 189
Piroxicam	NSAID, analgesic, arthritis, neoplasias	36322-90-4	15.45	243, 344	331	91, 332, 137, 152, 266
Progesterone	Contraceptive	57-83-0	12.32	240	315	316, 144, 193
Propranolol	β-Blocker	525-66-6	11.50	238, 292, 320	259	260, 276, 248, 255
Ramipril	Congestive heart failure	87333-19-5	19.43	255	417	418, 197, 113
Ranitidine	Histamine H2 receptor antagonist, peptic ulcer disease	66357-35-5	3.11	228, 316	350	351, 118, 137, 208, 227
Reserpine	Antiadrenergic agent	50-55-5	12.97	219, 268	609	-
Salbutamol	β2-Adrenergic receptor antagonist (asthma)	35763-26-9	3.04	240, 277	239	103, 187, 240, 146, 164
Sertraline	Antidepressant	79617-96-2	3.67	267, 259	343	253, 166, 191, 203, 334, 344
Tramadol	Opioid-centrally action	27203-92-5	9.58	272, 370	300	208, 301, 178, 107, 150, 165
Warfarin	Anticoagulant	81-81-2	17.65	220,281, 306	308	117, 309, 92, 204

NSAID - Non-steroidal anti-inflammatory drug; SAID - Steroidal anti-inflammatory drug; [1] Underlined wavelength used for quantification

2.2.2 Musks

The most used technique for the determination of the polycyclic musk fragrances (PMF) in wastewater and sludge samples of the WWTP is the headspace solid-phase micro extraction (SPME), followed by GC-MS analysis (Llompart *et al.*, 2003 and Ternes *et al.*, 2005). Due to their elevated lipophilicity ($\log K_{ow}$ = 5.90-6.35), PMF are, therefore, sorbed onto sludge and suspended matter. In literature, analytical methods are reported for analyzing polycyclic musk fragrances (PMF) in sewage sludge using soxhlet or pressurized liquid extraction (PLE) with dichloromethane, silica gel, alumina columns and gel permeation chromatography (GPC) as clean-up methodology previous to GC-MS analysis (Ternes *et al.*, 2004). In all the cases, several clean-up steps must be applied to the extracts before chromatographic analysis. The SPME is a solventless technique that simplifies the long and tedious processes of sample preparation and analyte extraction in a single step. Concentration levels for example for galaxolide and tonalide in municipal sludge have been reported to be in the range of 0.27 to 162 ng g^{-1} (Ternes *et al.*, 2003). Table 2.5 shows the analytical data of the most important musks. The SPME technique is a very sensitive technique that can be applied to adsorb the volatile and non-polar compounds released from the aqueous or solid phase to the headspace completely isolated where a fiber of an adsorbable material or can be immerged in the liquid sample to extract selectively the target compounds (Ternes *et al.*, 2003; Carballa *et al.*, 2004; Carpinteiro *et al.*, 2004). The fibers can be of polyacrilate (PA), polydimethylsiloxane (PDMS), divinylbenbenzene (DVB), PDMS/DVB and carboxen-PDMS (CAR-PDMS) and carbowax-DVB (CW-DVB), carboxen-PDMS-DVB (PDMS-DVB-CAR) and they are selected according to the characteristics of the compound that need to be extracted. For the musks, Carballa *et al*., (2004) used PDMS/DVB for the determination galaxolide and tonalide in WWTP as well as Ternes *et al.*, 2004. The head space technique is more used than the immerged fiber in the liquid phase due to the matrix characteristic of some samples that are inappropriate for the submerged fiber.

Sludge is a very complex sample and the extraction of the organic pollutants from the matrix usually implies solvent extraction of the dried sludge samples assisted by accelerated solvent extraction, sonication, microwave heating, solid phase extraction (SPE), simple agitation or solid phase micro extraction (SPME). Llompart *et al.*, 2003 studied the determination of musk compounds in sewage treatment plant sludge samples by SPME with different fibers for the determination of musks in sewage treatment plants sludge samples. The influence of extraction temperature, fiber coating, agitation, pH and salting out on the efficiency of the extraction along with the extraction kinetics was studied by Llompart in 2003. An extraction temperature of 100 ºC and sampling the

headspace over the stirred sludge sample using PDMS/DVB as fiber coating lead to best effective extraction of the musks in general. The method proposed was very simple and yields high sensitivity, good linearity and repeatability for all the analytes with limits of detection at the ng g^{-1} level. The total analysis time, including extraction and GC analysis, was only 40 min, and no manipulation of the sample was required (Llompart *et al.*, 2003).The GC-MS with MS in electronic impact (EI) mode analytical technique was the most appropriate for the identification and quantification of the polycyclic musk fragrances (PMF), Table 2.5.

Table 2.5 - Musks analysed by GC-MS: analytical data.

Compound	Application	CAS	t_r (min)	M_W	*m/z* (EI)[1]
Cashmeran (DPMI)	Health care product	33704-61-9	18.14	206	191, 135, <u>206</u>
Celestolide (ADBI)	Health care product	13171-00-1	20.80	244	244, 229, <u>244</u>
Galaxolide (HHCB)	Health care product	1222-05-5	22.41	258	243, 213, <u>258</u>
Phantolide (AHMI)	Health care product	15323-35-0	21.34	244	229, <u>244</u>, 115, 128
Tonalide (AHTN)	Health care product	1506-02-1	22.59	258	243, 159, <u>258</u>
Traseolide (AITI)	Health care product	68140-48-7	21.88	258	215, 115, 128, 141, <u>258</u>
Mirex	Internal Standard	2385-85-5	30.18	546	<u>272</u>, 237, 119

1) Underlined *m/z* was used for quantification

2.2.3 Metabolites and reaction products

The GC-MS, LC-DAD-MS and LC-MS/MS techniques are the most important techniques used for metabolite or reaction products identification (Agüera *et al.*, 2005; Kosjek *et al.*, 2011). Because of their selectivity and sensitivity, they are the most powerful methods for metabolite identification. Even when a definitive assignment of chemical structures is not possible and, therefore, only tentative degradation pathways can be proposed, GC-MS is so far the most frequently used tool of analysis for identifying transformation products (Winckler *et al.*, 2001, Marco-Urrea *et al.*, 2009). Winckler and Marco-Urrea used GC-MS for study the ibuprofen metabolites generated by biodegradation processes. Two

important advantages of GC-MS methods are the large amount of structural information they yield by the full scan mass spectra obtained under electronic impact (EI) ionization and the possibility of using commercial libraries, making identification of unknowns feasible. However GC-MS has important drawbacks because of its scan capability for analyzing the very polar, less volatile compounds typically generated by these photo-processes (Ternes *et al.*, 2011). Because of this limitation derivatization techniques should be considered for protection of the polar group by the chemical reaction for a specific period and temperature conditions to get a non-polar derivatized compound that is more compatible with the GC-MS analysis. Many compounds can be used as derivatized reagents to give this protection to the molecule (e.g. MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide), BSTFA (*N*,*O*-bis(trimethylsilyl)-trifluoracetamide), TMS (Trimethylsulfonium hydroxide solution) and MTBSTFA (*N*-*tert*-Butyldimethylsilyl-*N*-methyl)trifluoracetamide) depending on the chemical structure of the original compound to derivatize (De Witte *et al.*, 2011).

De Witte *et al.* (2011) found that the analysis of degradation products is a highly challenging task. First, the chemical structure of intermediates is unknown, although it can be assumed that primary degradation products are structurally related to the parent pharmaceuticals. Second, standard material for structure elucidation is seldom available. Third, degradation products are present at low concentrations.

Therefore, advanced and extended identification methodologies are needed for full structural elucidation of pharmaceutical AOP degradation products. Samples are typically separated by LC or GC, and either directly injected or pre-concentrated by SPE, lyophilization, evaporation, solvent extraction (e.g. liquid–liquid extraction), or SPME (Ternes *et al.*, 2003 and Carballa *et al.*, 2004). Many chromatographic techniques can be applied for product isolation prior to nuclear magnetic resonance (NMR) (Yi *et al.*, 2007, Marco-Urrea *et al.*, 2009). During GC or LC separation, degradation product retention times may provide the first source of identification information. One major point of attention during GC analysis is the thermal stability of pharmaceutical degradation products. High GC-inlet temperatures can decompose thermal labile compounds. They may be used to estimate the polarity and volatility of the degradation products and can be compared, if available, with the standards. For a more accurate identification, degradation product spectral data have to be collected by use of dedicated detection instruments such as UV spectroscopy or mass spectrometry (MS) (Kosjek *et al.*, 2010). When standard compounds are available, LC-UV or GC-MS spectral data of the unknown degradation products are compared with those of standard compounds. GC-MS also allows spectra comparison with extended databases (e.g., from NIST or Wiley). However, in the

majority of cases, standards or databases are not available and data on molecular weight, elemental composition, and chemical structure have to be collected by GC-MS, LC-MS, high resolution (HR)-MS, or multidimensional MS (MS^n). Analysis of the parent compound molecule and analogous products as well as isotope labeling strengthens identification. Next to these hyphenated techniques, direct UV photodetection, direct infusion (DI)-MS, and nuclear magnetic resonance (NMR) analysis (Yi *et al.*, 2007) may provide supplementary data to complement the chromatographic separation.

The MS analysis of the sample is frequently one of the most used techniques for AOP degradation product identification and also for the biotransformation products of PhACs studies without analysis of standards, parent molecules, or analogous products. This is possible since the structure of the parent molecule is also known. Another possibility can be as suggested by Doll and Frimmel (2003), where they made clear distinction between unequivocally identified pharmaceutical AOP degradation products, based on comparison of LC retention time and UV spectrum with standards, and tentatively identified degradation products, based on LC-MS fragmentation analysis with comparison with standards. The proposed techniques can be applied for a wide range of degradation products, i.e., biodegradation and photodegradation products from pharmaceuticals, as well as degradation products from other micro pollutants such as musks. Although standard compounds and GC-MS spectra may be readily obtained for transformation products resulting from AOP treatment or biotransformation metabolites of widely variable PhAC chemical structures and musks studied, these resources are frequently unavailable commercially to confirm the chemical structures for many other pharmaceuticals and some of this standards need to be synthesized in laboratory. Chromatographic separation by GC or LC is an indispensable part of the analytical procedure when multi residue analysis is the focus. The choice between both chromatographic techniques is especially based on the polarity and thermal stability of the target compound.

Another option to confirm the product and metabolite chemical structures is the use of combining the information of LC-DAD-MS and LC-MS/MS. This is a technique suitable for analytes with a wider range of polarities, and has been shown to be a powerful tool for the identification of by-products and unknown compounds in samples of many advanced oxidation studies (Razavi *et al.*, 2009, Yuan *et al.*, 2009, Kosjek *et al.*, 2011). Razavi *et al.*, (2009) studied the fibrate pharmaceuticals degradation by free radicals induced oxidative by pulse radiolysis; Yuan *et al.*, (2009) the photodegradation of antibiotics in UV and UV/H_2O_2 and Kosjek *et al.*, (2011) the phototransformatition products of ketoprofen. Agüera *et al.*, (2005) studied the phototransformation of diclofenac, a non-steroidal anti-inflammatory drug (NSAID) commonly used as an analgesic, antiarthritic

and antirheumatic by the use of the technique LC-(ESI+)MS/MS. The presence of diclofenac has been reported in natural waters and in wastewater treatment plant effluents as a consequence of its incomplete elimination with conventional wastewater treatment (Xue *et al.*, 2010, Jélic *et al.*, 2011). Xue *et al.*, (2010) and Jélic *et al.*, (2011) also found low diclofenac biodegradation and high effluent concentrations. Nakada *et al.* (2005) found high removal efficiencies for ibuprofen in the WWTP varying from 84-98% for influent concentrations of 69-1080 ng L^{-1}. Direct photolysis can produce photo transformation products that are commonly analyzed by GC-MS, LC-MS and LC-MS/MS techniques combined in order to get confidence in the chemical structures produced during the process.

LC-MS and LC-MS/MS allow the separation of semi-polar and polar degradation products without extensive derivatization. Moreover, aqueous samples can be directly injected when concentrations of intermediates are high compared to the instrument detection limit. Therefore, LC-MS and LC-MS/MS is also frequently applied than GC for analysis of pharmaceutical intermediates formed by AOPs (Razavi *et al.*, 2009, Yuan *et al.*, 2009, Kosjek *et al.*, 2011) but it always depend of the nature compounds used in the studies. The retention time of the degradation products can provide information on degradation product polarity. This is a tool for product identification in addition to stronger identification methods.

Kosjek *et al.* (2011) also agree that mass spectrometry (MS) is considered a principal tool for identifying new products of AOP, especially since it enables an efficient analysis of trace amounts of analytes in complex organic mixtures. Such complicated environmental or biological samples require separation of components prior to mass spectrometric analysis, which justifies the use of hyphenated techniques such as GC-MS or LC-MS. The single-stage quadrupole (Q) and an ion trap (IT) both hyphenated to a GC, and a quadrupole-time-of-flight (QTOF) MS coupled to an LC can also be used. In the Q mass analyzer, the ions generated in the source undergo electron impact (EI) fragmentation, which results in complex, ambiguous spectral data and hence in non-selectivity that is its main disadvantage (Kosjek *et al.*, 2011). In contrast, the IT mass detector has the unique ability to isolate and to accumulate ions. By iterating ion trapping and scanning, it allows the generation of collision-induced dissociation (CID) spectra of the parent and fragment ions (and their fragment ions), thus increasing the level of confidence in assigning a particular structure (Kosjek *et al.*, 2011). Alternatively, the hybrid QTOF, in which the final resolving mass filter of a triple Q is replaced by a TOF analyzer, not only allows MS^2 operation but also has the necessary accuracy and resolution to give exact-mass measurements (Kallio *et al.*, 2010, Yuan *et al.*, 2011, Kosjek *et al.*, 2011). Together with

MS methods, both chromatographic techniques complement each other to account for a wide range of polarity, acidic-basic characteristics and different functional groups formed during UV degradation or other oxidation techniques. LC-MS methods can also be used for the identification of metabolites produced by organisms like diclofenac in fish bile with electrospray ionization quadrupole-time-of-light mass analyser (QTOF) (Kallio *et al.*, 2010).

Kosjek *et al.,* (2009) studied the metabolism of diclofenac and clofibric acid in activated sludge bioreactors. The biodegradation of clofibric acid revealed one metabolite in the ESI(-)-QTOF chromatogram, 4-chlorophenol, which is known to exhibit a higher toxicity than the parent compound. This study confirms that further research is needed on the formation of stable metabolites both during wastewater treatment and in the environment. It also highlights the need for parallel toxicity testing. In addition, this study suggests that more needs to be known about the environmental fate of pharmaceuticals so that we are able to provide a comprehensive risk assessment.
Winckler *et al.,* (2001) studied the degradation of ibuprofen and clofibric acid in river biofilm systems and identified two ibuprofen metabolites by GC-MS.
The combined use of both GC-MS and LC-MS analysis for detection of pharmaceutical AOP degradation products targets two different purposes: (a) increasing the range of detectable degradation products or (b) confirmation of suggested degradation products.
A large range of degradation products was detected by Pérez-Estrada *et al.* (2005), who used GC-MS for detection of non-polar degradation products and LC-MS for semi-polar and polar degradation products during advanced oxidation of diclofenac and dipyrone. The second strategy was applied by Huber *et al.*, (2005) combining LC-MS, GC-MS, and GC-MS/MS analysis for identification of one of the carbamazepine ozonation products. For GC-MS confirmation of a large range of intermediates, whose identity was first suggested by LC-MS analysis, it was necessary to decrease degradation product polarity by derivatization, leading to increased complexity of the analysis. In this way, Huber *et al.*, (2005) identified 17α-ethinylestradiol (EE2) ozonation products.

The improvement of analytical methods confirms that for the majority of the organic trace contaminants, microbial degradation does not lead to mineralization but rather to the formation of a multitude of transformation products (Quintana *et al.*, 2005; Kosjek *et al.*, 2009; Kern *et al.*, 2010; Radjenovic *et al.*, 2009). In order to evaluate whether an organic contaminant was transformed to non-toxic products or even mineralized, it is important to know the transformation pathways. Modern hybrid mass spectrometry systems provide the accurate masses of the new products and deliver information of mass fragments which

can be used to identify the chemical structure. However, with the exception of very simple reactions (e.g. hydrolysis of amides and esters) the MS spectra are often not sufficient to obtain and confirm the chemical structures of the transformation products (Ternes *et al.*, 2011). In general, there are a couple of structural modifications which lead to products with the same accurate masses and similar mass fragments of the parent compound. Without the knowledge of chemical/microbial reactions and/or measurements with alternative methods, the suggested product chemical structure could be incorrect. One possible solution for structural confirmation of the transformation products is nuclear magnetic resonance spectroscopy (NMR). However, a drawback of NMR is the elevated quantity needed of a relatively pure isolated standard, not very easy to achieve for the low concentrations used in the lab-scale reactors and it is still a challenge.

Only a few studies have investigated the biological transformation of emerging organic micro pollutants in contact to activated sludge at aerobic conditions and even less at anoxic or anaerobic conditions (Ternes *et al.*, 2011). So far, the following enzyme-catalyzed reactions turned out to be quite commonly involved in the transformation of emerging organic micro pollutants: mono- and dihydroxylation, alcohol and aldehyde oxidation, ester and amide hydrolysis, *N*-dealkylation and decarboxylation. Even though these basic reactions are known to be catalyzed by many different ubiquitous and constitutive enzymes with wide substrate specificity, certain structural differences can discriminate between alternate transformation pathways (Ternes *et al.*, 2011).

2.3 Variability and removal mechanisms of PhACs and musks in full scale WWTP

Since PhACs and musks were first detected in water and treated wastewater in the 1990s, there have been vast developments in analytical methods and the application of these methods. The many chemical structures of PhACs and musks make it difficult to understand the behavior of these compounds in the biological treatment process of the WWTP. The PhACs have been investigated in WWTP for identification and quantification and also to determine the biodegradation removal in WWTP (Joss *et al.*, 2005; Göbel *et al.*, 2005; Clara *et al.*, 2005a). Many studies have been conducted in order to investigate the occurrence and fate of the PhACs and musk fragrances in WWTP (Joss *et al.*, 2005, Göbel *et al.*, 2005; Santos *et al.*, 2009; Plósz *et al.*, 2010). The fate of PhACs and musks (Joss *et al.*, 2005; Carballa *et al.*, 2007; Clara *et al.*, 2010) and quantification of concentrations in full scale pollutant and fluxes based on samples from sewers were also investigated.

Ort *et al.*, (2010a) studied the sampling strategies of PPCPs in WWTP by establishing different sampling methods, mainly the high frequency of grab samples and different composite sample modes. Distinct toilet flushes and wastewater packets from household expected to have high variable PPCP patterns in the sewers. The relatively long sampling intervals may also not be suitable to representatively sample for PPCPs in sewers given the unknown and potentially high short-term concentration variations according to Ort *et al.*, (2010). The influent wastewater at WWTP seems to be a continuous stream, but actually it is composed of a number of variable discharged, individual wastewater from household, industries, or in the sewer systems. The resulting heterogeneity can cause significant short-term variations of pollutant loads. The sampling intervals of 5 min or shorter may be required to properly account for temporal PPCP variations in influents of WWTP according to Ort *et al.*, 2010a, however this small sampling interval is not feasible in practical to the engineers to monitor micro pollutants in the plants and other studies should be performed in order better understand the behavior of the variability of PhAC and musk in the WWTP influent. Representative samples are important for providing meaningful analytical results and the number of samples should be adequate to get accuracy of the chemical analysis and to apply adequate statistical methods.

Clara *et al.*, (2011) studied the occurrence of polycyclic musk (galaxolide, tonalide, cashmeran, traseolide, celestolide and phantolide) in wastewaters (influent, effluent and sludges), surface waters discharged from the plant and sediments. The major musks detected include the galaxolide, tonalide and cashmeran. These compounds were mostly detected on the excess in the concentration ranges of 400-2650 and 4200-21000 $\mu g kg^{-1}$ dry matter for tonalide and galaxolide respectively, and digested sludge in the concentration ranges of 1100 to 2900 and 8500 to 20000 $\mu g kg^{-1}$ dry matter for tonalide and galaxolide respectively. Tonalide and galaxolide were also detected in the wastewater effluents in the ranges <0.38 to <0.50 and <0.80 to 1.1 $\mu g L^{-1}$ respectively.
Göbel *et al.*, (2005) studied the occurrence of some antibiotics (sulfonamides, macrolides and trimethoprim) in WWTP by collecting different samples at influent, effluent and secondary and digested sludge using flow proportional composite samples over 24h. With this study, authors wanted to evaluate the occurrence of these compounds in the different parts of the plant and find that sorption of these compounds was not significant and the elimination by the plant was incomplete.

The design of conventional WWTPs is not optimized to remove micro pollutants but the technology used to remove carbon, nitrogen and phosphorus used nowadays in the plant, can effectively improve the elimination of micro pollutants by means of sorption,

biotransformation, volatilization, and/or stripping (e.g., Ternes, 1998; Nakada *et al.*, 2006; Plósz *et al.*, 2010). In the most commonly used activated sludge technologies, biotransformation is important but insufficient to remove hormones and antibiotics (Carballa *et al.*, 2004). To increase the selection of bacteria capable of oxidizing micro pollutants in WWTPs, Clara *et al.* (2005b) show that values of the operating solids retention time (SRT), should be selected higher than 10 days, calculated to a reference liquid temperature of 10 °C. In the conventional systems, the recommended minimum SRT value corresponds to the requirements of meeting the effluent ammonia criteria by effectively retaining autotrophic nitrifying bacteria populations in activated sludge (Clara *et al.*, 2005b). Biodegradation rates for hormones and antibiotics can vary under aerobic and anoxic conditions, prevailing in most activated sludge systems, and the impacts of redox conditions on the overall biotransformation efficiency can be significant for most estrogens and antibiotics compounds (Plósz *et al.*, 2010a and b).

Joss *et al.*, (2005) studied the removal of PhACs and musks (galaxolide and tonalide) in biological wastewater treatment and conclude that the main removal mechanisms of the WWTP for the musks was the sorption to the sludges and for the PhAC was the biological for sludge ages between 10 and 60-80 days however the variability found in the removal was not completely explained. The influent samples were collected within the mixing of primary clarifier with sampling frequency < 60 min enough to assess the influent loads. The importance of getting representative 24 h samples was also considered in his study. Some of these compounds are not completely degraded by the conventional activated sludge process and when the wastewater is subjected to UV radiation for disinfection purposes, they are also not completely removed.

The enhanced tertiary treatment processes, such as, ozonation (Ternes *et al.*, 2003), UV radiation (Vogna *et al.*, 2004a and b; Nakada *et al.*, 2005, Kim *et al.*, 2009), in combination with a biological treatment, can be a robust engineering solution to eliminate the residual micro pollutants derived from biological systems (Plósz *et al.*, 2010b, Xue *et al.*, 2010). Since intensive daily routine monitoring of micro-pollutants is not feasible in WWTPs, the implementation of an appropriate monitoring plan should be considered because the micro pollutant discharge can exhibit seasonal, weekly and diurnal variations. Sewers, including influents to WWTPs, exhibit often unique transport characteristics, highly dynamic flows, and variable pollutant loads. Preservation and laboratory specific measures to prevent and control contamination are very important to get confidence in the results obtaining when monitoring micro pollutants (Ort *et al.*, 2010a).

Several removal mechanisms have been reported in different studies in WWTP such as biodegradation or biotransformation (Joss *et al.*, 2005), adsorption (Carballa *et al.*, 2007),

volatilization (Tan *et al.*, 2007) and oxidation (Ternes *et al.*, 2003, Nakkada *et al.*, 2005) in disinfection systems for PhACs and musks. In most of the cases, incomplete removal is observed for the PhACs and musks. Another important point in monitoring these kinds of compounds is the sampling point selection and the monitoring strategies. Strategies for monitoring PhACs and musks have been developed by many authors (Joss *et al.*, 2005; Göbel *et al.*, 2005, Ort *et al.*, 2010a and b, Plósz *et al.*, 2010) in order to try to establish sampling frequencies and preservation techniques to get confidence in the results expected in the ranges of ng L^{-1} or µg L^{-1}.

In many cases, the influence of the variability of the occurrence of PhACs and musks in the influent of the WWTP have been studied and in other cases mass balances have been carried out to predict the impact of the discharges of the effluent treated wastewater in the environment (Joss *et al.*, 2005, Göbel *et al.*, 2005, Carballa *et al.*, 2007, Weissbrodt *et al.*, 2009). Göbel *et al.*, (2005) also perform mass balances to the antimicrobials (sulfamethoxazole, trimethoprim and clarithromycin) including primary, secondary (conventional activated sludge process) and tertiary (sand filtration) treatment and also the digestion. In this study, no completely removal was obtained even with the tertiary treatment and also an important fraction of this antimicrobials was removed by sorption to the sludges. More research is needed to extent the knowledge for other PhACs and musks in the WWTP that may be not completely removed after the tertiary treatment and optimization of this treatment process with different technologies should be considered as well as the sorption to the sludge was not completely studies yet and more studies should be performed in order to understand the fate of PhACs and musks in the WWTP. To assess the occurrence and behavior of micro pollutants in sewage and treatment systems, optimal sampling time resolution and sampling sites need to be selected with some criteria. In sewer systems and in the WWTP, for the assessment of average micro pollutant load, in addition to an accurate flow meter, the substance's load pattern, the sampling frequency and the length of the composite sample are decisive (Ort and Gujer, 2006). Many studies in monitoring full scale WWTP show that grab samples can only serve to obtain preliminary results in mostly screening studies. For calculating the loads or mass-fluxes in WWTPs, 24-hour composite sample collection is recommended (Ternes and Joss, 2006, Plósz *et al.*, 2010a; Ort *et al.*, 2010).

2.4 Sampling of PhACs and musks in full scale WWTP

Until now, there is no online instrumentation to analyze for PPCPs directly in sewers and the monitoring plan can be found similar to the norms and guidelines already used for

macro pollutants. Due to the high analytical costs per sample, 24 h composite samples are most commonly reported (75% of analyzed papers) (Ort *et al.*, 2010a and b).
There are also events in sewers and WWTPs that influence the pollutant concentrations. Some authors explicitly mention that samples were taken during dry weather conditions, knowing that rain can impact the occurrence and fate of pollutants, particularly in combined sewer systems. The sewer systems, toilet flushes and wastewater from other household contain the majority of the pharmaceutical load and can be considered sometimes as "events". These events can lead to significant short-term variations in combined and separate sewers in the range of minutes. This integrated volume is sufficient to calculate the environmental pollutant flux from an average concentration in a sample after sampling. However, if flow variations occur within the sampling period, they must be taken into account during sampling to weight individual subsamples and obtain a representative 24 h composite sample (average concentration) (Ort *et al.*, 2010a).

The sampling frequency should not be reduced just because the storage capacity in the available sampling device is limited. This may lead to an inadequate sampling frequency and cannot be compensated for with a large number of samples, or a sophisticated analytical technique or statistical analysis. The time and money spent on chemical analysis and subsequent interpretation of data, it is counterproductive to attempt to save costs for sampling personnel and equipment.
Ort *et al.*, (2010a) suggests performing sampling campaigns over a number of consecutive days, rather than nonconsecutive days. The different sampling locations should consider the comparison of the WWTP influent and effluent (effect of hydraulic or sludge retention time) and WWTP influent (variable travel times in sewers) (Ort *et al.*, 2010a). In the case of WWTP, there is usually more freedom to choice the best location to collect the samples. The untreated raw wastewater can theoretically be collected only at the influent of a WWTP, it may, for many studies, also be opportune to take samples after the primary clarifier where concentration variations are expected to be attenuated. Depending on the layout and the hydraulic retention time longer sampling intervals should be considered for attenuation of the concentration variability. Some particulate matter is removed in the first treatment step and the concentrations of dissolved compounds are unlikely to be significantly affected. Filtration of samples before chemical analysis also removes particulate matter which is often not analyzed any further such as the case for the analysis of organic compounds dissolved in the wastewater. In the case of existing internal recirculation flow, entering before the effluent of the primary clarifier, it might need to be taken into account because it will affect the mass balances of influent loads, according to the Ort *et al.*, (2010a) suggestions.

Municipal micro pollutants discharge can exhibit seasonal, weekly and diurnal variations (Plósz *et al.*, 2010). To assess the occurrence and behavior of micro pollutants in WWTP, optimal sampling time resolution and sampling sites are recommended. To assess the daily variation in influent concentrations, 8 h composite samples of primary effluent though only during one day, and the data obtained was not proportional to flow (Joss *et al.*, 2005, Plósz *et al.*, 2010). Göbel *et al.* (2005) assessed diurnal variations in WWTP influent using three 8-hour flow-proportional composite samples obtained in one sampling day, and suggested that daily antimicrobials loads can correlate with the respective water flows and ammonium loads. The diurnal variations in the influent antimicrobial load can correlate with the theoretically expected distribution pattern influenced by the half-life in the human body and the typically prescribed oral administration pattern (Göbel *et al.*, 2005). The results obtained by Göbel *et al.*, (2005) illustrate a possible daily variance for antimicrobials where the flow proportional composite samples at least during 24 h is crucial to assess the fate and occurrence in WWTP. Mass balances are also an important tool used for many authors to predict impacts of the variability in the plants to help in management of the WWTP (Göbel *et al.*, 2005, Carballa *et al.*, 2007; Weissbrodt *et al.*, 2009).

Carballa *et al.*, (2007) perform mass balances with two different methods in the WWTP. The methodology applied in the mass balances can have implications in the results obtained. Combining the influent, effluent and sludge loads to get the mass balances to all plant, sometimes negative degradation results can be obtained for example for estrone and β-estradiol (Carballa *et al.*, 2007). Similar results were obtained by other authors (Göbel *et al.*, 2005), but the reason for this is still unclear, even using 24 h composite sampling.

2.5 Biodegradation and oxidation studies of PhACs

2.5.1 Biodegradation studies

Many other studies have been carried out with PhACs and musks in lab scale reactors in order to more easily understand some of the biological transformations of these compounds under controlled conditions. The transformation of this group of compounds can also generate higher toxicity compounds to the aquatic organisms in the environment. Different technologies have been applied in these studies like Sequencing Batch Reactors (SBR) (Evangelista *et al.*, 2010; Marco-Urrea *et al.*, 2010), Membrane Bioreactors (MBR) (Clara *et al.*, 2005; Kimura *et al.*, 2007), Biofilm reactors (Winckler *et al.*, 2001; Zwiener *et al.*, 2003), and continuous biological reactors (Yi *et al.*, 2007; Kosjek *et al.*,

2009). Marco-Urrea *et al.* (2010) studied the oxidation of atenolol, propranolol, carbamazepine and clofibric acid by white-rot fungus *Trametes versicolor* to evaluate the biodegradation of this PhAC and the metabolite formation. Evangelista *et al.*, (2010) also studied the effect of secondary carbon sources (glucose) on the microbial degradation of chorophenoxy acids (e.g. clofibric acid) with different bacteria cultures. Other works reported the effect of nitrifier cultures on PhAC removal, 17α-ethinylestradiol (Yi *et al.*, 2007) and clofibric acid, diclofenac, carbamazepine and propyphenazone (Tran *et al.*, 2009). Almost all the lab-scale biological reactors studied the biodegradation kinetics and metabolites produced when the compounds are not completely biodegraded by varying the operational conditions (e.g. pH, type of carbon source, carbon source concentration, SRT, HRT, and temperature).

Yi *et al.*, (2007) studied the link between nitrification and biotransformation of 17α-ethinylestradiol (EE2) using enriched cultures of autotrophic ammonia oxidizer bacteria (AOB). The degradation of EE2 was mainly by conjugation and hydroxylation of the ring of the chemical structure of steroid corresponding to the electrophilic initiator of the reaction. In this study a linear relationship between nitrification and EE2 removal in enriched nitrifying cultures was obtained. The EE2 biotransformation can be cometabolically mediated under operating conditions with enriched nitrifiers (Yi *et al.*, 2007).

Tran *et al.*, (2009) studied the degradation of PhAC (clofibric acid, diclofenac, carbamazepine and propyphenazone) by enriched nitrifier cultures. Results showed high degradation of ibuprofen and partial degradation of other selected pharmaceuticals in the presence of allylthiourea (ATU), an ammonia monooxygenase inhibitor, reflecting the activity of heterotrophic bacteria, while without ATU addition, the contribution of the nitrification was dominant. The results suggest that nitrification can enhance the biotransformation of pharmaceutical substances. The contribution of autotrophs and heterotrophs in the biotransformation of the pharmaceuticals by the enriched nitrifier culture was successfully estimated by the addition of inhibitors in this study. Furthermore, high removal was obtained for clofibric acid, diclofenac, ibuprofen, fenoprofen, carbamezaepine when organic substrate (e.g. acetate) was added. Clofibric acid, carbamazepine and propyphenazone did not exceed the 25% removal even in the presence of 100 ppm of acetate concentration.

Evangelista *et al.*, (2010) studied the effect of structure and a secondary carbon source (e.g. glucose) on the biodegradation of chorophenoxy acids (e.g. clofibric acid, methylchlorophenoxypropionic acid and 4-chloro-2-methylphenoxyacetic acid). The formation of metabolites was observed in the presence of different microbial cultures

such as *Pseudomonas putida, Aspergillus niger, Bacillus subtilis, Pseudomonas fluorescens, Sphingomonas herbicidovorans* and *Rhodococcus rhodochrous.* The metabolite, 4-chloro-2-methylphenol, was only identified in trace amounts for methylchlorophenoxypropionic acid and 4-chloro-2-methylphenoxyacetic acid degradation in the presence of *S. herbicidovorans* and glucose in Evangelista´s study in 2010. The presence of an easily degradable carbon source such as glucose was able to biotransform the original compound to metabolites even in low concentrations. In order to understand the biodegradation and biotransformation of the refractory compounds such as clofibric acid and the metabolites produced during the process, further studies should be performed with other bacterial consortia. In the same way, different operational conditions should be tested and optimized to help the environmental engineers to deal with this problem.

Marco-Urrea *et al.*, (2009) screening four white-rot fungi (*Trametes versicolor, Irpex lacteus, Ganoderma lucidum* and *Phnerochaete chrysosporium)* for the degradation of ibuprofen, clofibric acid, carbamazepine after 7 days of incubation for a concentration of 10 ppm. Ibuprofen was found degraded by all the fungi studied but clofibric acid and carbamazepine was only affected by some strains of fungi. The biodegradation metabolites were also studied and identified for the ibuprofen and additional should be done for the identification products of the biodegradation of clofibric acid and carbamazepine.

Other strategies used in the biodegradation studies involve the acclimatization of the activated sludge, collected in municipal WWTP, for long periods of time to the reactors by spiking the PhAC and supplemented with an extra carbon source. Control studies are also performed in combination without spiking the PhACs in this case. The biodegradation studies involve changes in operating conditions e.g. hydraulic retention time, substrate composition and concentration (Kosjek *et al.*, 2009). The light and temperature are also controlled in the reactors to avoid interference effects of these two parameters.

Biofilm reactors have also been used for PhAC biodegradation studies in rotating annular reactors operating with raw river water serving as continuous inocula and nutrient source. Then, without any previous biomass acclimatization to the biodegradation of any specific compound, the PhAC was added to the reactor. Other bioreactors were fed with artificial wastewater over a long period of acclimatization time with exposure to the PhAC (Winkler *et al.*, 2001, Kraigher *et al.*, 2008). Studies focus on the operation of lab scale reactors has also included the biomass characterization (Winkler *et al.*, 2001; Zwiener *et*

al., 2003, Kraigher *et al.*, 2008). Zwiener and Frimmel, (2003) studied the biodegradation in biofilm reactors and including the sorption of the PhAC in the biofilms without and with acetone to a toxic level. In this study clofibric acid and diclofenac were eliminated only in 5% and ibuprofen was eliminated in 60% with the biofilm reactor operating with oxic and anoxic conditions. The biofilm was created in the reactor with biomass from activated sludge. However, the anoxic conditions tend to favor the removal of the clofibric acid, ibuprofen and diclofenac. Some of the most studied PhAC and microbial cultures used in lab scale reactors are listed in Table 2.6.

Table 2.6 - Some of the most studied PhAC and microbial cultures in lab scale reactor.

Author	Compounds	Microorganisms
Yi *et al.*, 2007	17α-ethinylestradiol	Nitrifiers
Kraigher *et al.*, 2008	ibuprofen, naproxen, ketoprofen, diclofenac and clofibric acid	Nitrifiers (*Nitrospira sp.*) Betaproteobacteria (*Thauera, Sphaerotilus, Ideonella* and *Acidovoraz spp.*)
Tran *et al.*, 2009	clofibric acid, carbamazepine, diclofenac, propyphenazone	Nitrifiers
Marco-Urrea *et al.*, 2009	ibuprofen, clofibric acid, carbamazepine	White-rot fungi (*Trametes versicolor, Irpex lacteus, Ganoderma lucidum* and *Phnerochaete chrysosporium*)
Marco-Urrea *et al.*, 2010	clofibric acid, carbamazepine, atenolol and propranolol	*Trametes versicolor*
Evangelista *et al.*, 2010	clofibric acid, methylchloro-phenoxypropionic acid, 4-chloro-2-methylphenoxyacetic acid	*Pseudomonas putida, Aspergillus niger, Bacillus subtilis, Pseudomonas fluorescens, Sphingomonas herbicidovorans* and *Rhodococcus rhodochrous*

Other strategies for the operation of these reactors include starvation experiments with different changes in PPCP feeding periods to study this effect on the biodegradation of phenol, such as 4-chorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol (Buitrón *et*

al., 2011). Biodegradation kinetic studies of mixture of 4-chlorophenol (reported as metabolite generated from the clofibric acid biotransformation (Kosjek *et al.*, 2009), in sequencing batch reactors (SBR) subject to starvation and shock load had a transient effect on the microorganisms degradation rate show very fast removal of this compound after 15 h led to 90% removal of 4-chlorophenol and it is used as cometabolite in the clofibric acid biodegradation. The small concentration of this metabolite was too low to be characterized. In the same way, Buitrón *et al.*, (2011) suggests that the substrate removal rate decreases when perturbations are applied to the SBR. The specific dehydrogenase activity of suspended biomass was higher than in the biomass attached before starvation. Increasing the starvation period did not affect significantly the removal and only the dehydrogenase activity was more affected. The operation of SBR under variable influent concentrations need to be further studied in order to understand the effects on the removal of the compounds.

The identification of the new products formed (metabolites) during the biotransformation process (Winkler *et al.*, 2001, Kosjek *et al.*, 2009, Marco-Urrea *et al.*, 2009, Evangelista *et al.*, 2010) and the metabolic biodegradation pathways is one of the most studied subjects in the biological reactors (Yi *et al.*, 2007, Marco-Urrea *et al.*, 2009 and 2010, Evangelista *et al.*, 2010). Winkler *et al.*, (2001) identified two metabolites of ibuprofen biodegradation, hydroxyibuprofen and carboxyibuprofen by GC-MS analysis. Kosjek *et al.*, 2009 identified biotransformation products of diclofenac by UPLC-ESI(+)-QTOF-MS and clofibric acid by UPLC-ESI(-)-QTOF-MS. Seven main metabolite products were detected but only one chemical structures have been proposed for diclofenac, 1-(2,6-dichlorophenyl)-1,3-dihydro-2*H*-indol-2-one) and 4-chlorophenol was identified as the main metabolite of clofibric acid biotransformation. Marco-Urrea *et al.*, (2009) also identified three biodegradation products of ibuprofen, 2-hydroxyibuprofen, 1-hydroxy-ibuprofen, 1,2-dihydroxyibuprofen by GC-CIRMS (combustion isotope ratio mass spectrometry) and ^{1}H and ^{13}C NMR. Evangelista *et al.*, (2010) identified ethyl clofibrate as metabolite of clofibric acid and other metabolites have been detected but not identified. The results obtained from this studies justify that further studies should be done in order to understand the metabolites generated from the different PhAC in biological treatment process.

2.5.2 Advanced Oxidation techniques

To reduce the input of pharmaceuticals into the environment, physicochemical end-of-pipe technologies such as advanced oxidation processes (AOPs) are indispensable after

the biological treatment in the WWTP (De Witte *et al.*, 2011). AOPs are characterized by the generation of hydroxyl radicals at ambient conditions. Degradation of pharmaceuticals and endocrine disrupting agents by AOPs, with focus on parent compound removal and kinetics, has gained a lot of attention. Full mineralization of pharmaceuticals by AOPs is reported to be not cost effective. Partial degradation, with formation of more biodegradable or less toxic intermediates, is the key to optimize treatment. This involves careful process control because intermediates can still have endocrine activity, antibiotic activity, or other biological effects. Moreover, degradation products can be more toxic than the parent compound. For example, Vogna *et al.*, (2004a) found that mutagenic and carcinogenic acridine intermediates are formed during UV/H_2O_2 treatment of carbamazepine.

Many studies using oxidation techniques have been applied to the transformation of PhAC in lab scale reactors with pure water and wastewater (Radjenovic *et al.*, 2009); surface water (Pereira *et al.*, 2007a) and ground water, drinking water (Canonica *et al.*, 2008) and tap water (Kim *et al.*, 2009), resulting in recent emerging concerns of the safety of drinking water, ground water, surface water, reclaimed wastewater and aquatic ecosystems. Pilot plant studies on ozononation and UV have also been applied to study pharmaceuticals, iodinated X-ray contrast media (ICM) and musk fragrances from municipal wastewater (Ternes *et al*., 2003). Ultraviolet (UV) light radiation is an established method for drinking-water disinfection and also a technology for wastewater purification (Canonica *et al.*, 2008). It has long been recognized that even at such (relatively low) UV doses organic compounds dissolved in water may undergo photochemical transformation. Since surface waters are often used as a source for drinking-water production, and treated wastewaters are utilized to recharge groundwater tables or reused for washing facilities or other purposes, the question about transformation products is still an unclarified problem when oxidation technologies are applied. Canonica *et al.*, 2008 studied the extent of the degradation (expressed as depletion of the parent compound) of four selected pharmaceuticals in a UV drinking water treatment system for disinfection purposes. They determined the pH dependence on direct phototransformation, quantum yields and how they can be used to quantify the extent of degradation of the pharmaceuticals under low-pressure (LP) and medium-pressure (MP) mercury lamp UV irradiation conditions. It was an important contribution to understand the extent of degradation of pharmaceuticals at higher UV doses used in disinfection of drinking water and in the UV treatment of wastewaters.

The limited removal of some PhACs in the biological process of WWTP could be responsible for the contamination in the water environment, resulting in recent emerging

concerns with the application of oxidation technologies as tertiary treatment (e.g. ozonation, UV radiation, chlorine) for disinfection. These technologies used for the inativation of microorganisms could also lower the concentrations of other micro pollutants. Vogna *et al.*, (2004b) have conducted a study on diclofenac oxidation with UV/H_2O_2 and ozone, showing that both ozonation and UV/H_2O_2 systems proved to be effective in inducing diclofenac degradation. In other study, they have reported that UV/H_2O_2 process could degrade carbamazepine very effectively, while UV alone process was not effective for reducing carbamazepine concentration (Vogna *et al.*, 2004b). The potential effectiveness of UV and UV/AOP (UV coupled with another advanced oxidation process) as drinking water remediation technologies for PhACs most commonly found in surface waters has been studied (Pereira *et al.*, 2007b). Kim *et al.*, 2009 studied the removal of six PhACs such as carbamazepine, naproxen, clofibric acid, ciprofloxacin and ketoprofen from surface water to evaluate their degradation by the use of fundamental photodegradation parameters in laboratory-grade water during UV and UV/H_2O_2. Canonica *et al.*, (2008) have evaluated the extent of photodegradation of four selected pharmaceuticals such as 17α-ethinylestradiol, diclofenac, sulfamethoxazole and iopromide in UV drinking water treatment for disinfection purpose. Nakada *et al.*, (2005) studied NSAIDs (Ibuprofen, naproxen, mefenamic acid and ketoprofen) removal in WWTP, and showed that UV radiation, used for disinfection, could also increase the PhAC removal when compared with other disinfection techniques, such as chlorination, and in particular for ketoprofen.

Rosario-Ortiz *et al.*, (2010) studied the effect of low pressure UV light coupled with hydrogen peroxide (UV/H_2O_2) on the removal of six pharmaceuticals (meprobamate, carbamazepine, dilantin, atenolol, primidone and trimethoprim) in the effluent wastewater of 3 WWTP and obtained a range from zero removal to >90%. The efficacy of UV/H_2O_2 treatment for the removal of pharmaceuticals from wastewater was a function of not only the concentration of the effluent dissolved organic matter but also its inherent reactivity towards the hydroxyl radicals. The removal of pharmaceuticals also correlated with reductions in ultraviolet absorbance at 254 nm. The advanced oxidation processes (AOPs) have been shown to be effective at the removal of various organic contaminants from drinking water and wastewater (Huber *et al.*, 2003; von Gunten, 2003; Rosario-Ortiz *et al.*, 2010). Ozone, ozone with hydrogen peroxide (H_2O_2), and UV with H_2O_2 are three of the most commonly studied AOPs. During ozone and ozone with H_2O_2, pharmaceutical oxidation occurs via reactions with molecular ozone and the hydroxyl radical. Both of these oxidants contribute to the oxidation of trace contaminants.

Ozonation using 5-15 mg L^{-1} of ozone is appropriate to oxidize pharmaceuticals, musk fragrances, estrogens and to simultaneously inactivate relevant microorganisms studied in

a pilot plant (Ternes *et al.*, 2003). Even not optimized, ozone based AOPs (O_3/H_2O_2 and O_3/UV) slightly increased the oxidation efficiency for some iodinated contrast media in comparison to ozonation (Ternes *et al.*, 2003). The ability of a particular treatment process to remove organic contaminants depends mostly on the structure and concentration of the contaminant. In addition, the operational parameters of the process (e.g., oxidant dose and contact time) will also determine the degree of attenuation of a particular contaminant (Ternes *et al.*, 2003). Trace levels of hormones and pharmaceuticals are ubiquitous contaminants of municipal wastewater effluents. The detection of these chemicals is a direct function of analytical detection limits (Ternes *et al.*, 2003). Therefore, more and more trace contaminants will continue to be discovered. Water treatment processes have various levels of efficacy in the attenuation of these contaminants. In drinking water, oxidation provides a cost effective means for disinfection and simultaneous contaminant removal (Falconer, 2006, Canonica *et al.*, 2008). In an evaluation of estrogenicity as a class of toxicity, the estrogenicity of common food items is far beyond that of any wastewater or drinking water evaluated. The relative risk factors of common exposure to endocrine disrupting compounds (EDCs) through foods and beverages appear to be far greater than the exposure through drinking water (Falconer, 2006). The potential risk to health exists, however the estrogenic contamination of drinking water is very unlikely to result in physiologically detectable effects in consumers (Falconer, 2006). More research is needed to adequately address relevance of EDCs and pharmaceuticals to human health, but it is likely that most drinking waters do not provide substantial concentrations that are expected to be detriment to human health. Very few studies, using oxidation technologies for the study of PhAC and musks, combine the kinetic parameter determination, the energy supplied to the phototransformation process and the by-product identification in wastewater. Most of the studies carried out are applied to pure water or to drinking water.

CHAPTER 3

ANALYSIS OF 65 PHARMACEUTICALS AND PERSONAL CARE PRODUCTS IN 5 WASTEWATER TREATMENT PLANTS IN PORTUGAL USING A SIMPLIFIED ANALYTICAL METHODOLOGY

3. Analysis of 65 pharmaceuticals and personal care products in 5 wastewater treatment plants in Portugal using a simplified analytical methodology

Pharmaceuticals and personal care products (PPCPs) are becoming increasingly recognised as important micropollutants to be monitored in wastewater treatment plants (WWTPs), since WWTP effluents represent an important point source to natural aquatic systems. In this study, the abundance of 65 PPCPs was analysed in 5 Portuguese WWTPs during the spring and autumn. Due to the fact that analytical approaches normally used to quantify the abundance of these compounds are labour intensive and require various specific procedures, this study proposes a set of simplified analytical methods for the quantification of pharmaceutically active compounds (PhACs) and polycyclic musks in liquid and sludge samples. The analytical methods were validated using influent wastewater matrices, showing comparable limits of detection and quantification as literature values for most PPCPs, with the exception of the estrogenic compounds. The PhACs concentrations detected in the WWTP survey were in the range of 0.050 - 100 μg L^{-1} in the influent and up to 50 μg L^{-1} in the effluent, where the non-steroidal anti-inflammatory drugs (NSAIDs) were the most abundant and frequently detected group. Some musks were detected up to 11.5 μg L^{-1} in the influent and 0.9 μg L^{-1} in the effluent, and adsorbed in the sludge up to 22.6 μg g^{-1}.

3.1 Introduction

Pharmaceuticals and personal care products (PPCPs) are commonly occurring micropollutants with a potentially significant environmental impact. The impact in the environment and public health arises not only from wastewater effluents discharged in aquatic media (Bartelt-Hunt *et al.*, 2009), but also from sludge application in agriculture, since they can desorb and contaminate the groundwater (Carrara *et al.*, 2008). Therefore, it is important to monitor these compounds to know their concentration in the liquid and solid phases after treatment in wastewater treatment plants (WWTP). While many studies have been carried out in different countries and geographical locations (Comeau *et al.*, 2008; Okuda *et al.*, 2008; Santos *et al.*, 2009), the occurrence of PPCPs in wastewater and environmental samples is highly dependent on the local diseases, treatment habits and market profiles, thus, the pollution profile and can vary significantly between different countries (Zuccato *et at.*, 2006). This was the motivation for the present study, since little information on the occurrence of PPCPs in WWTPs in Portugal is available.

The most common PPCPs are the pharmaceutical active compounds (PhACs) and the polycyclic musk fragrances. PhACs include the antidepressives, anticonvulsants, non-steroidal anti-inflammatory drugs (NSAID), steroidal anti-inflammatory drugs (SAID), drugs for asthma and allergic diseases, antihypertensives, β-blockers, lipid regulators, antibiotics, and estrogens.

Due to the high diversity of compounds displaying a wide variance of chemical structures, many previous studies have elected to perform a combination of analytical methods targeting specific families of compounds (Sacher *et al.*, 2001; Ternes *et al.*, 2001). While this strategy can be advantageous for the analysis of each target group, the time-consuming and labour-intensive nature of the analytical procedures makes numerous methodologies undesirable when the goal is to make an overall assessment of PPCPs present in environmental samples and WWTPs. This work proposes a simplified methodology adapted from previously published analytical methods that can be applied to wastewater and sludge samples for the detection of 65 PhACs and musks. All PhACs were analysed through LC-DAD-(ESI+)MS with the same set of conditions after solid-phase extraction (SPE) using two different materials for either neutral or acidic compounds. The musks were analysed by GC-MS after solid-phase microextraction (SPME). Sludge samples were pre-treated with an ultrasonication step prior to PhAC and musk analysis.

The methodology proposed in this study was applied to the influent, effluent and sludge samples from 5 Portuguese WWTPs. The validity of the simplified analytical methodology was assessed using the influent of the different plants. The PhAC compounds covered in this survey were selected based on the top-ranking sales figures for 2003 and 2007 provided by INFARMED (Portuguese Authority for Medication and Health Products), which is a similar approach as adopted by e.g. Erickson (2002) and Zuccato *et al.* (2006).

3.2 Materials and methods

3.2.1 Chemicals and reagents

HPLC-grade acetonitrile, methanol, *n*-hexane and formic acid were purchased from Panreac (Portugal), all pharmaceutical active compound standards from Sigma-Aldrich (Steinheim, Germany) and the musks from LGC-Promochem (Spain). Stock solutions (1

mg mL^{-1}) of each pharmaceutical and musk were prepared in methanol or hexane, respectively, and stored at 4 ºC.

3.2.2 Sampling collection and properties of the WWTPs

The characteristics of the 5 WWTPs assessed in this study are presented in Table 3.1. All plants contained screening and primary clarification prior to the secondary treatment process, as well as secondary clarifiers. Grab samples were collected at the influent (prior to primary treatment), the final effluent and the sludge (in the recycle from the secondary clarifier). 5 L samples were collected in plastic (PET) bottles and preserved at 4 ºC during transportation. The samples were filtered by 0.45 µm glass fiber membranes (GF 6, Whatman, England) and stored at -20 ºC.

Table 3.1- Characteristics of the WWTPs.

	Setúbal	Cucena	Valdeão	Quinta da Bomba	Fernão Ferro
Average Influent Flow (m^3d^{-1})	11 195	773	1634	15 536	3579
Wastewater	Domestic + Hospital + Industrial (8%)	Domestic + Industrial (20%)	Domestic + Hospital	Domestic	Domestic
Process	Activated sludge [1]	Activated sludge	Activated sludge	Trickling filter	Trickling filter
Volume biological reactor (m^3)	765 * 7000 ** 2083 ***	696 *	1352 *	9120	628
Hydraulic retention time (h)	33.6 h	11 h	20 h	14 h	7.6 h
Sludge age (d)	15	9	-	-	-
Sludge waste flow (m^3d^{-1})	354	40	No wastage	28	12

*Aerobic; ** Anaerobic; *** Anoxic, [1]Process includes tertiary treatment by UV-radiation

3.2.3 Extraction and clean-up

3.2.3.1 Acidic and neutral pharmaceuticals by SPE

SPE was used for the extraction and clean-up of the liquid wastewater samples. Oasis HLB cartridges (60 mg, 30 µm, Waters, Eschborn, Germany) were used for the acidic PhACs and RP-$C18_{ec}$ cartridges (500 mg, 50 µm, Waters, Milfort, U.S.) for the neutral PhACs. Each cartridge was previously conditioned with 1 mL methanol followed by 1 mL of MilliQ water, then dried in a N_2-stream. For the acidic PhACs, 200 mL of filtered

wastewater and 10 μL of meclofenamic acid (as internal standard) were passed through the Oasis HLB cartridges at pH 2-3. For the neutral PhACs, 500 mL of filtered wastewater and 50 μL of meclofenamic acid were passed through the RP-$C18_{ec}$ cartridges at pH 7-7.5. Samples were passed through the SPE cartridges at a flow rate of 20 mL min^{-1} and vacuum pressure of -5 psi, and the cartridges were eluted four times with 1 mL of methanol. The methanol extracts were evaporated to 1 mL by a N_2-stream. Then, 50 μL of extract was injected into the LC-MS.

3.2.3.2 Sludge samples – ultrasonic solvent extraction prior to SPE

The secondary sludge collected in the WWTPs was centrifuged for 5 min at 10 000 rpm. 2 g of the centrifuged sludge was mixed with 4 mL methanol in an ultrasonic bath for 5 min. The slurry was then centrifuged for 1 min at 10 000 rpm. The supernatant was collected in a separate vial and 2 mL of methanol were again added to the sludge. Centrifugation and supernatant collection were then repeated. To ensure the extraction was complete, 2 mL of acetone were then added to the sludge and the same procedure (i.e. ultrasonic bath, centrifugation, supernatant collection) was repeated. Then, the 4 extracts (2 x 2 mL of methanol and 2 x 2 mL of acetone) were combined and evaporated to a volume of ca. 1 mL. The concentrated extract was diluted in 150 mL of MilliQ water prior to SPE.

3.2.3.3 Polycyclic musk fragrances by SPME

The extraction of musks from the wastewater and sludge samples was carried out by solid phase micro extraction (SPME) with 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibers (Supelco, Spain). The fibres were pre-conditioned prior to use for 30 min at 250 ºC. 2 g of wastewater or sludge was added to 0.5 g NaCl and 10 μL of mirex as internal standard. The PDMS/DVB fibre was exposed to the sample headspace in a sealed vial with a Teflon lid for 15 min at 90 ºC. The fibre was thermally desorbed and analysed by GC-MS.

3.2.4 Analytical procedures

3.2.4.1 Detection of acidic and neutral PhACs by LC-MS

Reverse-phase chromatography (LiChroCART 250-4 Purospher Star RP18 column, Merck) was performed with a diode array detector (DAD) from 200 to 400 nm with a 0.6

mL min^{-1} flow, using a degassed mobile phase with 0.1% water/formic acid (A) and acetonitrile (B). The following binary gradient was used: start with 2 min, 15% B at 0.6 mL min^{-1}; 20 min, 100 % B at 0.6 mL min^{-1}; 25 min, 100 % B at 1.0 mL min^{-1}; 27 min, 15 % B at 1.0 mL min^{-1} and 35 min, 15 % B at 0.6 mL min^{-1}. HPLC-DAD-(ESI+)MS was carried out in a HPLC system (Waters) coupled with a pump and controller (Waters 600), an in-line degasser (X-Act-4 channels, Jour Research), an autosampler (Waters 717 plus), a photodiode array detector (DAD, Waters 996) and a quadropole VG Platform spectrometer (Micromass, UK) equipped with an electrospray ionisation (ESI) source operating in positive mode. A split ratio of 1:10 was used between the HPLC column and the mass spectrometer. The capillary temperature was 100-120ºC, the scanning cone voltage was 35-100 V and the capillary voltage was 3.5 kV. Nitrogen was used as drying and nebulising gas at 300 mL min^{-1} and 10 mL min^{-1}, respectively. The mass/charge spectrum range used was 100-450 amu with a MassLynx™ software data acquisition system. All samples were analysed in triplicate.

3.2.4.2 Detection of polycyclic musk fragrances

Analysis was performed using a Hewlett-Packard 5890 GC fitted with a QMD1000 Carlo Erba mass spectrometric detector. The injection port was operated in splitless mode. A DB-5MS, 5% phenyl and 95% dimethylpolysiloxane fused-silica capillary column (30 m x 0.32 mm i.d., 0.25 μm film thickness, Agilent-J&W Scientific, Spain) was used with helium as carrier gas at a flow rate of 1.5 mL min^{-1}. The injection port temperature was 250 ºC. The ion source and the transference line were kept at 200 and 310 ºC, respectively. The oven temperature was maintained at 60 ºC for 3 min, increased to 250 ºC at 10 ºC min^{-1}, then raised to 310 ºC at 20 ºC min^{-1}, and held for 13 min. The MS spectra were obtained with 70 eV, mass range *m/z* 50-500 amu and using MassLab™ software (Micromass). The injector temperature during SPME splitless analysis was 250 ºC for PDMS/DVB.

3.2.5. Method validation

3.2.5.1 Determination of recoveries

Samples were spiked with analytes dissolved in a stock solution (each at 1 mg mL^{-1} methanol). The influent wastewater samples were spiked with 100 μg L^{-1} of analyte and internal standard (i.e. meclofenamic acid). After spiked, the samples were stirred for homogenisation and to enable a sufficient contact of analytes and standards with the

matrix. Relative recoveries were determined relative to a MilliQ water standard solution, also spiked with 100 μg L^{-1} of analyte and internal standard. Recoveries of the pharmaceuticals in the individual clean-up steps were determined by SPE in wastewater influent matrices and in MilliQ water, and analysed by LC-DAD-MS as described above. The relative recoveries were calculated from the analyte areas in the influent matrix, subtracting the analyte area quantified in the original unspiked matrix, divided by the area of the MilliQ standard sample.

3.2.5.2 Calibration curves, limits of detection (LOD) and quantification (LOQ)

Standards were prepared from the stock solutions diluted in methanol. A six-point calibration curve was used for each compound, ranging from 5-200 ng L^{-1}. The regression coefficient of the resulting calibration curves was >0.95 for all compounds. Ten blank samples were analysed by LC-MS (with methanol) and GC-MS (with *n*-hexane) to determine the lowest signal/noise ratio of each analyte. Limits of detection (LOD) for the analytes were calculated by the formula 3xSD/*m*, where SD is the standard deviation of the lowest signal/noise ratio of the analyte and *m* is the slope of the calibration curve. Limits of quantification (LOQ) were calculated as 10xSD/*m*.

3.3. Results and discussion

3.3.1 Validation of the Analytical Approach

The analytical methodology selected for the PhAC survey carried out in this work was based on SPE followed by LC-DAD-(ESI+)MS. Although two different SPE materials were used to best enrich either acidic or neutral pharmaceuticals, the analysis of both extracts was done using the same LC-MS conditions (see methods), in order to detect the highest number of compounds with the lowest analytical effort. The selection of positive mode for the MS electrospray ionisation was based on preliminary tests using standards of the target compounds. For the majority of the compounds it was found that, when compared to ESI-, the ESI+ resulted in precursor molecular ions ($[M+H]^+$ or $[M-H]^-$, respectively for ESI+ or ESI-) with higher relative peak intensity, thus ESI+ was selected. The LOD, LOQ and recoveries obtained with this approach are presented in Table 3.2 for the compounds detected in this study. The results showed that the analytical procedure for PhACs enables the detection of a substantial number of pharmaceuticals with LOD and LOQ comparable to those reported in literature using a combination of analytical methods designed for specific groups of compounds. The estrogens are the main exception, where

tandem MS should be used in order to detect these compounds to a concentration that is relevant to assess their potential environmental impact (Ternes, 2001). However, some other compounds, namely carbamazepine, showed lower LOD and LOQ with SPE (RP-C18) followed by LC-DAD-(ESI+)MS when compared to results obtained with SPE (RP-C18) and GC-MS (Sacher *et al*., 2001). The LOQ for carbamazepine in this study was similar to that found by Ternes (2001) through LC-MS/MS. In Sacher *et al.* (2001), three separate analytical methods were used for antibiotics, whereas in this study, antibiotics were analysed together with the other acidic compounds. While Sacher *et al.* (2001) found lower LOD and LOQ for amoxicillin, their recovery was lower than in this study (36% vs. 83%). The remaining compounds that were not detected in this study had LOD values ranging from 1-25 ng L^{-1} and LOQ values ranging from 3-83 ng L^{-1}.

Table 3.2 - Limits of detection and quantification, and recovery of the PhACs found in this study. The results are compared with literature studies.

Compound	LOD ng L^{-1}	LOQ ng L^{-1}	Recovery* %	LOD ng L^{-1}	LOQ ng L^{-1}	Recovery %	LOQ ng L^{-1}	Recovery %
Neutral PhACs								
Atenolol	3	10	**>94±5**	2.4[a]	8.2[a]	67[a]	50[b]	98[b]
Caffeine	27	91	**>82±1**					
Carbamazepine	2	7	**>75±1**	9.6[a]	32[a]	74[a]	10[b]	92[b]
Clorazepate	17	57	-					
Dimethylamino-phenazone	29	95	**>83±3**	4.3[a]	14[a]	66[a]	100[b]	93[b]
Domperidone	3	9	-					
Etofenamate	20	67	-					
Fentiazac	-	-	-					
Fluoxetine	17	57	**>60±6**					
Fluticasone	25	85	**>87±1**					
Hydroxyzine	18	60	**73±1**					
Indapamide	6	18	**>86±1**					
Nimesulide	14	46	**>82±6**					
Paroxetine	27	89	**>86±12**					
Piroxicam	-	-	-					
Ramipril	9	31	-					
Salbutamol	11	36	**>94±1**	2.6[a]	9.1[a]	66[a]	50[b]	61[b]
Tramadol	20	67	**>86±2**					

* signifies that the recoveries were obtained with WWTP influent. The value presented is the minimum value obtained from the 5 WWTPs.[a] Sacher *et al.* (2001); recoveries obtained with surface water.[b] Ternes (2001); recoveries obtained with WWTP effluent.[c] Ternes *et al.* (2005); recoveries obtained with groundwater.[d] Smyth *et al.* (2008)

Table 3.2 - (cont.) Limits of detection and quantification, and recovery of the PhACs found in this study. The results are compared with literature studies.

Compound	LOD ng L^{-1}	LOQ ng L^{-1}	Recovery* %	LOD ng L^{-1}	LOQ ng L^{-1}	Recovery %	LOQ ng L^{-1}	Recovery %
Acidic PhACs								
Captopril	5	15	**66±10**					
Clofibric acid	15	50	**>98±1**	5.3[a]	18[a]	103[a]	50[b]	82[b]
Diclofenac	7	24	**>79±5**	8.7[a]	29[a]	70[a]	50[b]	89[b]
Enalapril	8	28	**>88±3**					
Flurbiprofen	18	58	**>65±1**					
Furosemide	19	63	-					
Ibuprofen	14	46	**>89±9**	3.5[a]	12[a]	110[a]	50[b]	81[b]
Indomethacin	7	23	**>96±5**	5.4[a]	18[a]	114[a]	50[b]	90[b]
Ketoprofen	21	69	**>86±2**	4.8[a]	16[a]	104[a]	50[b]	94[b]
Naproxen	18	59	**102±6**	3.8[a]	13[a]	105[a]	50[b]	91[b]
Meclofenamic acid	4	14	**>74±4**				50[b]	89[b]
Antibiotics								
Amoxicillin	13	43	**>83±4**	4.6[a]	15[a]	36[a]		
Ampicillin	3	11	**>67±1**					
Estrogens								
17α-Ethinylestradiol	21	69	-				1[b]	76[b]
Estrone	18	60	**104±12**				1[b]	82[b]
β-Estradiol	4	12	-				1[b]	76[b]
Musks								
Cashmeran	1	4	**83±3**	21[d]				
Celestolide	2	6	**85±4**	16[d]				
Galaxolide	1	4	**94±2**	11[d]			20[c]	82[c]
Phantolide	1	4	**97±2**	17[d]				
Tonalide	1	2	**82±3**	8.4[d]			20[c]	78[c]
Traseolide	2	6	**85±4**	13[d]				
Mirex	1	2	**89±3**					

* signifies that the recoveries were obtained with WWTP influent. The value presented is the minimum value obtained from the 5 WWTPs.[a] Sacher *et al.* (2001); recoveries obtained with surface water.[b] Ternes (2001); recoveries obtained with WWTP effluent.[c] Ternes *et al.* (2005); recoveries obtained with groundwater.[d] Smyth *et al.* (2008)

PhACs recoveries for the analytical process (SPE followed by LC-DAD-MS) were obtained with samples of influent wastewater for the compounds that were detected in that plant. These tests aimed at assessing the effect of the wastewater matrix on the analytical method as well as interferences due to the manipulation associated with the extraction and analytical process. The precision and reproducibility of the method indicated a relative standard deviation varying from 1 to 12 %. Despite some variation of the PhACs recoveries with the matrix (results not shown), the results were above 70% except for four compounds (Table 3.2). Fluoxetine showed the lowest recovery (60±6%)

with wastewater from Valdeão, suggesting that a different SPE material or GC after derivatisation should have been used for better detection of this compound. Nevertheless, the recoveries of fluoxetine for other WWTPs were >79±1%. With this methodology, a very wide range of compounds with different structures were covered, using a reduced number of analytical processes.

3.3.2 Measurement of PPCPs in wastewater influents

Table 3.3 - Pharmaceuticals and musks detected at the influent, effluent and secondary sludge of 5 different WWTPs in Portugal during the spring (23 May to 7 July) and autumn (2 to 25 October).

Compound	Influent Occurence	Influent minimum ng/L±SD	Influent maximum ng/L±SD	Effluent Occurrence	Effluent minimum ng/L±SD	Effluent maximum ng/L±SD	Secondary Sludge Occurrence	Secondary sludge minimum ng/g±SD	Secondary sludge maximum ng/g±SD
Neutral PhACs									
Atenolol	**5/10**	65± 5	4757±25	**4/9**	119±25	1297±14	**0/9**		
Caffeine	**10/10**	258±13	36160±36	**4/9**	437±20	4392±22	**3/9**	1788±12	8423±29
Carbamazepine	**2/10**	664±49	994±11	**1/9**	238±17	238±17	**0/9**		
Clorazepate	**1/10**	6227±16	6227±16	**0/9**			**1/9**	181± 9	181± 9
Dimethylamino-phenazone	**4/10**	158± 8	3664±19	**6/9**	252±65	4278±12	**3/9**	158± 8	1361±17
Domperidone	**1/10**	163±67	163±67	**0/9**			**0/9**		
Etofenamate	**7/10**	58±12	7333±26	**2/9**	229± 6	1620±33	**2/9**	24785±121	134431± 438
Fentiazac	**1/10**	5297±19	5297±19	**0/9**			**0/9**		
Fluoxetine	**5/10**	85± 1	1704±15	**0/9**			**1/9**	77±10	77±10
Fluticasone	**3/5**	196± 1	1298±82	**3/9**	33±0.3	2848±14	**2/9**	1473±17	2330±42
Hydroxyzine	**1/10**	9344±80	9344±80	**0/9**			**1/9**	43339±86	43339±86
Indapamide	**3/10**	177±19	1236±23	**2/9**	90± 2	329±13	**3/9**	47± 3	1362±31
Nimesulide	**1/10**	6911±13	6911±13	**0/9**			**0/9**		
Paroxetine	**3/10**	182±19	1312±15	**3/9**	224±15	3367±30	**0/9**		
Piroxicam	**2/10**	2575±49	9298±34	**0/9**			**0/9**		
Ramipril	**1/10**	5445±49	5445±49	**0/9**			**1/9**	488±62	488±62
Salbutamol	**3/10**	104±21	2158±13	**1/9**	572±27	572±27	**2/9**	12±0.6	104±21
Tramadol	**2/5**	158± 2	1344±17	**2/9**	51± 3	134± 4	**0/9**		
Acidic PhACs									
Captopril	**5/10**	32± 2	13335±26	**1/9**	1376±56	1376±56	**3/9**	875±16	5516±49
Clofibric acid	**9/10**	40± 1	6785±21	**8/9**	198± 3	7286±19	**4/9**	117±7	15655±18
Diclofenac	**7/10**	207±44	6674±24	**2/9**	26± 2	1612±18	**3/9**	2259± 5	17785±48
Enalapril	**4/10**	51± 4	10238±32	**3/9**	624±21	19888±22	**1/9**	61±13	61±13
Flurbiprofen	**5/10**	918± 2	9631±69	**3/9**	684± 2	3011±56	**2/9**	1018±84	3544±18
Furosemide	**2/10**	3618±29	15244±48	**0/9**			**1/9**	3602±30	3602±30
Ibuprofen	**8/10**	550±33	106490±42	**8/9**	518±33	43653±54	**3/9**	550±33	3398±16
Indomethacin	**5/10**	240±17	8899±17	**2/9**	1470±53	2393± 5	**2/9**	20± 3	88± 3
Ketoprofen	**10/10**	260±41	14275±98	**5/9**	20± 4	160±13	**5/9**	47±10	21989±52
Naproxen	**2/10**	283± 2	3894±28	**0/9**			**0/9**		

Table 3.3 - (cont.) Pharmaceuticals and musks detected at the influent, effluent and secondary sludge of 5 different WWTPs in Portugal during the spring (23 May to 7 July) and autumn (2 to 25 October).

Compound	Influent Occurence	Influent minimum ng/L±SD	Influent maximum ng/L±SD	Effluent Occurrence	Effluent minimum ng/L±SD	Effluent maximum ng/L±SD	Secondary Sludge Occurrence	Secondary sludge minimum ng/g±SD	Secondary sludge maximum ng/g±SD
Antibiotics									
Amoxicillin	**4/10**	232± 5	5698±99	**2/9**	1097±35	4801±45	**2/9**	112± 5	166±28
Ampicillin	**3/10**	306± 2	4120±19	**1/9**	410± 5	410± 5	**0/9**		
Estrogens									
17α-Ethinyl -estradiol	**2/10**	103±12	106± 1	**0/9**			**1/9**	221± 8	221± 8
Estrone	**2/10**	189±27	2484±15	**1/9**	25± 2	25± 2	**2/9**	8±0.5	181±18
β-Estradiol	**1/10**	344±10	344±10	**0/9**			**0/9**		
Musks									
Galaxolide	**10/10**	55±17	11463±20	**10/10**	4± 1	889±61	**6/6**	119±63	22649±154
Tonalide	**10/10**	1±0.1	2933±51	**10/10**	1±0.1	225±20	**6/6**	11± 3	5968±47
Cashmeran	**10/10**	50± 2	7206±125	**10/10**	4±0.4	640±11	**5/6**	35±13	1865±108
Celestolide	**5/10**	5± 1	338± 2	**0/10**			**0/6**		

In this study, two sampling campaigns were performed for the 5 WWTPs analysed, in the spring and in autumn (Table 3.3). The results showed that 33 out of 59 pharmaceuticals were detected in the influents to the plants, at varying frequencies of occurrence. The concentrations detected were in the range of approximately 50 ng L^{-1} to 100 μg L^{-1}. The most dominant class of compounds present in the WWTPs were the non-steroidal anti-inflammatory drugs (NSAIDs), which appeared at significantly higher concentration levels than the other PhACs groups (Figure 3.1).

The total quantity of the major PhACs families present in the influent of most plants surveyed in this study (with the exception of Quinta da Bomba, see Figure 3.1) was found to be between 30 and 70 μg L^{-1}, and went up to 120 μg L^{-1} in one sample. In previous surveys covering a wide range of PhACs in other countries, the total PhAC concentration reported was approximately 23 μg L^{-1} in a Japanese study (Okuda *et al.*, 2008), 143 μg L^{-1} in a Spanish study (Rosal *et al.*, 2009) and 320 μg L^{-1} in the U.K. (Kasprzyk-Hordern *et al.*, 2009).

The most commonly detected and abundant NSAIDs found in the WWTP analysed were ibuprofen, ketoprofen, flurbiprofen, diclofenac and indomethacin. Caffeine was present in all plants and generally detected in high concentrations. Santos *et al.* (2009) also found ibuprofen, ketoprofen, and caffeine in the μg L^{-1} range of concentrations in WWTP influents of Seville, Spain, which is consistent with the present study. In addition to the NSAIDs, the antihypertensive (enalapril, captopril and furosemide) and lipid regulator groups were also occasionally found at μg L^{-1} levels, whereby clofibric acid represented

the sole lipid regulator detected. No readily observable pattern was found between the frequency of detection and quantity of PhACs measured in influent samples collected in the spring or autumn from the different plants (Figure 3.1).

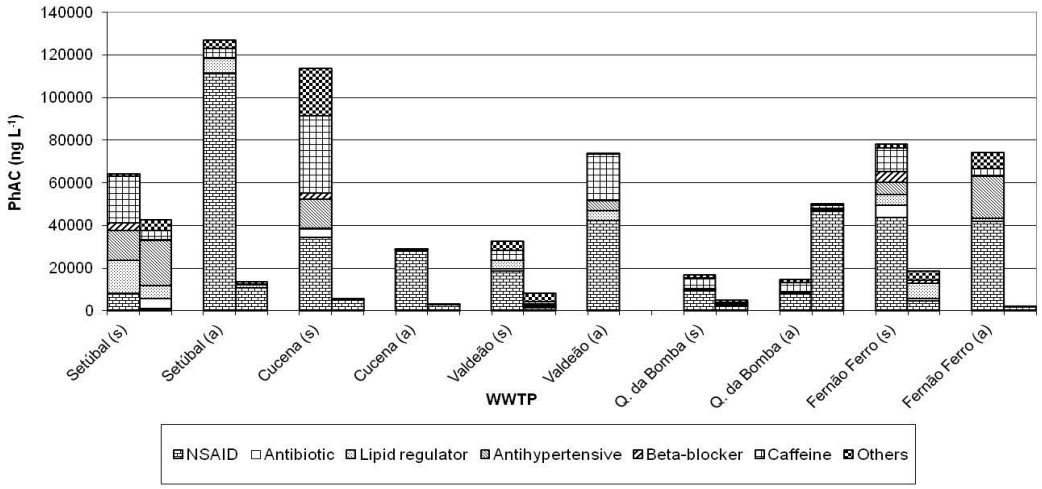


Figure 3.1- Occurrence of the most important PhAC families observed in the spring (s) and autumn (a) campaigns in the 5 studied WWTPs in the influent ('left' column of each pair) and effluent ('right' column of each pair).

Three of the musks analysed (galaxolide, tonalide and cashmeran) were found in each plant (Table 3.3), while celestolide also appeared punctually, and traseolide and phantolide were not detected. In this case, the results show that in the spring the concentrations are, in general, higher than in the autumn (Figure 3.2). The concentration of the musks ranged from 117 ng L^{-1} to 5.0 μg L^{-1}, going up to 21.6 μg L^{-1} in one sample. In literature studies, galaxolide and tonalide are generally the most frequently detected musks, as found in this study. Most previous reports indicate that the total musk concentrations are within a similar range for domestic/industrial wastewater. For example, in a study of 11 polycyclic musks in 6 WWTPs, Smyth *et al.* (2008) found them in a concentration range of 2-40 μg L^{-1} in the influent to the plants, while other literature studies report similar values or lower (Bester, 2004; Rosal *et al.*, 2009).

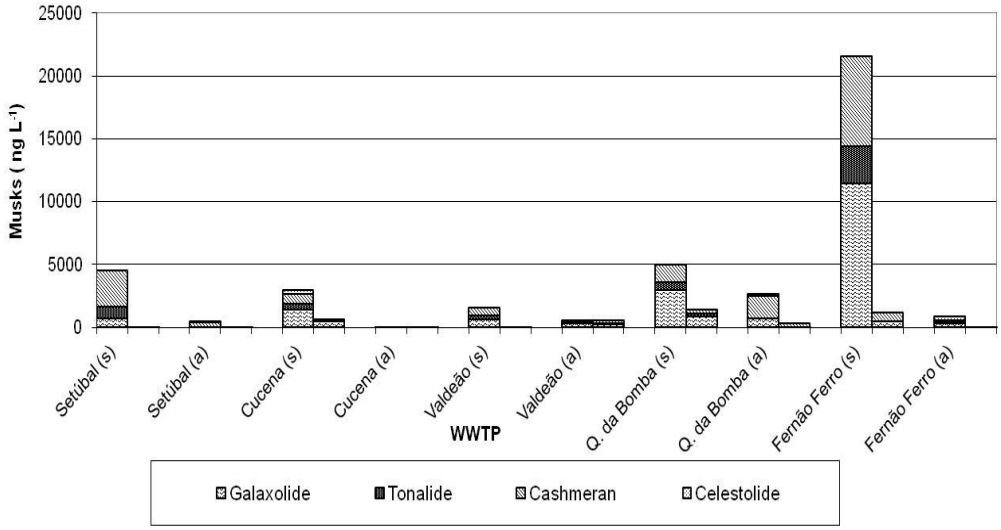


Figure 3.2 - Occurrence of the musks observed in the spring (s) and autumn (a) campaigns in the 5 studied WWTPs in the influent ('left' column) and effluent ('right' column).

3.3.3 Measurement of PPCPs in wastewater effluents and adsorption to the secondary sludge

As expected, the effluent concentrations of most PhACs were generally much lower than the influent concentrations, suggesting at least some degree of transformation via biological degradation, UV degradation or adsorption to the sludge (Table 3.3). Also, the diversity of compounds detected was substantially lower, as well as the frequency in their occurrence. It is noteworthy that clofibric acid and dimethylaminophenazone were present at a similar frequency and abundance in the influent and effluent of most plants (Table 3.3), suggesting that these compounds were difficult to degrade by the WWTPs. Clofibric acid has previously been found to have a very high persistence in the environment (Winkler *et al.*, 2001), which is consistent with the results of this study.

The range of total PhAC concentrations found in the effluent of the different plants varied between 2-50 μg L^{-1}. When compared with surveys of WWTP effluents in other countries, a similar range of total PhAC concentrations were reported. The total PhAC concentrations observed in the effluent in some other locations is as follows: in Japan, 2-10 μg L^{-1}; in Atlantic Canada, 2 μg L^{-1}; in Spain, 26 μg L^{-1}; and Italy, 3 μg L^{-1} (Zuccato *et al.*, 2006; Comeau *et al.*, 2008; Okuda *et al.*, 2008; Rosal *et al.*, 2009). In the U.K., an activated sludge effluent contained 43 μg L^{-1} of PhACs, while a trickling filter plant contained 93 μg L^{-1}, where the influent concentration to each plant was similar (Kasprzyk-Hordern *et al.*, 2009). In this study, no apparent differences were observed in the range of total effluent concentrations of the activated sludge plants (3-43 μg L^{-1}),

versus the trickling filters (2-50 μg L^{-1}). In addition, similarities were observed between the main families of compounds detected in literature surveys (i.e. NSAIDs, antibiotics, β-blockers, lipid regulators, analgesics and caffeine), though each geographical region generally displayed differences in the particular compounds that were most abundant.

In relation to the musks, galaxolide, tonalide and cashmeran were detected in the effluent of each plant, but at lower concentrations than in the influent (Table 3.3). Celestolide was not detected in the effluent of any of the WWTPs. The range of musk concentrations in the effluent were between 9 ng L^{-1} and 1.4 μg L^{-1}, from which 5 ng L^{-1} to 1.1 μg L^{-1} were either galaxolide or tonalide. Previous studies reporting galaxolide and tonalide concentration in WWTP effluents found on average 1.3 μg L^{-1} (Rosal *et al.*, 2009) or 4.8 μg L^{-1} (Smyth *et al.*, 2008), which is a comparable range to that found in Portuguese plants.

The high frequency of occurrence of musks in the sludge suggests that adsorption was one of the important removal mechanisms, which agrees well with previous studies (Bester, 2004; Ternes *et al.*, 2005). The total galaxolide and tonalide found in the sludge ranges from 0.130-28.6 μg g^{-1}, while Bester (2004) found 4.5 μg g^{-1} and Ternes *et al.* (2005) observed 2.3-8.5 μg g^{-1}. The high adsorption levels detected for the musks are due to their high hydrophobicity. Some pharmaceuticals were also found in the secondary sludge at high levels, however, they were less persistent since they were not detected as frequently in all of the plants tested. The most abundant PhACs in the sludge were diclofenac, ketoprofen, etofenamate, clofibric acid and hydroxyzine, which were present at levels above 10 μg g^{-1}.

3.4 Conclusions

The results of this study show that the adapted analytical methodology employed in this work was effective for monitoring pharmaceutical and personal care products in wastewater treatment plants. This method reduced the analytical effort necessary to cover a wide range of compounds with different natures, and still achieved good LOD and LOQ levels (with the exception of the estrogens), with high recoveries in influent wastewater. The total PhAC and musk concentrations found in this work were in a similar range as previously reported studies. The most abundant PhACs were the NSAIDs (particularly ibuprofen), while the antihypertensives (particularly enalapril), caffeine, and clofibric acid were also present in relatively high concentrations in the influent and effluent. Clofibric acid represented one of the few compounds present at a similar range of

concentrations in the influent and effluent of the plants, suggesting that little biodegradation and adsorption of this compound took place.

CHAPTER 4

ASSESSING THE DIURNAL VARIABILITY OF PHARMACEUTICAL AND PERSONAL CARE PRODUCTS IN A FULL-SCALE ACTIVATED SLUDGE PLANT

4. Assessing the diurnal variability of pharmaceutical and personal care products in a full-scale activated sludge plant

An intensive sampling campaign has been carried out in a municipal wastewater treatment plant (WWTP) to assess the dynamics of the influent pharmaceutical active compounds (PhACs) and musks. The mass loadings of these compounds in wastewater influents displayed contrasting diurnal variations depending on the compound. The musks and some groups of PhACs tended to follow a similar diurnal trend as compared to macropollutants, while the majority of PhACs followed either the opposite trend or no repeatable trend. The total musk loading to the WWTP was 0.74 ± 0.25 g d^{-1}, whereas the total PhACs mass loading was 84.7 ± 63.8 g d^{-1}. Unlike the PhACs, the musks displayed a high repeatability from one sampling day to the next. The range of PhAC loadings in the influent to WWTPs can vary several orders of magnitude from one day or week to the next, representing a challenge in obtaining data for steady-state modelling purposes.

4.1 Introduction

Pharmaceuticals and personal care products (PPCPs) are xenobiotics that can be detected in wastewater treatment plants (WWTPs) at the influent, effluent and in waste sludges (Ternes *et al.*, 1999; Kolpin *et al.*, 2002; Richardson *et al.*, 2005; Zuccato *et al.*, 2005; Ternes *et al.*, 2005; Jones *et al.*, 2007; Miége *et al.*, 2009; Sim *et al.*, 2010; Santos *et al.*, 2009). Many studies have been previously performed concerning the occurrence and fate of PPCPs in WWTPs from various countries. However, only few studies have attempted to assess the variability that can be expected in the diurnal PPCP loadings in the influent to WWTPs (Joss *et al.*, 2005; Göbel *et al.*, 2005; Weissbrodt *et al.*, 2009; Plósz *et al.*, 2010). Wastewater treatment plants are known to receive discharges that vary widely according to the time of day. Diurnal variations (in particular peak loads) are important to consider for successful WWTP design and operation, not only with respect to flow, but also the pollutant loading rates in order to achieve sufficient effluent quality. Further, peak pollutant loads can have toxic or inhibitory impacts on the WWTP sludge. The inhibition of heterotrophs and nitrifiers by pharmaceutical active compounds has previously been shown (Dokianakis *et al.*, 2004; Carucci *et al.*, 2006; Wang *et al.*, 2008) and can reduce treatment efficiency.

The typical dry weather diurnal variations of organic matter, ammonia, phosphate and wastewater flow consist of a relatively large morning peak, smaller variations throughout the day and low levels overnight (Tchobanoglaus and Burtan, 1995; Almeida *et al.*, 1999). It is still unknown if a similar-type pattern exists with respect to PPCP

concentrations, and if so, how repeatable this pattern is from one day to the next. Such information is important in order to incorporate PPCP compounds into WWTP models.

Thus far, the studies that have addressed the diurnal variability of PPCPs in the influent of WWTPs have generally observed lower overnight concentrations, consistent with the wastewater flow variation (Joss *et al.*, 2005; Göbel *et al.*, 2005; Weissbrodt *et al.*, 2009; Plósz *et al.*, 2010). Most of these studies collected the influent sample after primary clarification, dampening the effect of diurnal flow and PPCP loading variations. However, since the monitoring plan of WWTPs generally requires that pollutant characterisation is done at the sewer entrance of the plant (and treated effluent), it is desirable to understand quantitatively how raw influent loadings fluctuate diurnally, and indeed, how repeatable is this assessment from one day to the next in a WWTP.

In this study, 79 PPCPs, including 73 pharmaceutical active compounds (PhACs) of different families and 6 musk fragrances, have been monitored for characterising their diurnal variation in the raw influent prior to primary sedimentation. This analysis was performed along 2 days per week over 2 consecutive weeks at a municipal WWTP in Portugal in order to investigate the repeatability of the profiles. The goals of this study were to assess the diurnal variations of PPCPs in the influent, as well as the reproducibility of the mass loading of PPCPs detected during the different days and weeks analysed. To the best of our knowledge, this is the first study to investigate the diurnal variations of this broad range of PPCPs in the raw influent of municipal wastewater.

4.2 Materials and methods

4.2.1 Influent WWTP sampling and sample preservation

The influent samples were collected at the WWTP of Fernão Ferro (Seixal, Portugal), which has a design capacity for 32 700 population equivalents and treats 2790 m^3 d^{-1} of domestic municipal wastewater. Two consecutive 48 hour periods over two successive weeks have been monitored during dry-weather conditions. Samples of the influent (1L) were collected on Monday from 10 a.m. until Wednesday 10 a.m. (a sample was collected every 2 hours) using a refrigerated auto-sampler. The samples were transported to the laboratory in a refrigerated isothermal container and immediately extracted and stored at -20 ^{o}C until the analysis was performed. The samples were prepared for analysis of two different classes of pharmaceutical compounds (acidic and neutral) and of polycyclic musk fragrances according to the procedures described below.

4.2.2 Chemicals and reagents

HPLC-grade acetonitrile, methanol, and formic acid were purchased from Panreac (Portugal). Ultrapure water was obtained from a MilliQ water purification system (Millipore, Bedford, MA., USA). All of the pharmaceutical active compound (PhAC) standards were purchased from Sigma-Aldrich (Steinheim, Germany), while the musks were purchased from LGC-Promochem (Spain). Stock solutions (1 mg mL^{-1}) of each PhAC or musk were prepared and diluted in methanol or *n*-hexane, respectively. All samples were analysed in triplicate.

4.2.3 Extraction and analysis

4.2.3.1 Acidic and neutral pharmaceutical compounds

Solid phase extraction (SPE) was used for clean-up and concentration of the samples, as detailed in Salgado *et al.* (2010). All wastewater samples were filtered through glass fiber filters (GF 6, <1 μm pore diameter, Whatman, England. 350 mL of filtered wastewater were spiked with an internal standard (meclofenamic acid) to a final concentration of 100 μg L^{-1}. SPE was carried out on the filtered and spiked wastewater samples with Waters Oasis HLB cartridges (60 mg, 30 μm, Waters, Eschborn, Germany) for the acidic pharmaceutical compounds, while Waters RP-C18 cartridges (500 mg, 50 μm, Waters, Milfort, U.S.) were used for the neutral compounds. The pH of the samples was adjusted to 2 for the acidic compounds and 7 for the neutral compounds. The wastewater samples were passed through the cartridges at a flow rate of approximately 20 mL min^{-1}. The solid-phase material was then dried through a continuous nitrogen stream for 1 h and then the analytes were eluted four times with 1 mL methanol (total 4 mL). The extracts were evaporated to 1 mL by a gentle nitrogen stream.

The acidic and neutral PhACs extracts were analysed through high performance liquid chromatography coupled to mass spectrometry (HPLC-MS). For HPLC, a reverse-phase column was employed (LiChroCART 250-4 Purospher Star RP18 endcapped, 5 μm column, Merck) using a degassed mobile phase of water/formic acid 0.1% (A) and acetonitrile (B). The following binary gradient was used: 2 min, 15% B at 0.6 mL min^{-1}; 20 min, 100 % B at 0.6 mL min^{-1}; 25 min, 100 % B 1.0 mL min^{-1}; 27 min, 15 % B at 1.0 mL min^{-1} and 35 min, 15 % B at 0.6 mL min^{-1}. The HPLC system (Waters) was coupled with a pump and controller (Waters 600), an in-line degasser (X-Act-4 channels, Jour Research), an auto-sampler (Waters 717 plus), a photodiode array detector (DAD, Waters

996, used at 200-400 nm) and a quadropole VG Platform (Micromass, UK Ltd) spectrometer equipped with an electrospray ionisation (ESI) source operating in positive mode. An accurate splitter (split ratio of 1:10) was used between the HPLC column and the mass spectrometer. Capillary temperature was kept between 100 ºC and 120 ºC, using a scanning cone voltage from 35 to 100 V and capillary voltage of 3.5 kV. Nitrogen was used as drying and nebulising gas at 300 mL·min^{-1} and 10 mL min^{-1}, respectively. Spectra mass/charge range used was 100-450 amu with a MassLinx^{TM} software data acquisition system.

4.2.3.2 Polycyclic musk fragrances

The extraction of the musks was carried out by headspace solid-phase microextraction (SPME) using fibres coated with 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB, Supelco, Spain). Two grams of wastewater sample were combined with 0.5 g NaCl and spiked with Mirex (internal standard) to a concentration of 100 µg L^{-1} in 8 mL glass vials with magnetic stirring. The PDMS/DVB fibre was exposed to the sample headspace in the sealed glass vial for 15 min at 90 ºC. The fibre was inserted into the injection port of the GC-MS during 3 min, where the volatile compounds were desorbed.

Analysis was performed using a Hewlett-Packard 5890 gas chromatographer fitted with a QMD1000 Carlo Erba mass spectrometric detector (GC-MS). The injection port was operated in splitless mode. A DB-5MS, 5% phenyl and 95% dimethylpolysiloxane fused-silica capillary column (30 m x 0.32 mm i.d., 0.25 µm film thickness, Agilent-J&W Scientific, Spain) was used, with helium as carrier gas at a flow rate of 1.5 mL min^{-1}. The injection port temperature was 250 ºC. The ion source and the transference line were kept at 200 and 310 ºC respectively. The oven temperature was maintained at 60 ºC for 3 min, raised to 250 ºC at 10 ºC·min^{-1}, and then to 310 ºC at 20 ºC·min^{-1}, where it was held for 13 min. The MS spectra were obtained with electron energy 70 eV, mass range *m/z* 50-500 amu and using MassLab^{TM} software (Micromass).

4.2.4 Determination of recovery, LOD and LOQ

Recoveries, limits of detection (LOD) and limits of quantification (LOQ) were determined as detailed in Salgado *et al.* (2010). In brief, for determination of the recovery percentages, samples were spiked with standards of each of the PPCPs studied (100 µg L^{-1} in methanol) and an internal standard (meclofenamic acid, also at 100 µg L^{-1}). After

homogenisation for 30 min, the extraction and analysis was performed as detailed above. Relative recoveries were determined relative to MilliQ water.

The LOD for each of the PPCPs were calculated by the formula 3·SD/m, where SD is the standard deviation of the lowest signal/noise ratio of the analyte and m is the slope of the calibration curve. The LOQ were calculated as 10·SD/m. Ten blanks were analysed by LC/MS (with methanol) and GC-MS (with *n*-hexane) to determine the lowest signal/noise ratio of each analyte.

4.2.5 Statistical analysis

Contingency tables were used to determine if the results obtained in this study with respect to the frequency of detection of the PhACs was statistically significant from one day to the next. The entries of the contingency tables for each compound consisted of the number of detection and non-detection events in each of the four sampling days (12 sampling events per day). The corresponding χ^2-test for each compound was performed and the results are presented in Table 4.1. Further detail regarding this analysis is provided in appendix I.

4.3 Results and Discussion

4.3.1 Most frequently detected compounds

The number of PhACs detected in each sample over the four days (2 consecutive Mondays and Tuesdays) of the campaign is plotted in Figure 4.1. It can be observed that a small, relatively constant number of compounds were found throughout the first day (5±1), which increased to 23 compounds at 10am of Day 2. After 4pm, the number of compounds detected decreased steadily to 10-12 until midnight, where afterwards it decreased further overnight (7-8 compounds). In the second week, a high number of compounds were detected throughout Day 3 (21±3) and Day 4 (17±4). The difference in the number of compounds observed between the 1st week and the 2nd week illustrates the high variability of PhACs, and the difficulty in obtaining a repeatable assessment of the compounds being discharged to the WWTP.

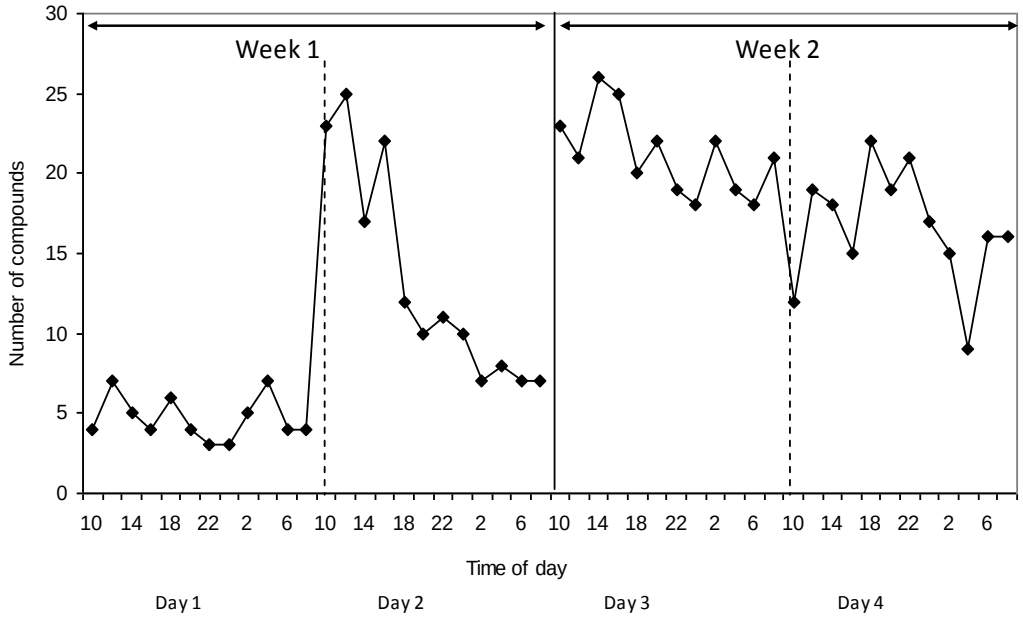


Figure 4.1 - Number of pharmaceutical compounds detected throughout the sampling campaign of 4 days: 2 consecutive Mondays and Tuesdays (mean and standard deviation: 1st week: Monday 5±1; Tuesday 13±7; 2nd week: Monday 21±3; Tuesday 17±4).

While different compounds were detected at different times of the day, some compounds were detected more frequently than others. The most frequently detected compounds are shown in Table 4.1, while those infrequently detected are listed in Table 4.2 and those never detected are listed in Table 4.3. From Table 4.1, it can be observed that diclofenac, ibuprofen, ketoprofen and clofibric acid were frequently detected at the WWTP influent during each sampling day. Etofenamate, clorazepate, hydroxyzine, indapamide, enalapril, captopril, atenolol, ampicillin, fluoxetine and estrone were all commonly detected in the second week of sampling, but were either not detected or were seldomly detected during the first week. Paroxetine was commonly detected on the first Tuesday (Day 2) and second Monday (Day 3) of the campaign, but not detected on the other two sampling days. Interestingly, 5 of the 6 musks studied (galaxolide, tonalide, cashmeran, celestolide and traseolide) were present in every sample analysed during the campaign. It should also be noted that the differences observed in the frequency of detection from one day to another were statistically significant for 14 of the 15 PhACs analysed (Table 4.1). This reflects the large differences in the PhACs composition of the wastewater observed between each sampling day.

Table 4.1 - Range of concentration, mean concentration, and frequency of detection of the 20 most frequently detected PhACs and musks analysed. Limits of detection and quantification, as well as the relative recovery are also shown.

Compound	Human effect	LOD (ng L^{-1}) (*n*=10)	LOQ (ng L^{-1}) (*n*=10)	Relative recovery WW (%) (*n*=3)	Week 1						Week 2					
					Monday			Tuesday			Monday			Tuesday		
					Range (ng L^{-1})	Mean (ng L^{-1})	Freq of Detection	Range (ng L^{-1})	Mean (ng L^{-1})	Freq of Detection	Range (ng L^{-1})	Mean (ng.L^{-1})	Freq of Detection	Range (ng.L^{-1})	Mean (ng.L^{-1})	Freq of Detection
Diclofenac	NSAID	7	24	65 ± 6	*n.d.*-26598	10898	11/12	11299-64479	38674	12/12	1257-16963	4534	12/12	*n.d.*-11742	5456	11/12
Etofenamate ***	NSAID	20	67	89 ± 4	*n.d.*	*n.d.*	0/12	*n.d.*-40168	7444	8/12	*n.d.*-3164	1541	11/12	*n.d.*-2979	1507	10/12
Ibuprofen *	NSAID	14	46	70 ± 2	*n.d.*-52201	9102	7/12	235-13905	4476	12/12	358-1795	1059	12/12	*n.d.*-1298	562	9/12
Ketoprofen*	NSAID	21	69	102 ± 13	1106-29496	28269	12/12	47-104114	9255	12/12	*n.d.*-211	71	8/12	*n.d.*-250	85	8/12
Clorazepate***	Anxiolytic	17	57	92 ± 2	*n.d.*	*n.d.*	0/12	*n.d.*-3332	416	5/12	*n.d.*-427	249	11/12	*n.d.*-463	316	11/12
Hydroxyzine***	Antihistamine	18	60	73 ± 5	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	162-1168	470	12/12	*n.d.*-570	216	8/12
Indapamide***	Antihypertensive	6	18	86 ± 1	*n.d*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*-15386	2668	8/12	*n.d.*-6737	3476	11/12
Enalapril***	Antihypertensive	8	28	88 ± 3	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*-4162	696	8/12	*n.d.*-6244	1532	10/12
Captopril***	Antihypertensive	5	15	66 ± 10	*n.d.*-978	82	1/12	*n.d.*-509	96	3/12	*n.d.*-4231	784	9/12	*n.d.*-2267	1015	11/12
Atenolol***	β-Blocker	3	10	115 ± 6	*n.d.*-4341	362	1/12	*n.d.*-427	36	1/12	141-944	476	12/12	77-1474	815	12/12
Clofibric acid**	Lipid mod. agent	15	50	96 ± 12	*n.d.*-41428	8461	6/12	137-1602	602	12/12	116-722	276	12/12	*n.d.*-1723	442	7/12
Estrone***	Estrogen	18	60	104±12	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*-177	82	12/12	*n.d.*-73	28	7/12
Ampicillin***	Antibiotic	3	11	68 ± 3	*n.d.*	*n.d.*	0/12	*n.d.*-126	11	1/12	*n.d.*-252	157	10/12	*n.d.*-240	100	7/12
Paroxetine ***	Antidepressant	27	89	86 ± 12	*n.d.*	*n.d.*	0/12	*n.d.*-39732	9676	11/12	*n.d.*-1927	251	10/12	*n.d*	*n.d*	0/12
Fluoxetine***	Antidepressant	17	57	41 ± 3	*n.d.*	*n.d.*	0/12	*n.d.*-3465	359	4/12	*n.d.*-971	219	6/12	*n.d.*-3090	946	10/12
Galaxolide	Musk	1	1	94 ± 2	119-1698	478	12/12	152-526	396	12/12	50-2780	465	11/11	53-617	204	11/11
Tonalide	Musk	1	1	82 ± 3	57-270	124	12/12	85-421	178	12/12	23-816	140	11/11	18-190	72	11/11
Cashmeran	Musk	1	1	83 ± 3	97-1275	599	12/12	127-2477	1362	12/12	93-4040	1447	11/11	66-2151	698	11/11
Celestolide	Musk	2	2	85 ± 4	6-428	216	12/12	288-1442	470	12/12	30-885	149	11/11	19-308	101	11/11
Traseolide	Musk	2	2	85 ± 4	35-165	87	12/12	125-676	207	12/12	7-309	53	11/11	10-79	37	11/11

n.d. – not detected

Differences in frequency of detection (occurrences per samples analysed) between sampling days are: * statistically significant ($p < 0.05$), ** very significant ($p < 0.01$), *** highly significant ($p < 0.001$).

Table 4.2 - Range of concentration, mean concentration, and frequency of detection of infrequently detected PhACs and musks. Limits of detection and quantification are indicated where available.

Compound	Human effect	LOD (ng L^{-1})	LOQ (ng L^{-1})	Monday			Tuesday			Monday			Tuesday		
				Range (ng L^{-1})	Mean (ng L^{-1})	Freq of Detection	Range (ng L^{-1})	Mean (ng L^{-1})	Freq of Detection	Range (ng L^{-1})	Mean (ng L^{-1})	Freq of Detection	Range (ng L^{-1})	Mean (ng L^{-1})	Freq of Detection
Allopurinol	Gout treatment	22	73	*n.d.*-8234	1498	3/12	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12
Dimethyl-phenazone	Analgesic; antiinflamatory	29	95	*n.d.*	*n.d.*	0/12	*n.d.*-221	18	1/12	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12
Paracetamol	Analgesic	3	9	*n.d.*	*n.d.*	0/12	*n.d.*-342	29	1/12	*n.d.*-91	8	1/12	*n.d.*-266	22	1/12
Codeine	Analgesic	-	-	*n.d.*	*n.d.*	0/12	*n.d.*-351.2	55	3/12	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12
Acetylsalicylic acid	Analgesic	-	-	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*-172	28	2/12	*n.d.*-404	88	3/12
Caffeine	CSN stimulant	27	91	*n.d.*-11751	1047	2/12	*n.d.*-2380	287	2/12	*n.d.*-4684	1205	10/12	*n.d.*-9175	1389	3/12
Omeprazole	Proton pump inhibitor	-	-	*n.d.*	*n.d.*	0/12	*n.d.*-148	21	3/12	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12
Domperidone	Antidopaminergic	3	9	*n.d.*-9145	1108	2/12	*n.d.*-77349	19150	12/12	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12
Propranolol	β-Blocker	4	15	*n.d.*	*n.d.*	0/12	*n.d.*-23446	2840	4/12	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12
Ramipril	Congestive heart failure	9	31	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*-2265	227	3/12	*n.d.*	*n.d.*	0/12
Betamethasone	Corticosteroid (SAID)	6	20	*n.d.*-61	5	1/12	*n.d.*-64	10	2/12	*n.d.*-343	145	7/12	*n.d.*	*n.d.*	0/12
Carbamazepine	Antiepileptic	2	7	*n.d.*	*n.d.*	0/12	*n.d.*-1950	461	4/12	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12
Nimesulide	NSAID	14	46	*n.d.*	*n.d.*	0/12	*n.d.*-671	70	3/12	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12
Naproxen	NSAID	18	59	*n.d.*	*n.d.*	0/12	*n.d.*-244	37	4/12	*n.d.*	*n.d.*	0/12	*n.d.*-78	16	3/12
Flurbiprofen	NSAID	18	58	*n.d.*-15480	4564	5/12	*n.d.*-1777	299	4/12	*n.d.*-18608	3645	5/12	*n.d.*-5385	752	6/12
Indomethacin	NSAID	7	23	*n.d.*	*n.d.*	0/12	*n.d.*-1639	254	5/12	*n.d.*-686	99	6/12	*n.d.*	*n.d.*	0/12
Fentiazac	NSAID	-	-	*n.d.*	*n.d.*	0/12	*n.d.*-317	-	1/12	*n.d.*-208	25	2/12	*n.d.*	*n.d.*	0/12
β-Estradiol	Estrogen	4	12	*n.d.*	*n.d.*	0/12	*n.d.*-937	97	4/12	*n.d.*	*n.d.*	0/12	*n.d.*-750	95	4/12
17α-Ethinylestradiol	Estrogen	21	69	*n.d.*	*n.d.*	0/12	*n.d.*-76	7	2/12	*n.d.*-80	39	10/12	*n.d.*	*n.d.*	0/12

n.d. – not detected

Table 4.2 - (cont.) Range of concentration, mean concentration, and frequency of detection of infrequently detected PhACs and musks. Limits of detection and quantification are indicated where available.

Compound	Human effect	LOD (ng L^{-1})	LOQ (ng L^{-1})	Monday			Tuesday			Monday			Tuesday		
				Range (ng L^{-1})	Mean (ng L^{-1})	Freq of Detection	Range (ng L^{-1})	Mean (ng L^{-1})	Freq of Detection	Range (ng L^{-1})	Mean (ng L^{-1})	Freq of Detection	Range (ng L^{-1})	Mean (ng L^{-1})	Freq of Detection
Escitalopram	Antidepressant	14	47	*n.d.*-32228	3506	2/12	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*-1104	381	8/12
Salbutamol	β2-Adrenergic receptor antagonist	11	36	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*-1301	317	4/12
Budesonide	Corticosteroid (asthma)	21	69	*n.d.*	*n.d.*	0/12	*n.d.*-12302	1051	2/12	*n.d.*-191	16	1/12	*n.d.*-51	4	1/12
Fluticasone	Glucocorticosteroid (asthma)	25	85	*n.d.*	*n.d.*	0/12	*n.d.*-7809	658	2/12	*n.d.*-569	67	4/12	*n.d.*-96	23	5/12
Tramadol	Opioid centrally action	20	67	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*-1130	94	1/12
Furosemide	Loop diuretic	19	63	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*-801	116	2/12	*n.d.*	*n.d.*	0/12
Alprazolam	Anxiolytic, tranquilizer	-	-	*n.d.*	*n.d.*	0/12	*n.d.*-4705	548	6/12	*n.d.*-89	12	2/12	*n.d.*-153	66	9/12
Oxazepam	Anxiolytic	-	-	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d*-36	-	1/12	*n.d.*-155	22	2/12
Bromazepam	Anxiolytic, tranquilizer	-	-	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*-36	5	2/12	*n.d.*	*n.d.*	0/12
Amoxicillin	Antibiotic	13	43	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*-569	252	9/12	*n.d.*	*n.d.*	0/12
Azithromycin	Antibiotic	3	11	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*-210	97	8/12
Ciprofloxacin	Antibiotic	1	3	*n.d.*	*n.d.*	0/12	*n.d.*-22074	1840	1/12	*n.d.*	*n.d.*	0/12	*n.d.*-15397	3985	4/12
Telmitarsen	Angiotensive; hypertension	-	-	*n.d.*	*n.d.*	0/12	*n.d.*-20	3	2/12	*n.d.*-187	18	2/12	*n.d.*	*n.d.*	0/12
Tiaprofencic acid	NSAID	-	-	*n.d.*	*n.d.*	0/12	*n.d.*-1175	131	3/12	*n.d.*-914	303	9/12	*n.d.*	*n.d.*	0/12
Lovastatin	Antideslipidemic	-	-	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*-251	145	10/12	*n.d.*	*n.d.*	0/12
Clofibrate ethyl	Lipid modifying agent	-	-	*n.d.*-15568	1398	3/12	*n.d.*-917	111	6/12	*n.d.*-56	20	6/12	*n.d.*-44	11	4/12
Salicylic acid	Analgesic	-	-	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*-2780	561	5/12	*n.d.*	*n.d.*	0/12
Penincillin G	Antibiotic	14	47	*n.d.*	*n.d.*	0/12	*n.d.*	*n.d.*	0/12	*n.d.*-127	11	1/12	*n.d.*	*n.d.*	0/12

n.d. – not detected

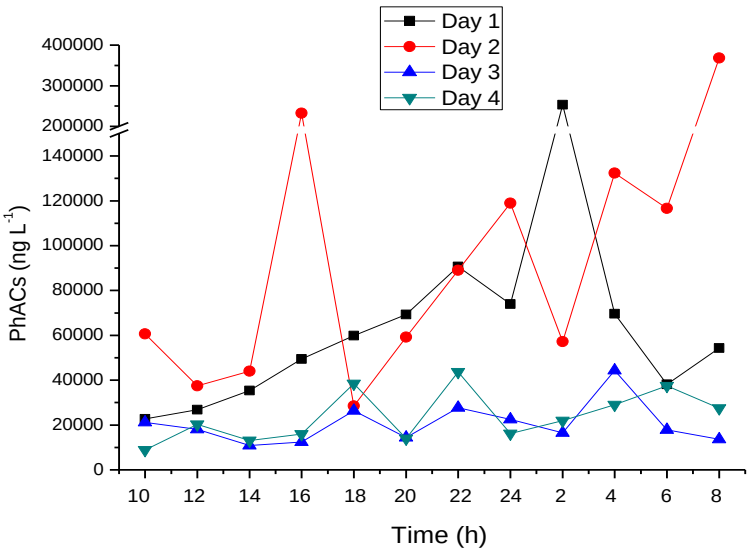
Table 4.3 - PhACs and musks that were never detected. Limits of detection and quantification are indicated when available.

Compound	Human effect	LOD (ng L^{-1})	LOQ (ng L^{-1})	Compound	Human effect	LOD (ng L^{-1})	LOQ (ng L^{-1})
Sertraline	Antidepressant	-	-	Reserpine	Antiadrenergic agent	8	28
Digoxin	Cardiac glycoside	25	83	Warfarin	Anticoagulant	-	-
Diazepam	Anxiolytic, Tranquilizer	-	-	Progesterone	Steroid contraceptive hormone	2	6
Diltiazem	Hypertensive, calcium blocker	6	18	Ecstasy (MDMA)	Psychoactive drug	18	61
Glibenclamide	Diabetes type II treatment	6	20	Tetrahydro-cannabinol	Psychoactive drug	17	58
Latanoprost	Ocular hypertension (glaucoma)	-	-	Triprolidine	Antihistaminic	-	-
Lorazepam	Anxiolytic	-	-	Zolpiden	Insomnia treatment	-	-
Nifedipine	Calcium blocker	19	62	Mexazolam	Anxiolytic, tranquilizer	-	-
Phenazone	NSAID	7	22	Valerian	Anxiolytic, tranquilizer	-	-
Piroxicam	NSAID	1	4	Mirtazepine	Antidepressant	-	-
Ranitidine	Histamine H2 receptor antagonist	-	-	Phantolide	Musk	1	1

4.3.2 Diurnal variations of the PhACs and musks in the WWTP

The diurnal variations of the total concentration of PhACs and musks detected in the filtered influent are shown in Figures 4.2a and 4.2b, respectively. In general, the total concentration of musks tended to follow a trend where higher concentrations were observed during the day and low concentrations were observed at night (with the exception of 2 outliers, the 4 a.m. sample on Day 3 and the 24 h sample on Day 2). Further, this trend was repeatable between the 4 sampling days, and the total concentration of musks measured was in a similar range in each case. However, the PhACs profile observed throughout each day was less repeatable as compared to the musks. On Days 1 and 2 (Monday and Tuesday from the first week of sampling), there appeared to be a higher PhAC concentration in the evening or night as compared to during the day. On Days 3 and 4 (Monday and Tuesday from the second week of sampling), there was a lower total concentration of PhACs compounds throughout the day with much smaller fluctuations. The reason for this difference is unclear, since the sampling strategy and weather conditions were consistent during each sampling day.

(a) PhACs



(b) Musks

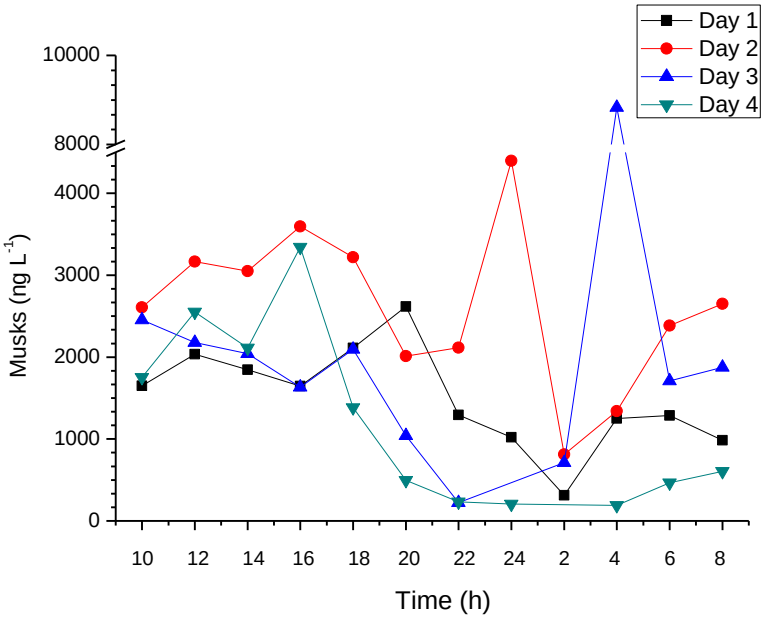


Figure 4.2 - Diurnal variations of the total concentration of PhACs (a) and the total concentration of musks (b) in the influent to the WWTP during each day of the campaign.

Figure 4.3 shows the relative contribution of the main PhAC (a) and musk (b) compounds towards the total concentration detected. It can be observed that diclofenac and ketoprofen were the most abundant of the analysed compounds, and usually responsible for the peak concentrations that were occasionally observed. Throughout the four sampling days analysed, diclofenac comprised an average of 40 ± 24 % of the total PhACs concentration and was regularly present in relatively high levels. This correlates well with sales data from the official Portuguese database (INFARMED, 2005), where diclofenac showed the highest sales when compared to the other compounds detected in this study. Ketoprofen

was present in high abundance more sporadically than diclofenac, comprising 36 ± 24 % of the PhAC concentration on Day 1, but only 1 ± 1 % on the other 3 days, excluding the 8 a.m. sample on Day 2 (36%). Overall, NSAIDs (such as diclofenac and ketoprofen) were the family of PhACs detected in highest abundance (55 ± 21 %), which is consistent with findings from some literature studies (Comeau *et al.*, 2008; Lin *et al.*, 2009; Miége *et al.*, 2009). Cashmeran was the dominant musk detected, forming on average 52 ± 18 % of the total musk concentration, while galaxolide was the second most abundant musk (22 ± 10 %), with both compounds consistently present in relatively high abundance.

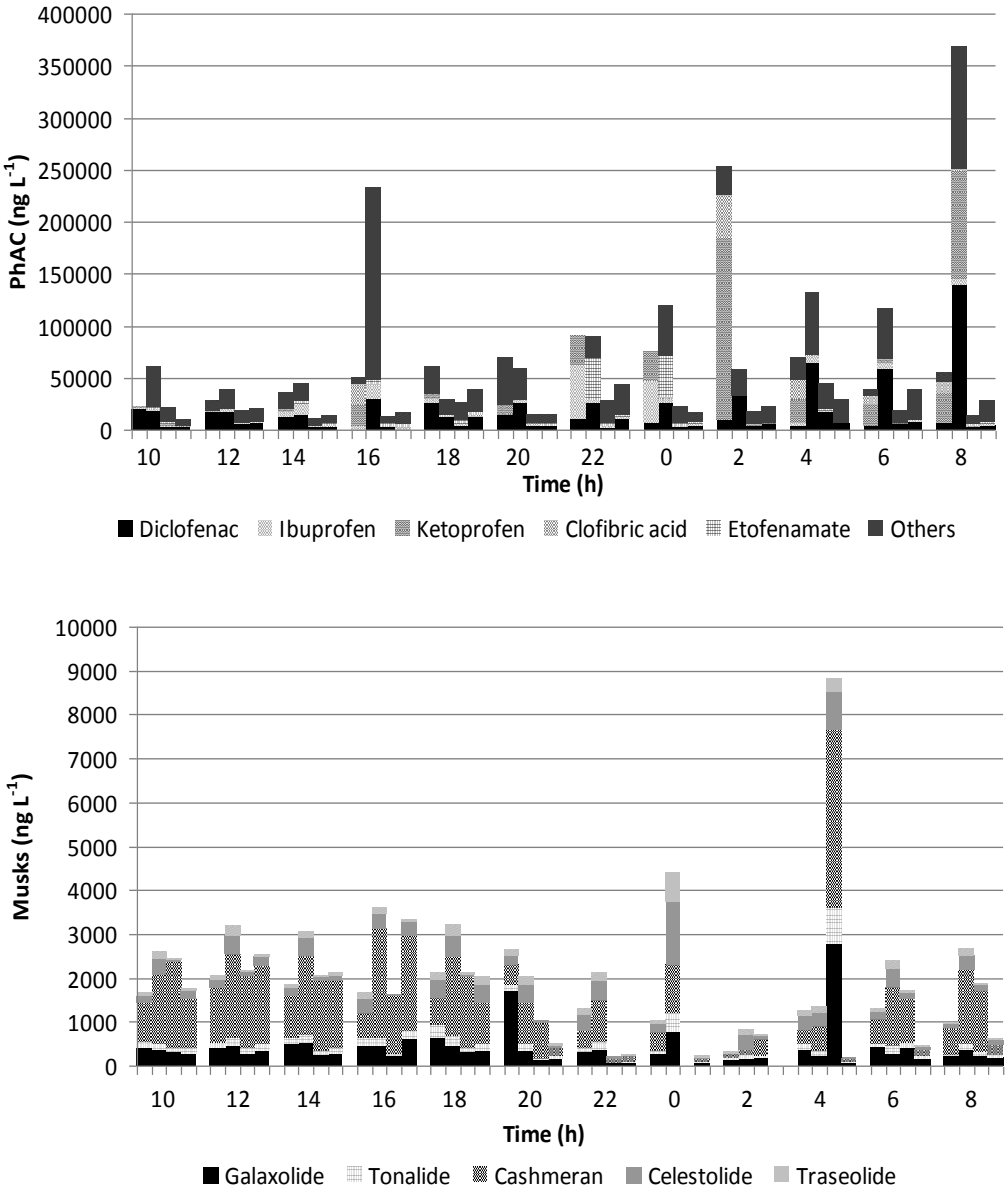


Figure 4.3 - Mean concentrations of the most frequently detected PhACs and musks throughout each sampling day (Days 1, 2, 3 and 4 are plotted for each 2 h-sample).

While a small number of PhACs generally constituted the bulk of the total PhAC concentration, occasionally some compounds that were only rarely detected appeared at high levels. One example of

this happened at 16 h on Day 2, where omeprazole was primarily responsible for the PhAC peak load observed at this time (67% of the total PhAC concentration).

Table 4.4 shows the diurnal variations in the influent throughout the periods of 8-16 h (day), 16-24h (evening) and 0-8 h (night). It can be observed that the total PhACs did not display a particular trend, while the musks were more abundant during the day. Also, the overnight mass loadings were generally quite low for the musks, with the exception of a peak load at 4 a.m. on Day 3. This pattern is similar to the wastewater flow. The WWTP influent flow rate (Table 4.4) shows that the wastewater flow was almost constant during the day, however, a notable decrease in flow was observed at night. Nevertheless, the total PhACs mass flow does not follow this pattern.

Table 4.4 - Diurnal variations of total pharmaceuticals and musks during the periods of 8-16 h, 16-24 h and 0-8 h for each day of the campaign.

		% of Total Mass Flow		
	Time	**8-16 h**	**16-24 h**	**0-8 h**
PhACs	Day 1	19	40	41
	Day 2	33	25	42
	Day 3	29	41	30
	Day 4	23	44	33
	Average	**26±6**	**38±8**	**36±6**
Musks	Day 1	43	41	16
	Day 2	43	39	17
	Day 3	38	20	42
	Day 4	74	17	9
	Average	**50±16**	**29±13**	**21±14**
Flow	$m^3 h^{-1}$	147±11	143±18	103±27

Figure 4.4 shows the relative contribution of the 15 most frequently detected PhACs throughout the sampling days. Eight of these 15 compounds average less than 25% of their total mass flow during the night period, including ibuprofen, etofenamate, clorazepate, atenolol, captopril, ampicillin, estrone and hydroxyzine. Etofenamate presented the lowest average mass flow of the PhACs at night (9 ± 8%), which could be related to the fact that this compound is administered as a gel, lotion or cream. This is similar to the musk compounds, and correspondingly, etofenamate appears to follow the same diurnal

pattern as most musks. Four compounds (ketoprofen, indapamide, paroxetine, enalapril) averaged > 50% of the total mass flow during the night period, thus displaying the opposite diurnal profile. The other three compounds (diclofenac, clofibric acid and fluoxetine) did not display any repeatable diurnal trend. The differences observed between the diurnal patterns of these PhACs likely reflect the varying frequencies of administration by consumers. Indeed, PhACs are administrated for specific medical reasons and the demand for these compounds is highly variable with time, as opposed to musks, which are more often used as part of routine hygiene habits.

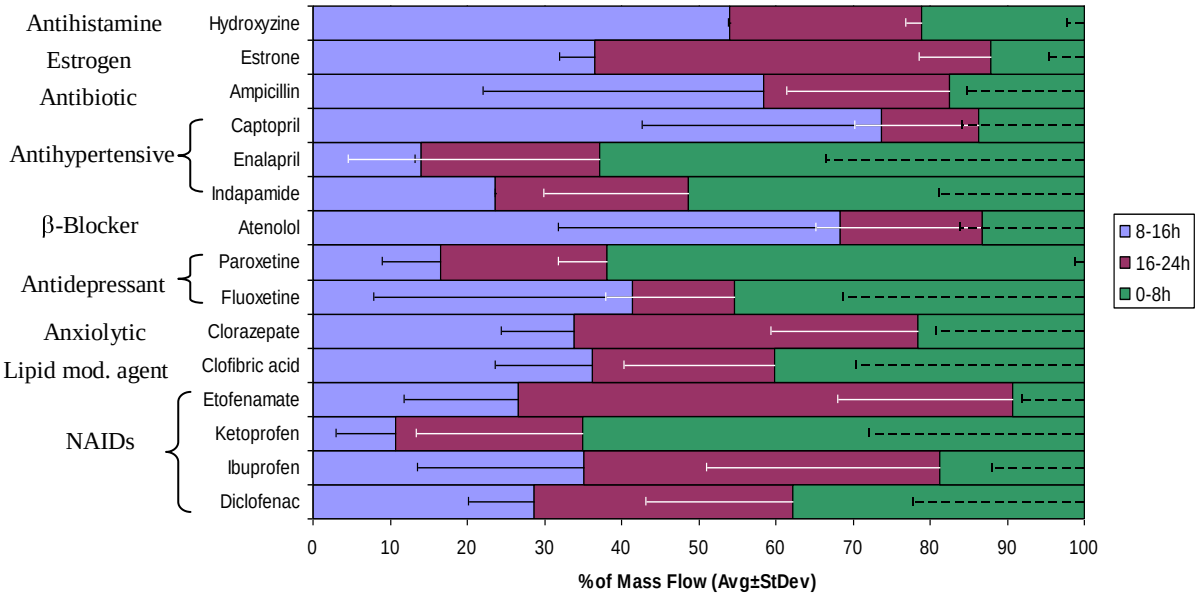


Figure 4.4 - Average percentage of PhAC mass flow that were detected during the periods of 8-16 h, 16-24 h and 0-8 h, for different families of the most frequently detected PhACs. Error bars indicate the standard deviation found for the 4 sampling days.

4.3.3 Mean and peak concentration of the PhACs and musks

The variability of the mean concentration of the 20 most commonly detected PhACs and musks for the 4 days of the campaign is shown in Figure 4.5. It can be observed that the 5 musks presented a relatively small variability in the mean concentration among the different days of the campaign as compared to the PhACs. The total musk loading to the WWTP was 0.74 ± 0.25 g d^{-1}, showing a 34 % relative standard deviation. By comparison, the total PhAC mass loading was 84.7 ± 63.8 g d^{-1}, a relative standard deviation of 75 %. Of the 5 PhACs that were detected each day of the campaign (diclofenac, ibuprofen, ketoprofen, clofibric acid and atenolol) the mean concentration varied between 1-3 orders of magnitude. Nine of the remaining 10 PhACs shown in Figure 4.5 were detected in both days of the second week, showing generally consistent mean concentrations for each compound on these days. Eight of these nine compounds were not detected during Days 1 and 2, thus, the loading of PhACs changed significantly for this WWTP from one week to the next. This implies that frequent

sampling campaigns will be required over time to obtain a representative description of the PhAC loading to the WWTP for e.g. modelling purposes.

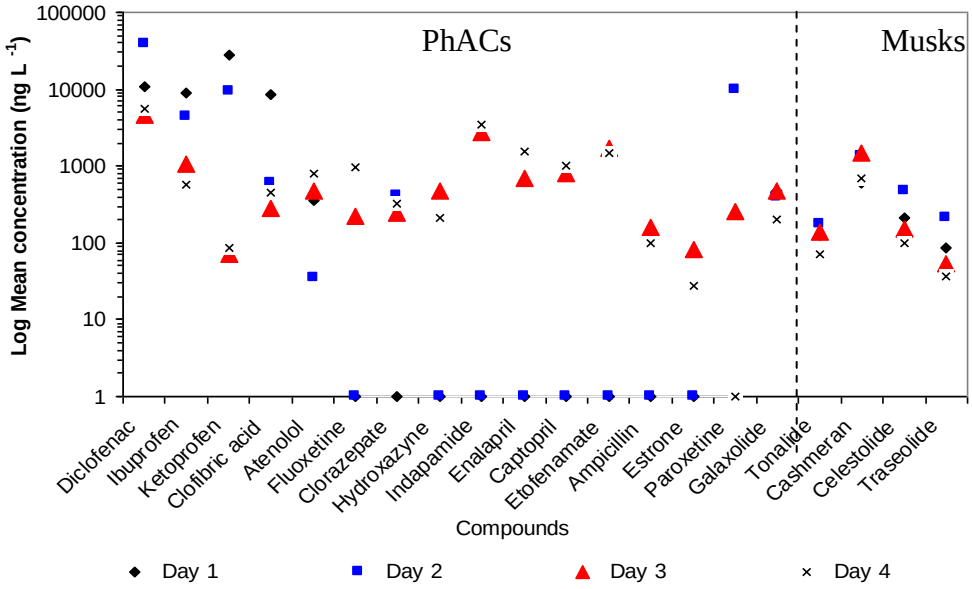


Figure 4.5 - Mean concentrations of the most frequently detected PhACs and musks during each sampling day of the campaign.

In contrast, relatively little variability was observed for the musk compounds detected in this study. The differences in the mean concentration from each day were always far less than one order of magnitude apart. The occurrence of musks was in the same concentration range or lower than the PhACs (Figure 4.5), showing that the higher repeatability observed with these compounds was not due to higher abundance. Rather, the fact that these compounds were ubiquitously present in the influent is likely reflective of their more regular and widespread usage than PhACs. This implies that obtaining a representative description of the musk compounds entering the WWTP is a far simpler task as compared to the PhACs.

The ratio of the maximum concentration detected to the mean concentration (max/mean) provides an indication of the occurrence of peak loads as well as their relative magnitude. Figure 4.6 shows the ratio of max/mean for the top 20 most frequently detected PhACs and musks. In general, the max/mean values are more variable for the PhACs as compared to the musks. For the musks, the max/mean was between 2 and 4 for three of the four sampling days, while a value above 6 was observed for most musks on Day 3, corresponding to the peak load at 4 a.m. of that day. For the PhACs, the max/mean ratio of the samples from the first week generally varied more than the second week, which was more consistent. The max/mean of ketoprofen approached the theoretical maximum on Day 2 (11.2) despite the fact that this compound was detected in every sample analysed on this day.

This shows that the ketoprofen maximum peak load on Day 2 (8 a.m.) far outweighed the concentration observed throughout the rest of the day; indeed, the concentration of this sample was greater than 2 orders of magnitude higher than the average ketoprofen concentration of the remaining samples. Paroxetine and clorazepate also displayed high variability in the max/mean ratio, where the highest value observed was approximately 8. On Day 1, captopril was only detected once, while atenolol was only detected once during Days 1 and 2, thus, at these times the max/mean ratios were the theoretical maximum for these two compounds.

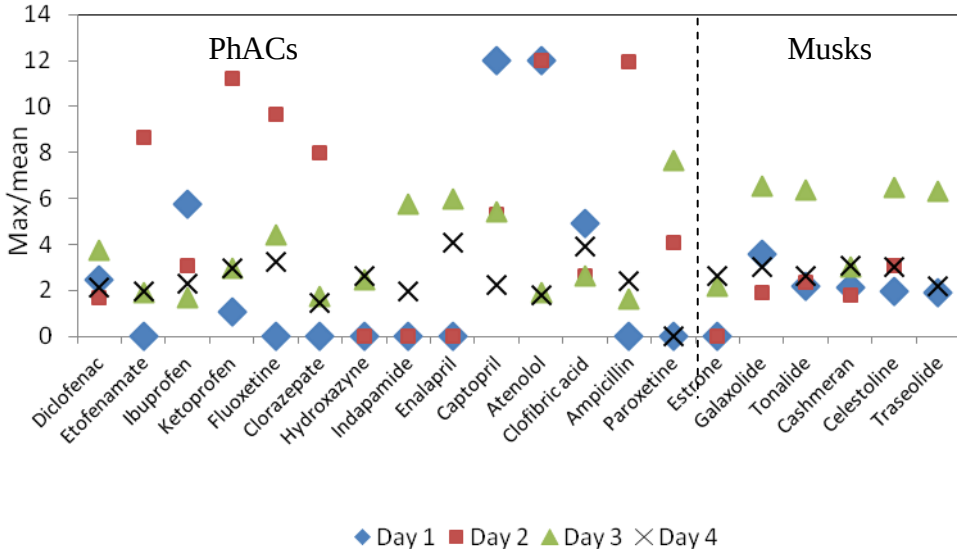


Figure 4.6 - Max/mean concentrations of the most frequently detected pharmaceuticals and musks for the 4 days of the campaign. Since 12 samples were analysed per day, the highest possible max/mean is 12, while the lowest possible ratio is 1, unless the compound was not detected on that day, in which case the max/mean value is represented as zero.

The max/mean generally ranges between 2 and 3 for BOD, suspended solids (SS), nitrogen and phosphorus in the influent to WWTPs (Tchobanoglaus and Burtan, 1995). While shock loads of these macropollutants are also known to occur occasionally, there is often a more repeatable pattern as compared to micropollutants such as PPCPs. As seen from Figures 4.3 and 4.6, the influx of most PhACs do not follow a repeatable pattern and peak loads of different compounds appear to occur sporadically. However, the results of this study suggest that musk compounds do, in general, follow the typical pattern exhibited by macropollutants (i.e. higher loadings throughout the day, lower at night) and are quite repeatable over the 4 sampling days, although occasionally higher peak loads can also be expected, such as those observed for most musks during day 3 (max/mean ~ 6).

4.3.4 Implications of the results and comparison with literature

Upon comparing the results from this study with those who have studied diurnal variations of PPCPs in literature, it can be observed that most studies have generally found that the micropollutants studied followed a similar pattern as compared to macropollutants; i.e. a clear decrease in the loading of these compounds was observed at night (Joss *et al.*, 2005; Göbel *et al.*, 2005; Plósz *et al.*, 2010). This study has investigated a higher number of compounds, many differing from those analysed in previous works. Thus, we have focussed this comparison with literature on either the same compounds, or similar compounds (i.e. from the same therapeutic family).

For example, Joss *et al.* (2005) studied the diurnal pattern of two musks also found in this study, galaxolide and tonalide. Our results indicate that these compounds, as well as the other musks detected, were generally less abundant at night (Figures 4.2 and 4.3), which agrees well with the results of Joss *et al.* (2005). Similarly, Plósz *et al.* (2010) found that estrone was also present in lower abundance at night, which was in accordance with our results (Figure 4.4). Previous studies investigating antibiotic compounds (Joss *et al.*, 2005; Göbel *et al.*, 2005; Plósz *et al.*, 2010) also observed a similar diurnal pattern. Although the specific antibiotic compounds detected in those studies were not widely detected at the Fernão Ferro WWTP, the one antibiotic that was frequently detected (ampicillin) was also present in low abundance at night (Figure 4.4). Thus, the results of this study are in accordance with literature findings. However, many other compounds were detected in this study that displayed differing diurnal patterns (e.g. ketoprofen, paroxetine, enalapril and indapamide) where the mass loadings were higher at night. This highlights the fact that patterns observed for some micropollutant groups cannot be readily extrapolated to other types of micropollutants. It is clear that different types of PPCPs display dissimilar diurnal variations, likely due to their varying administration patterns.

Furthermore, the results from this study show that even for the most commonly detected PhACs, variations in the mean concentration greater than 1 order of magnitude were found from one day to the next. The diurnal trend observed for PhACs was also variable between the first and second weeks and the occurrence of peak loads varied widely and was highly unpredictable. Obtaining a representative description of PhAC loading to WWTPs for modelling purposes is clearly a challenging task. Indeed, a "steady-state" did not exist for PhACs, unlike the musks. It is possible that obtaining a representative description of PhACs is not practically feasible due to the typically intermittent consumption of many different compounds, where some substances are consumed and excreted only on certain days by a small number of point sources (i.e. consumers). The high cost associated with analysing these compounds further inhibits the practical feasibility of performing multiple sampling campaigns. These

issues are important to be resolved in order to model the fate of these compounds in WWTPs, since wastewater influent characterisation is important for model calibration and application, and these models usually describe typical “steady-state” conditions of the WWTP. Nevertheless, it should be noted that a much higher repeatability was observed among the musks analysed, suggesting that less intensive monitoring is needed for acquiring the necessary data to model these compounds in WWTPs.

4.4 Conclusions

The dynamics of PPCPs in a WWTP was evaluated through an intensive sampling campaign covering a large number of pharmaceuticals and musks. It was found that the PhAC concentrations in the influent were subject to a wider variability than the musks, which were more repeatable. The typical diurnal pattern for macropollutants (i.e. higher loading during the day as compared to the night) was observed for the musks and some PhACs, while other frequently detected PhACs (e.g. ketoprofen) displayed the opposite trend or no trend. In general, the mean PhAC loadings varied between 1-3 orders of magnitude from one sampling day or week to the next, whereas the mean musk loadings were far less than one order of magnitude apart. This information is relevant to the design of sampling campaigns for modelling purposes.

CHAPTER 5

ASSESSING THE REMOVAL OF PHARMACEUTICAL AND PERSONAL CARE PRODUCTS IN A FULL-SCALE ACTIVATED SLUDGE PLANT

5. Assessing the Removal of Pharmaceuticals and Personal Care Products in a Full-Scale Activated Sludge Plant

This study aimed to investigate the removal mechanisms of pharmaceutical active compounds (PhACs) and musks in a wastewater treatment plant (WWTP). Biological removal and adsorption in the activated sludge tank as well as the effect of UV radiation used for disinfection purposes were considered when performing a sampling campaign. The fluxes of the most abundant compounds (13 PhACs and 5 musks) out of 79 compounds studied were used to perform a mass balance of the WWTP. Results show that incomplete removal of diclofenac was observed via biodegradation and adsorption, and that UV radiation was the main removal mechanism for this compound found in the highest abundance. The effect of adsorption to the secondary sludge was often negligible for the PhACs, with the exceptions of diclofenac, etofenamate, hydroxyzine and indapamide, however, the musks showed a high level of adsorption to the sludge. UV radiation reduced the concentration of some of the target compounds (e.g. diclofenac, ibuprofen, clorazepate, indapamide, enalapril and atenolol) not removed in the activated sludge tank. The removal of PhACs and musks studied in the WWTP was most often biological (45%), followed by adsorption (33%) and by UV radiation (22%). In the majority of the cases, the plant achieved > 75% removal of the most detected PhACs and musks, with the exception of diclofenac.

5.1 Introduction

The discharge of pharmaceutical active compounds and personal care products (PPCP) from wastewater treatment plants (WWTP) is a growing concern worldwide, due to their potentially harmful effects on aquatic life and tendency to infiltrate into drinking water supplies (Kreuzinger *et al.*, 2004, Andersen *et al.*, 2003 and 2005; Gagné *et al.*, 2006; Nakada *et al.*, 2005 and 2006, Oulton, *et al.*, 2010, Sim *et al.*, 2011).

Studies focused on their fate in WWTP have shown that these compounds are mainly a) adsorbed to the primary and secondary sludges (Carballa *et al.*, 2004 and 2007; Jélic *et al.*, 2011), b) biologically removed during secondary treatment (Clara *et al.*, 2005; Carballa *et al.*, 2007; Radienovic *et al.*, 2009; Jélic *et al.*, 2011); and c) removed in tertiary treatment via e.g. advanced oxidation processes (Méndez-Arriaga *et al.*, 2009, Naddeo *et al.*, 2009a and 2009b, Bundschuh *et al.*, 2011).

Artola-Garicano *et al.*, (2003), Joss *et al.* (2005) and Clara *et al.*, (2011) reported no substantial biodegradation of musks and that adsorption is the main mechanism that should be taken into account for these compounds. In contrast, biodegradation has often been the primary removal mechanism for some PhACs. Carballa *et al.*, (2007) observed 50-70% biodegradation for PhACs such as ibuprofen, naproxen and sulfamethoxazole, while Joss *et al.* (2005) and Plósz et al. (2010) observed a similar range for the biodegradation of naproxen and sulfamethoxazole, although ibuprofen was >80%

biodegraded. While many studies have investigated PPCP removal during secondary treatment (i.e. biodegradation and adsorption to secondary sludge), these studies are seldom combined with an evaluation of PPCP removal via tertiary treatment.

Advanced oxidation technologies have been carried out in WWTP and applied to the transformation of PhAC by the use of ozone (De Witte *et al.*, 2009), different chemical oxidants (Sharma, 2008) and sonolyis (Hartmann *et al.*, 2008) but only a few have considered the effect of UV radiation used for disinfection on the removal of PhACs (Nakada *et al.*, 2005) and musks. Most of the studies on UV radiation and UV combined with hydrogen peroxide have been carried out in lab scale reactors rather than in full-scale treatment systems.

Göbel *et al.*, (2005) studied the occurrence of sulfonamides, macrolides, and trimethoprim in wastewater samples from the raw influent to the final effluent as well as activated and digested sewage sludge samples in municipal wastewater treatment plants. These authors found that clarithromycin and trimethoprim were not biodegraded or adsorbed, but partly removed during sand filtration, while sulfamethoxazole was mostly removed biologically, and not removed via tertiary treatment. The removal mechanisms of biodegradation, adsorption and oxidation processes (e.g. UV) combined together and their effects on the final loads of PhAC and musks in the treated wastewater and sludge have seldom been investigated in detail in previous works.

The main goal of this study is to provide insight, through the use of mass balances, on the removal of PPCPs in a full-scale WWTP taking into account the raw influent, effluent prior to UV, effluent post-UV and secondary sludge loads. A sampling campaign was performed during 4 different days over two consecutive weeks in order to fulfill this objective. A large number of compounds were analysed (79 PPCPs, including 73 PhACs and 6 musks), whereby the mass balance was performed on the most abundant 13 PhACs and 5 musks.

5.2. Materials and methods

5.2.1 Full-scale wastewater plant

The WWTP of Fernão Ferro (Seixal, Portugal) has the design capacity to handle approximately 32 700 population equivalents (Figure 5.1).

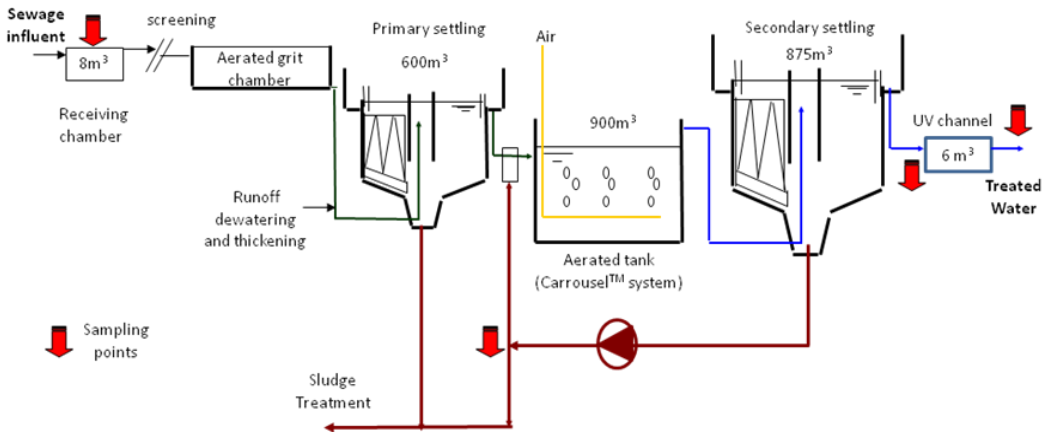


Figure 5.1 - Schematic diagram of the municipal wastewater treatment plant studied.

In the liquid stream, the treatment comprises, retention of coarse solids in a receiving chamber (8 m^3), two lines of mechanical pre-treatment (removal of sand and coarse solids and oil and grease), followed by the elevation of pre-treated effluent through pumps to the primary sedimentation tank. The activated sludge process in carried out in two aerobic reactors with a Carrousel® configuration of 900 m^3 each, where aeration is provided by two surface aerators. A selector is installed previous to the two biological reactors to combine the influent wastewater of the primary settler and the recycled sludge. The solid phase removed from the secondary settler is pumped and recycled to the aeration tank and a waste fraction is removed for sludge treatment with the primary sludge. Finally, the disinfection of the effluent is carried out in two channels, of 6 m^3 each, with UV lamps (low pressure, wavelength of 254 nm, operating at maximum flow rate of 760 m^3h^{-1} and total suspended solids (TSS) of approximately 25 mg L^{-1}).

5.2.2 Sampling collection

The influent composite samples were collected in the receiving chamber and effluent composite samples were collected at the inlet and outlet of the UV channel. Grab samples of primary and the secondary sludge were also collected for analysis. Two consecutive weeks were monitored. In both weeks, influent samples on Monday and Tuesday (24 h composite samples of sampling interval of 2 hour - see Salgado *et al.* (2011)) and Wednesday (24 h composite samples of sampling interval of 1 hour) were taken with an auto-sampler. The effluent samples (before and after the UV channel) consisted of 24h composite samples (sampling interval = 1 hour) collected with an auto-sampler. The effluent samples were taken one day following the inlet samples (i.e. Tuesdays, Wednesdays and Thursdays), which approximately corresponded to the hydraulic retention time (HRT) of the plant (0.9±0.1 d). Secondary sludge samples were collected on Tuesday and Thursday of the first week and

Wednesday and Thursday of the second week. Thus, biological removal *vs.* adsorption was calculated for the 4 days where sludge sampling was carried out, whereas the overall removal (influent - final effluent) was calculated for the 6 sampling days. The samples were transported to the laboratory in a refrigerated isothermal container and immediately extracted and stored at -20^{o}C until the analysis was performed. The samples were prepared for analysis of two different classes of pharmaceutical compounds (acidic and neutrals) according to the procedure described below.

5.2.3 Analytical methods

Analytical details, including information on materials and reagents used and limits of detection and quantification, are described for wastewater and sludge samples in Salgado *et al.* (2010). The compounds monitored in this study included those described in Salgado *et al.* (2011). Solid phase extraction (SPE) was used for PhAC analysis in the liquid phase (influent and effluent samples). SPE was carried out with Waters Oasis HLB for the acidic pharmaceutical compounds, while Waters RP-C18 cartridges were used for the neutral compounds.

The analysis of the PhAC and musk content of the secondary sludge was performed according to Ternes *et al.* (2005). Sludge samples were centrifuged at 10 000 rpm for 5 min to separate the solid from the liquid fraction. Ultrasonic solvent extraction (USE) was used as the extraction procedure of the PhAC from the sludge samples prior to SPE. The detection of acidic and neutral pharmaceutical active compounds by LC-MS (HPLC-DAD-(ESI)MS) in reverse-phase chromatography (LiChroCART 250-4 Purospher Star RP18 endcapped, 5 μm, column, Merck) of the samples was performed with DAD (diode array detector) detection.

The extraction of polycyclic musk fragrances was carried out by headspace solid-phase microextraction (HS-SPME). The detection of these compounds was performed by GC-MS using a Hewlett-Packard 5890 GC fitted with a QMD1000 Carlo Erba mass spectrometric detector. A DB-5MS fused-silica capillary column (30 m x 0.32 mm i.d., 0.25 μm film thickness) purchased from Agilent-J&W Scientific (Spain), was used for analytical separation.

5.2.4 Mass balance and removal calculations

In the mass balances carried out in this study, it was assumed that no biological or chemical reactions took place in the primary and secondary settlers. It was also assumed that the adsorption equilibrium of the PPCPs and primary sludge was attained in the sewer. The liquid influent PPCP load to the receiving chamber of the WWTP is therefore equal to the liquid PPCP load of the supernatant of the primary settler. The PPCPs concentrations in the solid fraction (C_s) of the activated sludge were measured in the sludge recycling from the secondary settler to the activated sludge tank.

The adsorption fraction was determined based on the new biomass generation rate in the aeration tank, which was assumed to be responsible for adsorbing the PhACs and musks that entered via the influent wastewater (Joss *et al.*, 2005). This new biomass generated is, thus, the new surface available for additional sorption, and can be estimated based on the purged sludge flow rate, according to equation 5.1:

$$SS_{new} = \frac{SS_{sld}.Q_w}{Q_{in}} \qquad \text{(eq. 5.1)}$$

Where Q_w is the excess sludge flow rate ($m^3\ d^{-1}$), Q_{in} is the influent wastewater flow rate ($m^3\ d^{-1}$), SS_{sld} is the suspended solids concentration in the excess sludge and SS_{new} is the sludge production per unit of treated wastewater ($kgSS\ m^{-3}$). The new biomass produced daily in the aeration tank was used in equation 5.2 to determine the load of PPCP eliminated by sludge wastage:

$$L_{sld} = C_w.\left(Q_w + Q_{in}.SS_{new}.\frac{C_s}{C_w}\right) \cong Q_w.C_w + Q_{in}.SS_{new}.C_s \qquad \text{(eq. 5.2)}$$

L_{sld} is the total load of the PPCP in the excess sludge ($\mu g\ d^{-1}$), C_s is the sorbed concentration of PPCP per amount of suspended solids ($\mu g\ kgSS^{-1}$), C_w is the soluble concentration of PPCP ($\mu g\ m^{-3}$).
The biological transformation was calculated from difference mass balance of the loads of the influent, effluent (prior to UV) and excess sludge:

$$L_{bio} = L_{in} - (L_{sld} + L_{out}) \qquad \text{(eq. 5.3)}$$

The removal by UV photolysis was obtained by the load difference before and after the UV chamber.

5.3. Results and Discussion

5.3.1 Removal mechanisms of PhACs and musks in the WWTP

5.3.1.1. Biological removal

Out of the 79 PPCPs assessed in this study, mass balances were carried out for the 13 most abundant PhACs and 5 musks with the aim of identifying the most relevant removal mechanism. The daily average inlet flow rate used to calculate the loadings into the WWTP had minimum and maximum values of 2485 and 3117 $m^3\ d^{-1}$, respectively. PhACs and musks biodegradation in the activated sludge

tank was quite variable for the different days analyzed (Table 5.1), but the overall level of biological removal could be classified into 3 separate groups as: a) low; b) medium and c) high biological removal.

Table 5.1 - Removal via biological degradation of the 13 most detected PhACs and 5 musks.

Day	Biological Removal (g d^{-1})						Level of removal by biodegradation $^{\Delta}$
	Day 1	Day 3	Day 9	Day 10	Average	StDev	
Diclofenac	-23.1	-4.1	-47.5	-24.1	**-24.7**	17.8	low
Etofenamate	-0.3*	-1.1	-2.6	12.4	**2.1**	6.9	medium
Ibuprofen	45.1	0.5	0.4	-0.3	**11.4**	22.5	high
Ketoprofen	83.3	-0.2	0.0	-0.3*	**20.7**	41.7	high
Fluoxetine	0.0*	0.0*	2.1	0.0*	**0.5**	1.1	high
Clorazepate	0.0*	0.2	-1.3	0.9	**-0.1**	0.9	low
Hydroxazine	-2.3*	-1.6*	0.3	1.1	**-0.6**	1.6	low
Indapamide	-0.9*	-0.1*	-22.8	-3.0	**-6.7**	10.8	low
Enalapril	0.0*	-0.3	3.1	0.0	**0.7**	1.6	medium
Captopril	2.1	-0.6	1.6	1.4	**1.1**	1.2	medium
Atenolol	12.8	2.8	2.0	0.9	**4.6**	5.5	high
Clofibric acid	49.9	0.2	-0.3*	0.0	**12.4**	25.0	high
Ampicillin	-0.1*	0.0*	0.2	0.5	**0.2**	0.3	high
Galaxolide	-0.4	-3.7	-32.0	-29.0	**-16.3**	16.5	low
Tonalide	0.2	-0.3	-3.8	-3.9	**-1.9**	2.2	low
Cashmeran	-0.8	-3.8	-64.3	-41.5	**-27.6**	30.7	low
Celestolide	0.5	-0.3	-3.9	-0.2	**-1.0**	1.9	low
Traseolide	0.2	-0.1	-1.1	-0.1	**-0.3**	0.6	low

$^{\Delta}$Low removal represents <25% removal from the average influent load, medium represents >25% and <75% removal, while high removal represents >75% removal;*Compound was below the detection limit in the influent.

The compounds were considered to display low biological removal when the average removal was < 25% of the average influent load for the 4 days analyzed. The low biological removal of some of the PhAC could be related with the influent load variability and sampling uncertainty in the influent, as discussed below. Medium biological removal corresponds to average removal loads between 25% and 75% of the average influent load.

Medium biological removal loads were found for etofenamate, enalapril and captopril. The high biological removal corresponds to situations when the removal was consistently high (>75%) relative to the average influent load during the campaign. The days where compounds were not detected in the influent (see Appendix II) were exempted from these calculations. The biological removal rates showed a large variability from one week to another in most of the compounds studied, and the total removal observed also varied within a pharmaceutical group. For example, within the therapeutical

group of the non-steroidal anti-inflammatory drugs (NSAIDs), diclofenac displayed low biological removal, etofenamate exhibited medium biological removal and ibuprofen and ketoprofen had high biological removal.

In some days within the study, the output loads were substantially greater than the influent loads (i.e. negative removal rates), such as diclofenac on days 1, 9 and 10, and indapamide on day 9, as well as the musks, particularly galaxolide and cashmeran on days 9 and 10. The reason for this could be due to either (*i*) sampling uncertainty, particularly in the raw influent where loading rates are more dynamic. Ort *et al.* (2010) found that time-dependent composite samples in the raw influent may be insufficient to adequately characterize PPCP removal in WWTPs; (*ii*) conjugate compounds not detected at the influent but retransformed into the original compound due to biological processes; (*iii*) desorption processes in the activated sludge tank of previously adsorbed PhACs and musks.

The results from previous studies have generally agreed well with the experimental results. Xue *et al.*, (2010) and Jélic *et al.*, (2011) also found low diclofenac biodegradation and high effluent concentrations. Nakada *et al.* (2005) found high removal efficiencies for ibuprofen in the WWTP varying from 84-98% for influent concentrations of 69-1080 ng L^{-1}. This result is in the same range of removal efficiencies observed by Joss *et al.* (2005) and Plósz *et al.* (2010) for ibuprofen. Ketoprofen was almost completely removed biologically (>80%) in Jélic *et al.*, (2011), which also agrees well with the results shown in Table 1. For the musks, the biological degradation of galaxolide and tonalide was reported as 15 and 30 %, respectively, for the influent loads of galaxolide of 602-671 g d^{-1} and tonalide of 248-402 g d^{-1} (Carballa *et al.*, 2007). Joss *et al.*, (2005) found that biodegradation of galaxolide was between 0-30% and tonalide was 0-10% in their study. In our study we also found low biological removal of both compounds.

5.3.1.2 Removal by adsorption in the activated sludge tank

In Table 5.2, removal by adsorption to the activated sludge is expressed taking into account that only the newly formed biomass has available adsorption sites (see Joss *et al.*, 2005) and the remaining sludge is fully saturated with the previously adsorbed PPCPs. Similarly to the biological removal loads, a scale was established to classify the adsorption removal loads of the PhAC and musks in three categories as: a) low, b) medium and c) high adsorption.

Table 5.2 - Removal via adsorption of the 13 most detected PhACs and 5 musks.

Day	Adsorption Removal (g d^{-1})						Theoretical $logK_{ow}$	Level of removal by adsorption $^{\Delta}$
	Day 1	Day 3	Day 9	Day 10	Average	StDev		
Diclofenac	1.3	1.0	14.9	12.7	**7.5**	7.3	4.0-4.7	medium
Etofenamate	0.1*	0.9	2.9	2.5	**1.6**	1.3	4.2	medium
Ibuprofen	0.0	0.2	0.2	1.1	**0.4**	0.5	3.5-4.0	low
Ketoprofen	0.0	0.0	0.0	0.1*	**0.0**	0.1	3.0-3.2	low
Fluoxetine	0.0*	0.0*	0.0	0.0*	**0.0**	0.0	1.8	low
Clorazepate	0.0*	0.0	0.7	0.1	**0.2**	0.4	2.1	low
Hydroxazine	1.1*	0.8*	0.0	3.1	**1.2**	1.3	2.4	medium
Indapamide	0.4*	0.0*	7.1	3.1	**2.7**	3.2	2.7	high
Enalapril	0.0*	0.1	0.0	0.4	**0.1**	0.2	-	low
Captopril	0.4	0.5	0.4	0.7	**0.5**	0.1	1.7	low
Atenolol	0.0	0.0	0.0	0.0	**0.0**	0.0	0.2-1.4	low
Clofibric acid	0.0	0.0	0.7	0.0*	**0.2**	0.3	2.6-3.6	low
Ampicillin	0.1*	0.0*	0.0	0.0	**0.0**	0.0	1.4	low
Galaxolide	0.9	1.8	14.7	13.2	**7.6**	7.3	4.6-5.9	high
Tonalide	0.1	0.1	1.8	1.8	**1.0**	1.0	4.8-5.7	high
Cashmeran	1.2	1.8	29.9	18.9	**12.9**	14.0	4.8	high
Celestolide	0.1	0.2	1.8	0.1	**0.6**	0.9	4.4	high
Traseolide	0.0	0.0	0.5	0.1	**0.2**	0.2	-	high

$^{\Delta}$Low removal represents <25% removal from the average influent load, medium represents >25% and <75% removal, while high removal represents >75% removal; Compound was below the detection limit in the influent.

The adsorption of PhACs to the secondary sludge was important for some NSAIDs (e.g. diclofenac and etofenamate) and some other PhACs such as hydroxyzine and indapamide. Adsorption clearly outweighs the biological removal for diclofenac, hydroxyzine, indapamide, galaxolide, tonalide, cashmeran and celestolide. Low adsorption was observed for fluoxetine, clorazepate, enalapril, captopril, and ampicillin. Occasionally high biological removal was found for ibuprofen, ketoprofen, atenolol and clofibric acid, thus only negligible amounts of these compounds remained undegraded and could be found adsorbed to the sludge (regardless their hydrophobicity).

The adsorption of PhACs and musks in the secondary sludge is generally in agreement with their hydrophobicity (expressed by the octanol - water partition coefficient K_{ow}). $LogK_{ow}$ values found in literature varies from 3.0 to 5.9 for musks (galaxolide, tonalide, cashmeran, celestolide and traseolide) and NSAIDs (diclofenac and etofenamate), which in most cases corresponded to high and medium

adsorption observed, with exception of the NSAIDs ibuprofen and ketoprofen that presented a high value of $logK_{ow}$ but low adsorption was observed. This effect could be due to the high biodegradation rates observed for these two compounds. Most of the other compounds detected in the influent wastewater have low octanol - water partition coefficients ($logK_{ow} < 3.0$) and low adsorption capacity to the sludge. Furthermore, this is in agreement with the low values of adsorption coefficients (K_d) for the PhACs and high values for the musks found in literature (Ternes *et al.*, 2004; Joss *et al.*, 2005; Clara *et al.*, 2011). These results suggests that only a few PhACs are removed by adsorption and that biodegradation is the most important removal mechanism, however the musks show the opposite trend and adsorption is the primary removal mechanism for this group of compounds.

5.3.1.3 Removal by UV radiation

The effluent loads before and after UV disinfection were also calculated in this campaign to evaluate the effect of this step on the removal of the PPCPs studied. The removal loads of the PhACs and musks in the UV channel of the WWTP are presented in Table 5.3.

Table 5.3 - Removal in the UV channel of the 13 most detected PhAC and 5 musks.

Day	UV Removal (g d^{-1})						Level of removal by UV photolysis$^{\Delta}$
	Day 1	Day 3	Day 9	Day 10	Average	StDev	
Diclofenac	10.2	1.0	28.2	6.6	**11.5**	11.8	medium
Etofenamate	0.0*	0.2	0.0*	0.0*	**0.1**	0.1	medium
Ibuprofen	0.8	1.1	0.2	0.1	**0.5**	0.5	medium
Ketoprofen	0.0*	0.1	0.1	0.0*	**0.0**	0.0	medium
Fluoxetine	0.0*	0.0*	0.0	0.0*	**0.0**	0.0	low
Clorazepate	0.0*	0.6	0.6	0.2	**0.4**	0.3	medium
Hydroxazine	0.0*	0.0*	0.1	0.0*	**0.0**	0.0	medium
Indapamide	0.0*	0.0*	15.9	0.0*	**4.0**	7.9	high
Enalapril	0.0*	0.9	0.8	0.1	**0.4**	0.5	high
Captopril	0.0*	0.0*	0.1	0.0*	**0.0**	0.1	high
Atenolol	0.0*	0.7	0.0*	0.0*	**0.2**	0.3	high
Clofibric acid	0.0*	0.0*	0.0*	0.0*	**0.0**	0.0	*n/a*
Ampicillin	0.0*	0.0*	0.0*	0.0*	**0.0**	0.0	*n/a*
Galaxolide	-0.01	0.05	0.01	0.02	**0.02**	0.02	medium
Tonalide	0.01	0.03	0.00	0.00	**0.01**	0.01	*n/a*
Cashmeran	0.07	0.00	0.03	0.01	**0.03**	0.03	medium
Celestolide	0.00	0.06	0.01	0.01	**0.02**	0.03	medium
Traseolide	0.01	0.02	0.00	0.01	**0.01**	0.01	*n/a*

$^{\Delta}$Low removal represents <25% removal from the average load entering the UV channel, medium represents >25% and <75% removal, while high removal represents >75% removal;*Compound was below the detection limit in the entrance to the UV channel; *n/a* – not applied

The highest removal by UV relative to the load entering the UV channel (secondary effluent) was detected for indapamide, enalapril captopril and atenolol. Medium removal by UV radiation was observed for diclofenac, etofenamate, ibuprofen, ketoprofen (the NSAIDs), clorazepate, hydroxyzine and the musks.

The removal of musks by UV was less clear due to the fact that most of these compounds were close to fully removed via adsorption. One of the PhACs most significantly removed in the UV radiation was diclofenac (11.5 g d^{-1}). This is likely due to the higher load of this compound in the influent to the UV channel when compared to the other NSAIDs, that were mostly removed biologically or by adsorption. After UV radiation, new chromatographic peaks were detected by LC-DAD-(ESI)MS analysis of the effluent, suggesting the formation of new compounds, which were not identified in the scope of this study. The UV radiation used for disinfection seems to have an important contribution to the removal of PhACs and musks that were not removed biologically or by adsorption in spite of the brief 5 minutes of hydraulic retention time (HRT) in the UV channel.

5.3.2 Overall removal of PPCPs in the WWTP

The relevance of each mechanism for the overall PPCP removal was assessed in Figure 5.2, which represents the percent number of compounds that were mainly removed via each mechanism: 45% of the PPCPs were mainly removed biologically, 33% of the compounds (mostly musks) were principally removed by adsorption, and the remaining 22% were mostly removed via UV. It should be noted that although UV was the least important mechanism, many of the compounds had already been removed biologically or by adsorption, thus UV represented an important effluent polishing step.

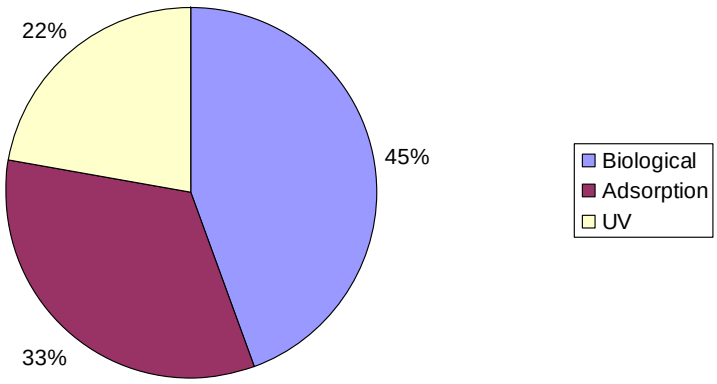


Figure 5.2 - Percentage of the PhACs and musk compounds removed principally by biological mechanisms, adsorption to the secondary sludge and UV photolysis in the WWTP.

Figure 5.3 shows the combined removal achieved biologically, by adsorption and by UV for the 13 PhACs and 5 musks that were most highly detected in this study. The box and whisker figure shows the raw influent minus the final effluent (after UV), where the top and bottom of the box represent the 75th percentile and 25th percentile, respectively, and the whiskers show the 95th and 5th percentiles, respectively.

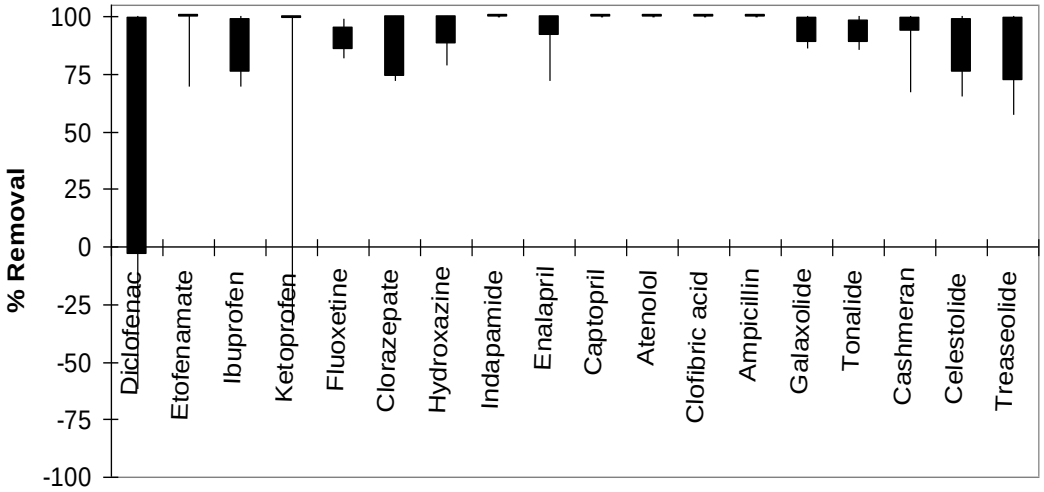


Figure 5.3 - Overall removal of the 13 most detected PhACs and the 5 musks in the WWTP for the six days over two consecutive weeks of the campaign. The lower and upper extremes of the boxes represent the 25th percentile and 75th percentile of % removal achieved, respectively, while the lower and upper extremes of the whiskers represent the 5th and 95th percentiles, respectively.

From Figure 5.3, it can be observed that most of the compounds were >75% removed in most cases, except for diclofenac, which varied more than all the other compounds studied. Indapamide, captopril, atenolol, clofibric acid and ampicillin have very consistent removals of almost 100% during the 6 sampling days. Other compounds present higher variability, though only ketoprofen exhibited an overall negative removal on one day, in addition to diclofenac. The remaining compounds achieved removal rates consistently higher than 75% of the influent loading rate. This level of removal is quite high as compared to that achieved for PPCPs in previous studies (Göbel *et al.*, 2005; Carballa *et al.*, 2007; Jelic *et al.*, 2011).

The treatment sequence of the WWTP in this study was able to remove all the studied PhACs and musks, with the exception of diclofenac. A prior study (Salgado *et al.*, 2010) carried out in this plant, yielded a similar removal efficiency (i.e. >75% removal of PPCPs in the spring and autumn). It should be noted that this study was conducted during the winter (January), suggesting that the observed removal efficiency was independent of temperature. Since biological removal was the dominant removal mechanism observed, this could suggest that a Carrousel-type configuration is an efficient configuration for PPCP removal. While this hypothesis requires further confirmation, is corroborates

the results of Joss *et al.* (2006), which suggest that a plug-flow configuration significantly improves the biological removal of non-sorbing and biodegradable micropollutants .

5.4. Conclusions

The combination of biodegradation, adsorption and removal by UV irradiation are each important mechanisms that lead to the transformation of PPCPs in WWTPs. Biodegradation was found to be the principal degradation mechanism, followed by adsorption and UV, which was found to be an effective effluent polishing step for PhAC and musk removal. In this study, >75% removal was usually found for 17 of the 18 most commonly detected PPCPs, with the sole exception being diclofenac, which often showed negative values for the biological removal rate in the activated sludge and was mostly degraded by UV photolysis. While further investigation should be focussed on ways to optimise PPCP removal in WWTPs, it is possible that the Carrousel-type configuration may be an efficient configuration for effective PPCP removal.

CHAPTER 6

BIODEGRADATION OF CLOFIBRIC ACID AND IDENTIFICATION OF ITS METABOLITES

6. Biodegradation of clofibric acid and identification of its metabolites

Clofibric acid (CLF) is the pharmaceutically active metabolite of the lipid regulator clofibrate, and it is considered environmentally persistent and refractory. This work studied the biotransformationof CLF in two sequencing batch reactors (SBR) with microbial mixed cultures, monitoring the efficiency of biotransformation of CLF and the production of metabolites. The inoculum for both SBRs was an enriched culture that degraded the herbicide propanil, a structurally similar compound as CLF. One SBR was fed only with clofibric acid and the other was fed with CLF and propanil as carbon sources in order to enhance the co-degradation of both substrates. In the reactor fed only with clofibric acid it was possible to remove only 15% of the CLF, while the reactor fed with clofibric acid and propanil achieved 51% removal. Kinetic studies were performed in order to investigate whether nitrifiers or heterotrophic bacteria were primarily responsible for the biotransformation of the CLF. Tests with nitrification inhibition showed only slightly lower removal as compared to tests without nitrification inhibition showing that the observed CLF biodegradation was mainly carried out by heterotrophic bacteria .Four transformation products of clofibric acid were identified by GC-MS, although one of them in quantities was below the level of quantification of HPLC-DAD. The main metabolites were α-hydroxyisobutyric acid, lactic acid and 4-chlorophenol. The latter is known to exhibit a higher toxicity than the parent compound, but did not accumulate in either SBR. α-hydroxyisobutyric acid and lactic acid accumulated for a certain period, where nitrite accumulation may have been responsible for inhibiting their degradation. A metabolic pathway for the biodegradation of CLF is proposed in this study.

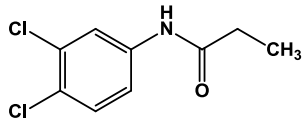
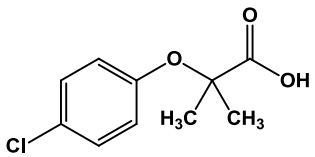
6.1 Introduction

Clofibric acid (CLF) is the active metabolite of clofibrate, a lipid regulator. This drug is resistant to biodegradation and, due to its polar nature, has a very high persistence in the environment (Hernando *et al.*, 2007, Winkler *et al.*, 2008). Clofibric acid is one of the most widely and routinely reported drugs found in open waters (Matamoros *et al.*, 2008a and 2008b). The presence of pharmaceutically active compounds (PhACs) and their metabolites in aquatic systems has become a concern in the past years due, in part, to their ubiquity in the environment. However, at present, the persistence and ecotoxicity of many of these compounds remains unknown. CLF was detected in most aquatic systems (e.g. rivers) where pharmaceutical contaminants were monitored and is reported to be persistent (Ternes, 1998, Winkler *et al.*, 2001, Joss *et al.*, 2006). In a survey study of 5 different Portuguese wastewater treatment plants (WWTPs), CLF was consistently detected in the influent in the ngL^{-1} to μgL^{-1} range (Salgado *et al.*, 2010). Peak loads of up to 41.4 $\mu g\ L^{-1}$ were found in one plant, representing one of the most abundant of all PhACs detected (Salgado *et al.*, 2011). Furthermore, a

mass balance performed on this plant suggested that CLF was mostly removed biologically (see chapter 5). Nevertheless, previous results revealed that clofibric acid was resistant to microbial degradation by several types of microorganisms, including wastewater treatment plant biomass (Kimura *et al.*, 2007, Evangelista, 2008, Evangelista *et al.*, 2008 and 2010). This apparent contradiction motivated the study of a CLF biodegrading culture and its metabolic pathway.

Carvalho *et al.*, (2010) obtained a microbial enrichment capable of converting propanil into dichloroaniline (DCA), its metabolite, and degrading both compounds completely. Propanil is an herbicide used on rice cultures, and structurally similar to clofibric acid since it contains both a chlorinated aromatic ring and a derivative of propionic acid connected to it (Table 6.1). Thus, this enrichment was used as an inoculum for attempting to generate a CLF-degrading microbial consortium.

Table 6.1 - Chemical structures of propanil and clofibric acid.

Compound	CAS	Formula	M_W	Chemical structure
propanil	709-98-8	$C_9H_9Cl_2NO$	218.08	
clofibric acid	882-09-7	$C_{10}H_{11}ClO_3$	214.65	

Biodegradation studies of clofibric acid (in the presence of propanil) were performed by exposing the compounds to aerobic cultures of microorganisms acclimatized for propanil biodegradation in sequencing batch reactors (SBRs). The objective of this study was to develop a microbial community capable of degrading CLF, which would be valuable for bioremediation or bioaugmentation purposes. Moreover, the metabolites generated from the CLF biotransformation were also identified and monitored. This study gives an important contribution towards the elucidation of biodegradation mechanisms of refractory PhAC that could affect the environment after discharge.

6.2 Materials and methods

6.2.1 Chemical and reagents

HPLC-grade acetonitrile, formic acid and phosphoric acid were purchased from Panreac (Portugal). Ultrapure water was obtained from a MilliQ water purification system (Millipore, Bedford, MA.,

USA). The clofibric acid ((2-4-chorophenoxy)-2-methylpropionic acid) standard was purchased from Sigma-Aldrich (Steinheim, Germany) and the metabolite standards, α-hydroxyisobutyric acid, lactic acid, 4-chlorophenol and also the derivatizing reagent, MSTFA (*N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide) ≥98.5% (GC grade) was purchased from Aldrich (Portugal). The allylthiourea (ATU) and the reagents used in the preparation of mineral media ≥98% (grade) of the reactors were all purchased from Panreac (Portugal).

6.2.2 Microbial enrichments

Microbial enrichments were initiated from a mixture of soil contaminated with several herbicides, including propanil, and soil from organic rice agriculture supplemented with $(NH_4)_2SO_4$ and propanil (Barreiros *et al.*, 2003). These cultures were further pre-enriched in a mixed culture that was used to inoculate a sequencing batch reactor (SBR), which was operated with a fed-batch strategy as detailed in Carvalho *et al.*, (2010). This culture was used to seed the SBRs employed in this study, which were supplied with either clofibric acid or a mixture of clofibric acid and propanil over a period of 20 months.

6.2.3 Composition of the feed solution of the SBR

6.2.3.1 Mineral media

The mineral media (phosphate buffer) was prepared with 2.620 g of $Na_2HPO_4.H_2O$; 1.162 g of KH_2PO_4 and 0.530 g of NH_4Cl dissolved in 1 L of MilliQ water. The pH was adjusted to 7.2 with NaOH, and then sterilized in an autoclave for 15 min at 120 ºC.

6.2.3.2 Nutrient Solution

The nutrient solution was prepared by combining solutions A, B and C in the following proportion: 0.1 mL of A, 0.6 mL of B and 0.1 mL of C per 100 mL of mineral media. *Solution A (Micronutrient solution):* 0.1 mM of $NiCl_2.6H_2O$; 0.15 mM of $NaMoO_4.2H_2O$; 0.5 mM of $ZnCl_2$; 1 mM of $MnCl_2.4H_2O$; 0.1 mM of $CuCl_2.2H_2O$; 0.8 mM of $CoCl_2.6H_2O$; 1 mM of H_3BO_3 and 10 mM of HCl (25% vol) dissolved in MilliQ water. This solution was sterilized in an autoclave for 15 min at 120 ºC. *Solution B (Macronutrients solution)*: 0.135 M of $MgCl_2.6H_2O$, 0.865 M of $FeCl_2.4H_2O$ and 1.26 M of NaCl was dissolved in MilliQ water. This solution was sterilized in an autoclave for 15 min at 120 ºC and was filtered through 0.45 μm glass fiber membranes (GF 6, <1 μm, diameter 47 mm from Wathman, England). *Solution C*: 0.2 M of $CaCl_2$ dissolved in MilliQ water.

6.2.3.3 Carbon source: clofibric acid and propanil

A clofibric acid stock solution of 1000 mg L^{-1} of concentration in MilliQ water and a 100 mg L^{-1} propanil prepared in mineral media and stored at 4 ºC to feed the reactors as carbon source.

6.2.4 Lab-scale SBR operation

6.2.4.1 SBR start-up and operation

Two 150 mL SBRs were fed with 1 mg L^{-1} clofibric acid and 3 mg L^{-1} NH_4^+-N in addition to mineral media, and nutrient solution. SBR A was fed with CLF as the sole carbon source and in SBR B, propanil was also added to a concentration of 3 mg L^{-1} in the reactor. The reactors hydraulic retention time (HRT) was approximately 9.3 days and no sludge wastage was made beyond sampling. 75% of the reactor media (supernatant after centrifugation) was replaced every 7 days. The two reactors were magnetically stirred, aerated through ceramic air dispersers, and kept in a air-conditioned room at temperature of 25±2 ºC, while the pH was uncontrolled.

After 6 months of reactor operation, CLF was added at 2 mg L^{-1} in both reactors and the propanil was increased to 6 mg L^{-1} in SBR B. After 9 months, both reactors A and B were supplemented with ammonium sulfate solution to get 20 mg L^{-1} NH_4^+-N in both reactors. In reactor B, propanil was spiked to get a 6 mg L^{-1} concentration in the reactor three times during the cycle period of 7 days. Once per day the pH of both reactors was adjusted to 7.0±0.1.
After replacing the supernatant, the biomass concentration was monitored through optical density measurements at 610 nm, and the pH was again measured in each reactor. Samples were periodically taken to monitor clofibric acid, propanil, ammonia, nitrite and nitrate and also the biodegradation metabolites of propanil and clofibric acid.

6.2.4.2 Test with non-limiting ammona concentration

After acclimatization to higher ammonia concentration, an experiment was carried out where the SBR B was operated with additional ammonia spikes during the cycle to prevent limitation. The SBR initially was fed with 2 mg L^{-1} of clofibric acid and 20 mg L^{-1} NH_4^+-N, as usual, but no propanil. The SBR was operated with pH controlled automatically to 7.0±0.1. Spikes of 5 mg L^{-1} NH_4^+-N were added on days 1 and 2 of operation and reactor performance was monitored for 5 days. Samples were taken daily to analyse for clofibric acid and metabolites, ammonia, nitrite and nitrate.

6.2.4.3 Nitrification inhibition test

The SBR was fed with mineral and nutrient and spiked with 2 mg L^{-1} clofibric acid and 20 mg L^{-1} NH_4^+-N ammonia but no propanil, as described above. Additionally, 1 mg L^{-1} of allylthiourea (ATU) was added to inhibit nitrification. After the reactors's performance was monitored throught 5 days, where the optical density was measured, while daily samples were collected to measure the clofibric acid, metabolites, ammonia, nitrite and nitrate concentrations.

6.2.4.4 Adsorption studies in the biomass

A fraction of biomass from SBR B was centrifuged and washed with mineral media. The biomass was ressuspended with mineral media and nutrient solution in the same proportion used in the reactor, and also 2 mg L^{-1} of clofibric acid was spiked. The initial biomass concentration was measured throught the optical density at 610 nm and a sample was collected to measure the CLF concentration in the supernatant as well as in the biomass. This procedure was repeated after 3 days. The samples were centrifuged and the liquid was directly analyzed by HPLC-DAD, while the extraction procedure detailed below was used to determine the amount of CLF adsorbed to the sludge.
The compounds adsorbed to the biomass were determined by the use of the modified method of ultrasonic solvent extraction described by Salgado *et al.*, (2011). In brief, 2x2 mL of methanol was added to the centrifuged biomass sample and the extraction was carried out for 5 min in an ultrasonic bath. The solvent/biomass mixture was centrifuged, and the supernatant collected in a vial and evaporated to 1 mL by a gentle nitrogen stream and the compounds extracted from the sludge were analyzed in HPLC-DAD.

6.2.4.5 Abiotic CLF degradation

Two 150 mL SBRs were fed with 1 mg L^{-1} clofibric acid and 20 mg L^{-1} NH_4^+-N in addition to mineral media, and nutrient solution. SBR A was fed with CLF as the sole carbon source and in SBR B, propanil was also added to a concentration of 6 mg L^{-1} in the reactor. No biomass was added in this tests in both reactors. The reactors's performance was monitored throught 5 days with daily samples collection to measure the clofibric acid, proanil, DCA, ammonia, nitrite and nitrate concentrations. Once per day the pH of both reactors was measured.

6.2.5 Analytical procedures

6.2.5.1 HPLC-DAD analysis

Samples of both reactors were centrifuged for 5 min at 10000 rpm and injected in a high performance liquid chromatography (HPLC) for quantification of the clofibric acid, propanil, nitrite, nitrate and dichloroaniline (DCA, the primary metabolite of propanil degradation). Standard solutions in the range of 0-2 mg L^{-1} for clofibric acid, and 0-10 mg L^{-1} for propanil and dichloroaniline were used to obtain the respective calibration curves. The quantification of the CLF metabolites was carried out by using the same procedure as for clofibric acid, using the metabolite standards. HPLC-DAD was carried out in a HPLC system (Waters) coupled with a pump and controller (Waters 600), an in-line degasser (X-Act-4 channels, Jour Research), an autosampler (Waters 717 plus), and a photodiode array detector (DAD, Waters 996). Reverse-phase chromatography (LiChroCART 250-4 Purospher Star RP18 endcapped, 5 μm, column, Merck) of the samples was performed with diode array detection from 200 to 400 nm with a 0.6 mL min^{-1} flow, rate of a degassed mobile phase with 70 % methanol and 30% water with pH adjusted to 3 with phosphoric acid, and diode array detection from 200 to 400 nm. The chromatograms were acquired with a MassLinx^{TM} software data acquisition system.

6.2.5.2 GC-MS analysis of CLF metabolites

In order to identify the metabolites generated during CLF biodegradation in the SBRs, samples of both reactors were collected and prepared to be analysed by gas-chromatograpy-mass spectrometry (GC-MS). Samples and standards were previously chemically derivatized.

6.2.5.2.1 Derivatization of the SBR samples

200 μL of sample of the SBR reactor and standard solutions of the metabolites were totally dried with a N_2 stream and then 20 μL of the derivatization reagent MSTFA was added to the vial. The reaction took place for 60 min at 60 ºC and it was then injected in the GC-MS.

6.2.5.2.2 GC-MS Analysis

GC-MS analysis was performed using an Agilent 6850 GC fitted with a 5975 VL MSD (Triple Axis Detector) Agilent mass spectrometric detector. The injection port was operated in splitless mode, during 5 min. A DB-5MS 5% phenyl and 95% dimethylpolysiloxane capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness) from Agilent was used, with helium as carrier gas at a flow rate of 1

mL min^{-1}. The injection port temperature was 250 ºC. The ion source, the quadrupole and the transference line were kept at 230, 150 and 280 ºC, respectively.

The oven temperature was maintained at 60 ºC for 3 min, programmed to 250 ºC at an increase of 10 ºC min^{-1}, then increased to 310 ºC at 20 ºC min^{-1}, and held for 13 min. The MS spectrum was obtained with electron energy 70 eV, mass range *m/z* 40-650 amu and using MSD ChemStation software (Agilent).

The identification of the metabolites was performed by the use of mass spectrum database libraries of NIST (2005) and Wiley (2005), that suggest possible chemical structures for metabolites, which were confirmed by the injection of the derivatized standard. The ChemStation library of the MS search uses probability based matching (PBM) algorithm and a reverse search technique. The PBM algorithm compares an unknown mass spectrum to the reference spectrum using a reverse search routine. A prefilter within the search routine assigns significance to each of the peaks in the unknown spectrum and uses these to find the most probable matches in the condensed reference library. The selected condensed spectra are then compared with the complete unknown spectrum. The prefilter immediately eliminates approximately 95% of the compounds in the database and greatly speeds up the search (when using the default strategy parameters). Each condensed spectrum selected from the database (reference spectrum) by the prefilter is compared with all the peaks in the unknown (which has been normalized). The probability is then calculated. Each condensed spectrum selected from the database (reference spectrum) by the prefilter is compared with all the peaks in the unknown (which has been normalized). The probability is then calculated. After identifying the metabolites generated in the reactors, standards of each compound were also injected to confirm the structures suggested from the libraries databases of the GC-MS. The standards previously identified in GC-MS were also analysed by HPLC-DAD to obtain the chromatographic characteristics of the standards (retention time and absorption spectra in the DAD). Calibration curves were done in order to quantify the metabolites that were generated. This was performed by using the same analytical strategy as for monitoring CLF. Three of the four metabolites were possible to quantify by HPLC-DAD (see section 6.2.5.1).

6.2.5.3 Ammonia analysis

Samples of both reactors were centrifuged, for 5 min at 10 000 rpm and the ammonia concentrations was measured by an ammonia selective electrode (Orion model 720A), where 20 µL of ionic strength adjustor (ISA) solution (951211 ISA, Orion ISE series) was added to 1 mL of sample. Standard solutions of ammonia in the range of 0-20 mg L^{-1} NH_4^+-N have been prepared to obtain the respective calibration curve.

6.2.6 Fluorescence in situ hybridisation (FISH)

Fluorescence *in situ* hybridization (FISH) was carried out according to Amann et al., (1995) in samples of the SBR B for characterization of the autotrophic bacteria. The probes, NSO1225 (Mobarry *et al.*, 1996) for ammonia oxidizing bacteria and Ntspa662 (Daims *et al.*, 2001) for nitrite oxidizing bacteria were tested against the probes for all bacteria, EUBmix (EU338, Amann *et al.*, 1990; EUB338-II and EUB338-III, Daims *et al.*, 1999). FISH samples were observed using an Olympus BX51 epifluorescence microscope.

6.2.7 Determination of the proposed biodegradation mechanism

The biocatalysis and biodegradation database (BBD) software developed by the University of Minnesota (UM) was used to simulate and predict the biodegradation pathway of CLF (Helbling *et al.*, 2010; Gao *et al.*, 2010).

The pathway prediction system (PPS) predicts microbial catabolic reactions using substructure searching by atom-to-atom mapping (Gao *et al.*, 2010). The system is able to recognize organic functional groups found in a compound and predict transformations based on biotransformation rules. The biotransformation rules are based on reactions found in the UM-BBD database or in the scientific literature. Thus, the PPS predicts plausible pathways for microbial degradation of chemical compounds. PPS predictions are most accurate for compounds that are (Gao *et al.*, 2010):

- similar to compounds whose biodegradation pathways are reported in the scientific literature;
- in environments exposed to air, in moist soil or water, at moderate temperatures and pH, with no competing chemicals or toxins; and
- the sole source of energy, carbon, nitrogen, or other essential element for the microbes in these environments, rather than present in trace amounts.

The simulation of the reaction was carried out under aerobic conditions, exposed to air, soil (moderate moisture) or water, at neutral pH, 25 °C, with no competing or other toxic compounds.

6.3 Results and Discussion

6.3.1 Clofibric acid biotransformation

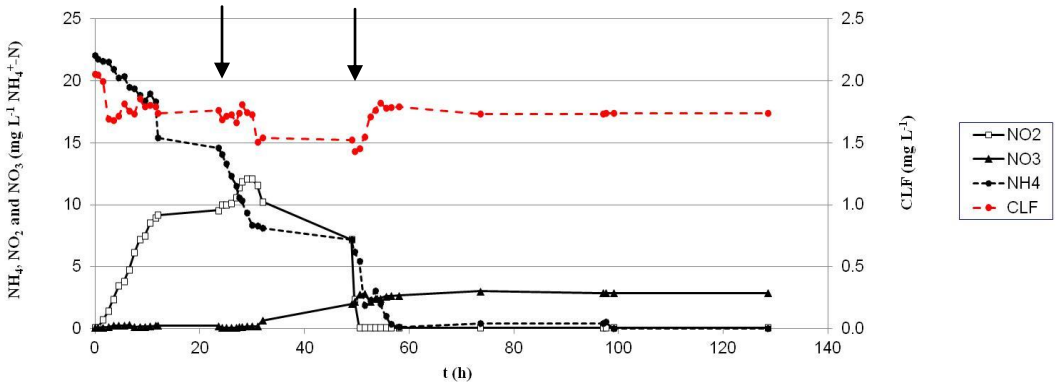
Initially, activated sludge from a WWTP was used as the inoculum for a sequencing batch reactor (SBR) fed with 300 µg L^{-1} CLF and glucose as a supplemental carbon source to facilitate biomass growth and potentially co-metabolism of CLF. This system was operated for more than 2 months,

where minimal CLF removal was observed (data not shown). At this time the CLF concentration in the feed was increased to 1200 µg L^{-1} in order to confirm that the CLF concentration was not limiting. However, CLF removal was still not observed after another 2 months of SBR operation (data not shown).

Thereafter, a microbial enrichment obtained from an agricultural soil exposed to the herbicide propanil, a compound that is structurally similar to CLF, was tested. This culture was shown to be able to degrade propanil as the only carbon source (Carvalho *et al.*, 2010)

Two sequencing batch reactors (SBRs) were inoculated with this enriched culture. In one SBR, clofibric acid was added as the sole carbon source (SBR A) and in the other, propanil and clofibric acid (SBR B) were added together to facilitate biomass growth and potentially co-metabolism. The initial CLF and propanil concentrations were 2 mg L^{-1} and 6 mg L^{-1}, respectively, Figure 6.1a) and 6.1b).

a)



b)

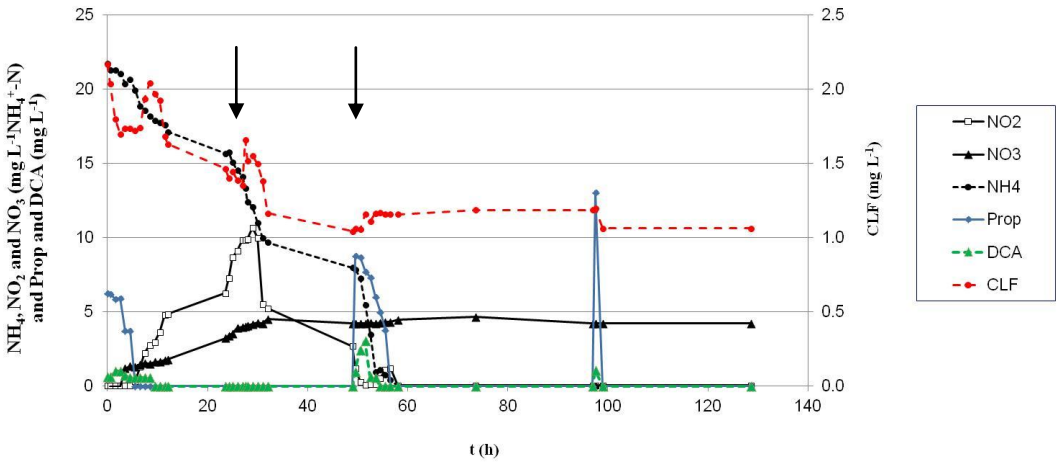


Figure 6.1 - Clofibric acid (CLF) degradation in SBR A (without propanil) (a) and SBR B (with propanil) (b). Arrows indicate pH adjustments.

SBR A showed lower CLF removal (15%) as compared to SBR B (51%), suggesting that the addition of propanil to the SBR increased the CLF degradation capacity. In separate sets of tests, the abiotic transformation of CLF (using the same conditions but no microbial culture added) was found to be negligible, as was the quantity of CLF adsorbed to the biomass (data not shown), suggesting that the removal observed in Figure 6.1 could be attributed to CLF biodegradation. In previous work, Tran *et al.* (2009) showed that the presence of acetate (100 mg L^{-1}), as an additional carbon source to the biotransformation study of CLF, increased its removal from almost 10% in the absence of acetate to 35% in its presence (Tran *et al.*, 2009). Similarly to our study, the addition of another carbon source likely induced biomass growth, leading to higher CLF removal. The biomass concentration of SBR B was more than double that of SBR A (1026 mg VSS L^{-1} vs 472 mg VSS L^{-1}, respectively). Despite this difference in biomass concentration, SBR B had a superior specific CLF removal as compared to SBR A. In SBR B, propanil biodegradation led to the temporary accumulation of its well known metabolite, 3,4-dichloroaniline (DCA), where the DCA was completely removed after one day.

Since substantial CLF biotransformation occurred in SBR B, further analysis was performed to investigate the mechanism responsible for this biotransformation. Biodegradation was observed until all of the ammonia was consumed in this reactor, suggesting that the nitrifiers may have been responsible for this biodegradation. Many previous studies have suggested a co-metabolic action of ammonia and aromatic compounds in nitrifying bacteria (Perkins *et al.*, 1994; Batt *et al.*, 2006; Yi *et al.*, 2007). Yi *et al.* (2007) proposed that the dioxygenase enzymes are capable of mediating the co-metabolic biotransformation of aromatic compounds (for biotransformation of 17α-ethinylestradiol), which was not only associated to nitrifiers but also to heterotrophic bacteria. The co-metabolism of PhACs (such as CLF) may be important because AMO (ammonium monooxygenase) has a relatively wide spectrum for the degradation of substrates (Tran *et al.*, 2009).

Since nitrification to nitrite and nitrate occurred in both reactors, FISH analysis was performed to study the autotrophic microbial community. FISH confirmed that both reactors were dominated by ammonia-oxidizing bacteria (AOB), which were targeted by FISH probe Nso1225. Each SBR also contained some nitrite oxidizing bacteria (NOB) as detected by the Ntspa662 probe, for the determination of the presence of *Nitrospira* and Nit3 for *Nitrobacter*. The high level of autotrophic nitrifiers is not surprising considering the low quantity of organic matter fed to each SBR and the relatively high content of ammonia.

In order to study whether or not nitrifiers were really responsible for CLF biodegradation, an additional batch test was performed on SBR B with a higher and non-limiting ammonia concentration (initially 20 mg $L^{-1}NH_4^+$-N, followed by two additional spikes of 5 mg L^{-1} each). The performance of SBR B without ammonia limitation is shown in Figure 6.2. Results revealed that a similar amount of CLF biodegradation could be observed (41%) in SBR B as compared to Figure 6.1b, suggesting that CLF biotransformation was not linked directly with the abundance of ammonia.

Further, the accumulation of nitrite was observed in both experiments (Figures 6.1 and 6.2). Nitrite has been frequently shown to be an inhibitor to many different types of bacteria (Zhou *et al.*, 2011). Thus, it was investigated if nitrite inhibition represented the limiting factor towards achieving CLF biodegradation.

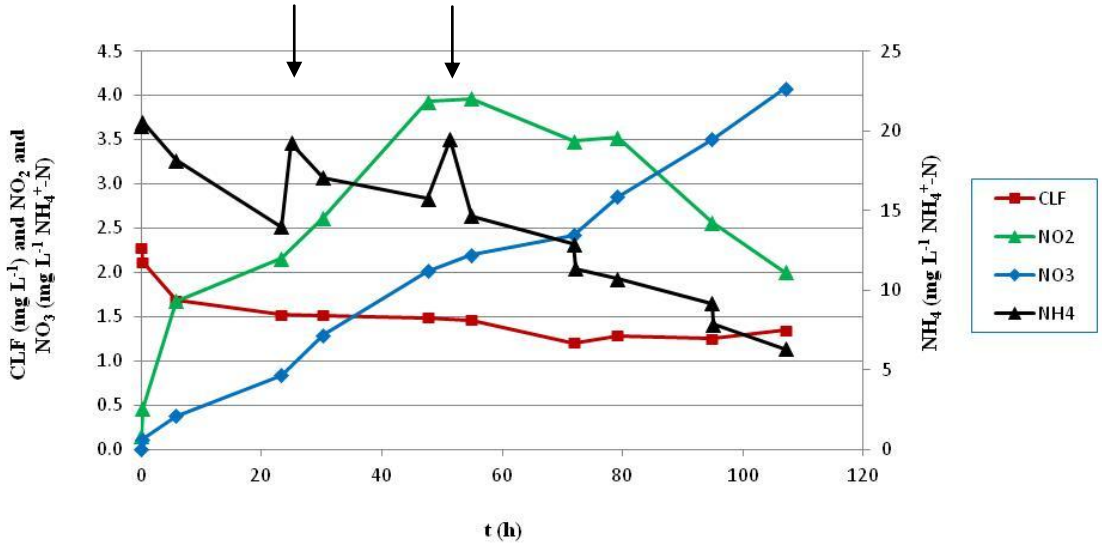


Figure 6.2 - Clofibric acid degradation in SBR B (with propanil) with no NH_4^+ limitation. Arrows indicate 5 mg L^{-1} NH_4^+-N spikes.

6.3.2 Nitrification inhibition

Figure 6.3 shows a batch test carried out in SBR B where nitrification was inhibited by ATU. In this test, negligible ammonia was consumed and nitrite and nitrate were not produced. Nevertheless, CLF biodegradation decreased only slightly (from 41% to 28%), suggesting that CLF removal was not mainly carried out by nitrifiers, but more likely by heterotrophs. Furthermore, since nitrite was not produced in this assay, the hypothesis of nitrite inhibition leading to the stop of CLF biodegradation was also discarded.

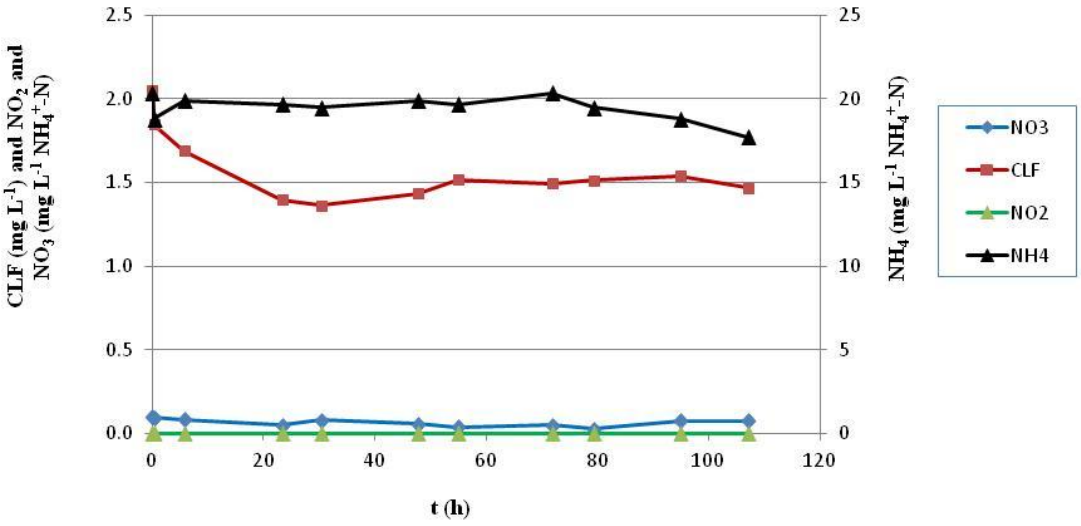


Figure 6.3 - SBR B operated with 1 mg L^{-1} ATU.

It is not clear why CLF biodegradation proceeded until a certain value and then did not continue (Figures 6.1-6.3). One possibility is that CLF removal is limited by an unknown nutrient present in the media or to the accumulation of an inhibitory metabolite in the culture medium. Analysis of spectra obtained by GC-MS along the reactor cycle showed that some metabolites (see section 6.3.3) temporarily accumulated in the culture media, but were later eliminated. Accumulation of these metabolites did not cause inhibition of CLF biodegradation, since even after being eliminated, the CLF concentration did not decrease further.

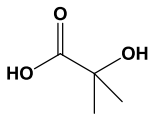
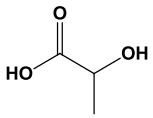
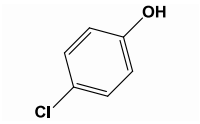
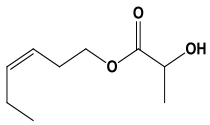
To the best of our knowledge, the percentage of CLF biodegradation obtained in this study is higher than those previously reported in the literature. While Evangelista *et al.*, (2010) found that >90% of a 100 mg L^{-1} CLF solution was transformed by *Rhodococcus rhodochrous* in 20 days after acclimatization, it was mainly transformed into its parent compound ethyl clofibrate, which accumulated in the solution. Winkler *et al.*, (2001) found that CLF was not removed at an initial concentration of 90 μg L^{-1} (over ~400 h) and only 27% removal was obtained at 11 μg L^{-1} (over ~95 h) in a biofilm reactor fed with river water. The elimination of CLF was less than 30% (200-500 μg L^{-1}) even with a high HRT of 48 h (Kosjek *et al.*, 2009). Similarly a maximum removal of ~25% was obtained for CLF with 200 mg L^{-1} $N\text{-}NH_4^+$ addition and an initial CLF concentration of 100 μg L^{-1} after 6 days of cultivation (Tran *et al.*, 2009).

6.3.3 Products of microbial clofibric acid transformation

Identification of the metabolites produced from clofibric acid degradation was done by GC-MS analysis of the bioreactor effluent samples. Table 6.2 shows the chemical structures of the 4 identified

compounds by GC-MS with their respective matches for the mass spectra comparison between the NIST and Wiley databases and the reactor samples.

Table 6.2 - Metabolite characteristics of chromatograms of GC-MS and HPLC-DAD analysis.

				GC-MS			HPLC-DAD	
Metabolite	**M_W**	**Formula**	**Chemical structure**	**t_r (min)**	***m/z* (%)***	**Match/R.Match/ Probability with spectra (%)**	**t_r (min)**	**λ (nm)**
α-hydroxyisobutyric acid (AHIBA)	104	$C_4H_8O_3$		7.98	45(12), 73(100), 117(80), 131(100), 147(60), 205(20), 233(12)	91.1/97.1/60.4	3.28	211, <u>250</u>
lactic acid (LA)	90	$C_3H_6O_3$		8.14	45 (20), 73(100), 117(75), 147(75), 162 (3), 191(12), 219(6)	94.9/97.1/78.8	3.48	258
4-chlorophenol (4-CP)	129	C_6H_5ClO		12.47	65 (30), 73 (18), 100 (12), 128 (100), 202 (3)	81.4/86.4/62.6	8.24	209, <u>281</u>
cis-3-hexenyllactate	172	$C_9H_{16}O_3$		7,01	45(100), 67(69) 73(12), 82(90), 99(12), 115(10), 172(10)	65.9/72.3/40.6	7.11	197, <u>277</u>

**m/z* obtained by derivatization with MSTFA, trimethylsilyl ethers; TMSi (-$Si(CH_3)_3$) *m/z* 73

GC-MS analysis was able to identify 4 metabolites generated in the reactors as well as the remaining CLF in solution. The match (similarities) between the sample and the MS database spectra during GC-MS analysis were higher than 81% with the exception of the metabolite, *cis*-3-hexenyllactate, which was only 66%.

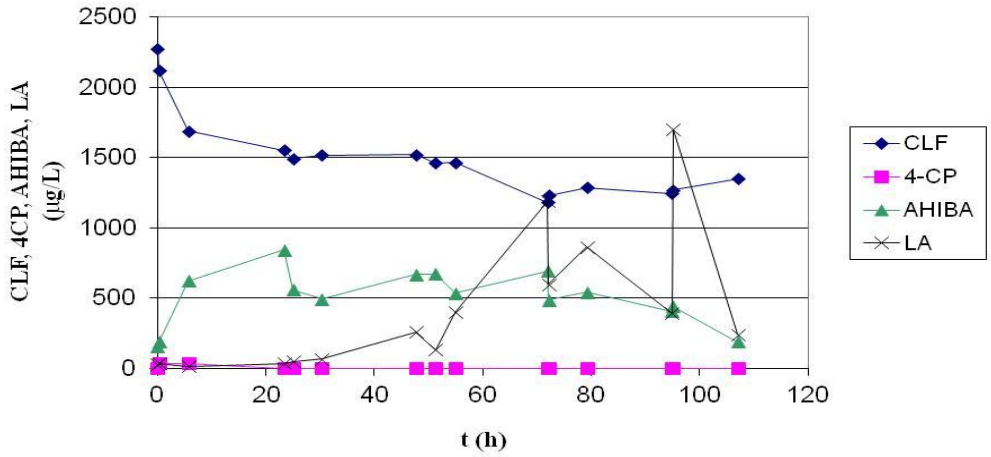
The metabolite *cis*-3-hexenyllactate did not appear in the HPLC-DAD chromatograms of the monitored reactor samples, suggesting that this compound was unstable and/or an intermediary metabolite present at concentrations below the level of quantification. The absence of *cis*-3-hexenyllactate was also in agreement with the GC-MS results, where low confidence was obtained in the identification of this compound.

The main metabolites detected were α-hydroxyisobutyric acid (AHIBA), followed by lactic acid (LA) and, in lower concentrations, 4-chlorophenol (4-CP). The quantification of the three main metabolites

by HPLC-DAD from the biotransformation of CLF in SBR B: AHIBA, LA and 4-CP, is plotted in Figure 6.4a and 6.4b.

4-chlorophenol was generated by the cleavage of the ether bond of CLF, and has previously been recognized as a metabolite of CLF by Kosjek *et al.*, (2009). 4-CP is known to have higher toxicity than the parent compound, CLF. In our experiments, 4-chlorophenol was temporarily detected in very low concentrations (low µg L^{-1}) throughout the SBR cycle (Figure 6.4a and 6.4b). Pérez *et al.*, (1997) found that chlorophenol was biodegraded, in their tests with *Phanerochaete chrysosporium*. Biodegradation was enhanced from 25% to 50% for an initial concentration of 100 mg L^{-1} of 4-chlorophenol with the addition of glucose (10 g L^{-1}) in that study. Similar results have been obtained by Buitrón *et al.*, (2011), where after 1 h of reactor operation, 18 mg L^{-1} of 4-chlorophenol was biodegraded.

a)



b)

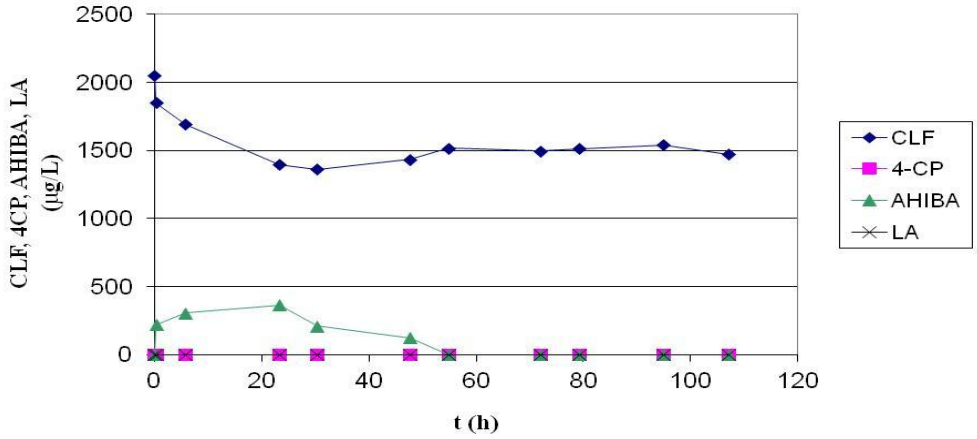


Figure 6.4 - Metabolites generated from CLF degradation in SBR B (with propanil) at 20 mg L^{-1} NH_4^+-N (a) and with 1 mg L^{-1} of ATU (b).

During the biodegradation of CLF, AHIBA was the most important metabolite formed initially, and appeared almost immediately after inoculation (Figure 6.4a and 6.4b). All of the metabolites were biodegraded in the SBR and a conversion of AHIBA into LA seemed to begin after 30 h when nitrification was not inhibited (Figure 6.4a). After 72 h, the CLF and metabolites biodegradation seemed to slow down and LA was the metabolite produced in the highest abundance overall when nitrification was not inhibited, but even this metabolite was mostly consumed by the end of the cycle (after 107 h). The metabolite 4-CP was produced in only low concentrations and was immediately consumed thereafter.

When the nitrification was inhibited with ATU (Figure 6.4b), there was a lower accumulation of metabolites detected during the experiment, only a small quantity of AHIBA was formed, whivh was then quickly consumed. The nitrification inhibition experiment showed that the generated metabolites were removed from the process at a higher rate, or that they were not generated in the first place. This could suggest that some of the metabolites result from partial CLF degradation concomitant to autotrophic activity, or that the nitrite produced when nitrification was uninhibited (Figure 6.2) caused inhibition to the metabolite degradation, but not the degradation of the parent compound (CLF).

Figure 6.5 shows the simulation of the biodegradation pathway for clofibric acid under aerobic conditions, using the UM-BBD software as described in section 6.2.7. Three of the metabolites suggested by the software are in agreement with the results obtained by GC-MS and HPLC-DAD: specifically AHIBA, LA and 4-CP. Two other metabolites proposed, the acetone, and an opening of the aromatic ring, (*Z*)-4-chloro-5-oxohex-2-enedioic acid, are hypothesized to be major compounds in the second step of the biotransformation of CLF. The acetone was not detected neither in the HPLC-DAD nor the GC-MS, likely because it is a structure that is very easy to biodegrade. The (*Z*)-4-chloro-5-oxohex-2-enedioic acid compound was also not detected, but could have been a limiting intermediate in the reaction with AHIBA that yields lactic acid, which was detected.

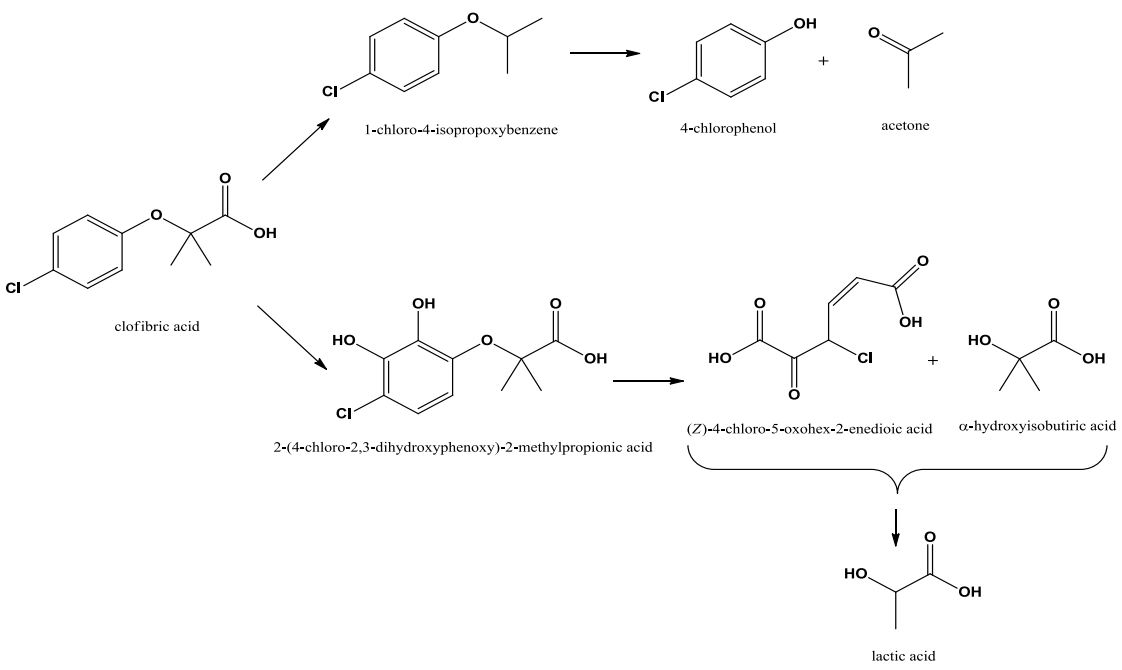


Figure 6.5 - Mechanism of biotransformation of clofibric acid proposed by the biocatalysis and biotransformation database of the University of Minnesota.

This proposal for the biotransformation of CLF under aerobic conditions, suggests that the formation of AHIBA and LA occurs at a faster rate than 4-CP, which was confirmed by HPLC-DAD results showing that greater AHIBA and LA accumulation was observed. This work, thus contributed towards understanding in mechanism of CLF biodegradation, where the main degradation pathway for CLF appeard to be the AHIBA – LA pathways.

6.4 Conclusions

In this study, CLF biodegradation was observed to a greater extent in a SBR fed with CLF and propanil as compared to CLF as the sole carbon source. Nevertheless, the presence of propanil was confirmed not to be essential towards co-metabolism of the clofibric acid. The heterotrophic populations within the bioreactor were likely more responsible for CLF biodegradation than the autotrophic populations, after testing without ammonia limitation and with inhibition of nitrification. Combining the information from GC-MS and HPLC-DAD, it was possible to identify and quantify three main metabolites produced in the reactors, including α-hydroxyisobutyric acid, lactic acid and 4-chlorophenol. The α-hydroxyisobutyric acid was the first important metabolite produced, and accumulated in the reactor, getting transformed into LA over time. The 4-chlorophenol was detected only in small concentrations, due either to fast biodegradation when compared to the other metabolites or due to only a small amount of CLF being channeled through the 4-CP pathway. It is unclear why

the biotransformation of CLF slows down before it is fully consumed, but could perhaps be due to the limitation of a specific nutrient in the media that regulates the biodegradation reaction.

CHAPTER 7

PHOTODEGRADATION KINETICS AND INTERMEDIATES OF KETOPROFEN, DICLOFENAC AND ATENOLOL IN PURE WATER AND TREATED WASTEWATER

7 Photodegradation kinetics and intermediates of ketoprofen, diclofenac and atenolol in pure water and treated wastewater

Pharmaceutical compounds such as ketoprofen, diclofenac and atenolol are frequently detected at relatively high concentrations in secondary effluents from wastewater treatment plants. Therefore, it is important to assess their transformation kinetics and intermediates in subsequent disinfection processes, such as direct ultraviolet (UV) irradiation. The photodegradation kinetics of these compounds using a medium pressure (MP) lamp was assessed in pure water, as well as in filtered and unfiltered treated wastewater. Ketoprofen had the highest time- and fluence-based rate constants in all experiments, whereas atenolol had the lowest values, which is consistent with the corresponding decadic molar absorption coefficient and quantum yield. Not surprisingly, the fluence-based rate constants of all compounds were lower in a wastewater matrix than in pure water. However, particulated organic matter had an enhancement effect on photolysis, probably due to the stronger indirect action of free radicals generated in this matrix due to the higher concentration of organic matter. Finally, the transformation products of ketoprofen, diclofenac and atenolol were identified and monitored throughout the irradiation experiments. This enabled the identification of persistent transformation products, which can be discharged from WWTP disinfection works using UV photolysis.

7.1 Introduction

In the past decade, there has been a growing concern about the discharge of pharmaceutical active compounds (PhACs) from wastewater treatment plants (WWTP) (Fatta-Kassinos *et al.*, 2011; Kolpin *et al.*, 2002). These compounds are designed to have an impact on life and are often resistant to biological degradation in the treatment systems. Therefore, it is important to assess the fate of PhACs in the tertiary treatment of WWTPs where physical/chemical methods are often used. UV irradiation is often employed for disinfection of drinking water and municipal WWTP (Oppenländer, 2003), and it has also been demonstrated to effectively reduce the concentration of recalcitrant organic compounds (Canonica *et al.*, 2008; Rosario-Ortiz *et al.*, 2010). However, this process can also generate photodegradation intermediates that are more recalcitrant or toxic than the parent compounds. The compounds selected for this study were widely used pharmaceutical compounds with different chemical structures: ketoprofen, diclofenac (non-steroidal anti-inflammatory drugs), and atenolol (a β-blocker). These compounds were found in relatively high concentrations (up to 21.6 µg L^{-1}, Salgado *et al.*, 2010 and Salgado *et al.*, submitted) in the effluent of the secondary settler of municipal WWTPs, thus reaching a subsequent disinfection process. The photolysis of ketoprofen, diclofenac and atenolol has been previously investigated, mostly in distilled water and surface water matrices (e.g. Pereira *et a.l*, 2007a and b, Szabo *et al.*, 2011, Canonica *et al.*, 2008). Baeza and Knappe (2011) recently studied

the kinetics of diclofenac using low pressure (LP) direct and indirect UV photolysis in ultrapure water, lake water and also in wastewater effluent and found that the degradation rate was similar across all matrices. Good LP/UV photodegradation of ketoprofen and diclofenac in treated wastewater was demonstrated by Kim *et al*. (2009, JHM), although atenolol only showed medium removal. Also Rosario-Ortiz et al. (2010) reported low atenolol removal in WWTP effluent through LP/UV photolysis. Nevertheless, very few studies reported the effect of treated wastewater on photodegradation using medium pressure (MP) lamps. LP systems are known by their high disinfection efficiency since they irradiate at a wavelength close to the maximum absorption of DNA. However, MP lamps may be preferred in compact treatment systems, since the UV intensity per lamp is higher than in LP systems (Oppenländer, 2003).

The motivation for this study was to investigate the degradation kinetics and extent of transformation of selected PhACs by UV radiation used for wastewater disinfection purposes (i.e. direct photolysis). UV photolysis can be strongly affected by the presence of other organic compounds (other PhACs or dissolved organic matter), or particulated matter. In the present study, the photodegradation kinetics of ketoprofen, diclofenac and atenolol were assessed in a reactor equipped with a MP lamp, in filtered and unfiltered treatment wastewater, and compared to the results obtained in pure water. Moreover, the photodegradation products of each compound were identified and monitored along irradiation time. To the best of our knowledge, this is the first study identifying the transformation products of ketoprofen, diclofenac and atenolol with direct photolysis.

7.2 Materials and Methods

7.2.1 Reagents

The PhACs used in this study were atenolol, diclofenac and ketoprofen (Discovery CPR, Sigma-Aldrich, Portugal). Atrazine (Discovery CPR, Sigma-Aldrich, Portugal) was used for actinometry of the UV medium pressure lamp. For each compound was prepared a solution standard 1.0 mg mL^{-1} in methanol and stored at 4 °C. The mobile phases used in HPLC were acetonitrile (HPLC grade, Panreac, Portugal) and ultra pure water obtained from MilliQ50 system water purification (Millipore, Bedford, USA), acidified with formic acid (analytical grade, Merck, Portugal). The derivatization reagent used for GC analysis was MSTFA (*N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide) (GC grade, Sigma-Aldrich, Portugal).

7.2.2 UV irradiation experiments

7.2.2.1 Photolysis with UV mercury vapour lamp, low pressure (LP)

LP/UV photolysis experiments were conducted in a collimated beam bench-scale reactor (Trojan Technologies Inc., Canada) using a low pressure Hg lamp that emits mainly monochromatic light at 254 nm.

100 mL of laboratory grade water were spiked with the appropriate volume of each pharmaceutical's stock solutions to achieve concentrations of 1 mg L^{-1}. 50 mL of sample were placed in a Petri dish and continuously stirred underneath the LP/UV lamp. The remaining 50 mL were used as control and kept in the dark under identical experimental conditions in order to determine possible PhACs losses due to evaporation or adsorption to the Petri dish walls. All experiments were conducted at room temperature (21±2 ºC). The lamp irradiance was measured using a calibrated radiometer (IL1700, International Light, Newburyport, MA) which was placed at the same height of the water level in the Petri dish and the solution transmittance was measured by a UV photometer (P254C, Trojan Technologies Inc.). UV fluences of approximately 0, 100, 500, 750, 1000 and 1500 mJ cm^{-2} were selected (taking into account the radiometer meter reading as well as Petri, reflection, water, and divergence factors) to establish the corresponding exposure times at which 200 μL of sample were taken to quantify the concentration of the compounds by HPLC-UV and LC-MS/MS analysis.

7.2.2.2 Photolysis with UV mercury vapour lamp, medium pressure (MP)

The photodegradation tests were carried out in a pear-shaped glass reactor with a maximum volume of 300 mL, using a medium pressure (MP) Hg lamp, Heraeus Noblelight model TQ 150 (nominal power 150 W) which emits radiation between 200 and 450 nm. The lamp was covered with a quartz cooling jacket, where pure water (with negligible light adsorption in the wavelength range of emitted radiation) was used as optical filter and to maintain a temperature of 25±1 ºC.

300 mL of pure water was added to the reactor and it was spiked with atenolol, ketoprofen or diclofenac to get a concentration of 1 mg L^{-1}, or a mixture of the three compounds with the same concentration of each. The homogenization of the mixture of the PhAC and the pure water was obtained by bubbling air in the UV reactor. In order to calibrate the MP/UV lamp emission spectrum of the lamp, and the irradiation parameters, atrazine was added in the experiments. The photolysis of a mixture of the three PhACs was also carried out in 300 mL of filtered and unfiltered secondary effluent of a biological WWTP (Fernão Ferro, Portugal).

2 mL samples were taken throughout the experiments to assess the photolysis of the PhACs in the different experimental conditions through HPLC analysis. Samples were also taken for identification of photolysis transformation products at critical points of the photodegradation experiments

(ketoprofen, LP - 2.5 h; ketoprofen, MP - 7.5 min; diclofenac - 1.5 min; atenolol - 17.5 min), where the highest relative area of new chromatographic peaks was detected.

Fluence rates under MP Hg lamp irradiation were determined by chemical actinometry at low optical density (Canonica *et al.*, 2008) using 4.6 µM aqueous atrazine as an actinometer, following the procedure described in Canonica *et al.*, (2008), i.e. assuming a wavelength-independent quantum yield and using the emission spectrum of the MP Hg lamp.

$$E_p^o(200-450nm) = \frac{k_{atr}}{2.303.\phi_{atr}.\sum_{200nm}^{450nm}(f_{f,\lambda}.\varepsilon_{p,\lambda})} \qquad \text{(Eq. 7.1)}$$

where E_p^0(200 – 450 nm) (einstein m^{-2} s^{-1}) is the photon fluence rate determine through atrazine actinometry in the wavelength interval of 200 to 450 nm, k_{atr} (s^{-1})is the pseudo-first-order rate constant of atrazine depletion, ϕ_{atr} is the quantum yield of atrazine depletion (= 0.046 mol $einstein^{-1}$, Canonica *et al.*, 2008), $f_{p,\lambda}$ is the emission spectrum of the lamp based on the photon flux and normalized to the chosen wavelength interval, i.e., $\sum_{200nm}^{450nm}(f_{f,\lambda}) = 1$and $\varepsilon_{atr,\lambda}$ (M^{-1} cm^{-1}) is the molar absorption coefficient of atrazine at wavelength λ (=3860M^{-1} cm^{-1}, Canonica *et al.*, 2008).

7.2.3 Analytical procedures

7.2.3.1 HPLC-DAD-MS analysis

HPLC analyses with a diode array detector (DAD) were used to monitor diclofenac, ketoprofen, atenolol and atrazine degradation kinetics. HPLC-DAD was carried out in a HPLC system (Waters) coupled with a pump and controller (Waters 600), an in-line degasser (X-Act-4 channels, Jour Research), an autosampler (Waters 717 plus), a photodiode array detector (DAD, Waters 996). Reverse-phase chromatography (LiChroCART 250-4 Purospher Star RP18 endcapped, 5 µm, column, Merck) of the samples (injection volume of 50 µL) was performed using a degassed mobile phase with 70 % water with 0.01% formic acid and 30% acetonitrile with a 0.6 mL min^{-1} flow rate, with DAD detection from 200 to 400 nm. The chromatograms were acquired with a MassLinx™ software data acquisition system. The (ESI+)MS was carried out a quadropole VG Platform (Micromass, UK Ltd) spectrometer equipped with an electrospray ionisation (ESI) source operating in positive mode. An accurate splitter (split ratio of 1:10) was used between the HPLC column and the mass spectrometer. Capillary temperature was kept between 100 ºC and 120 ºC, using a scanning cone voltage from 35 to 100 V and capillary voltage of 3.5 kV. Nitrogen was used as drying and nebulising gas at 300 mL min^{-1} and 10 mL min^{-1}, respectively. The mass/charge spectrum range used was 100-

450 amu with a MassLynx^{TM} software data acquisition system. Limits of detection of ketoprofen, diclofenac and atenolol were 23, 129 and 78 μg L^{-1}, respectively.

7.2.3.2 LC-MS/MS Analysis

Two LC-MS/MS systems were used for identification of the photolysis intermediates. The LC-MS/MS (BfG Lab, Koblenz, Germany) analysis of ketoprofen and diclofenac metabolites generated in LP UV lamp was carried out using an Agilent 1200 HPLC system. The reverse-phase chromatography (*Zorbac* XDB/*C8 column*, 4.62 x 150 mm, 5 μm*)* was operated at an oven temperature of 30 ºC. The samples (10 μL) were injected into the LC system (including degasser, quaternary pump and autosampler, Agilent Technologies, Waldbronn, Germany) using water/formic acid 0.2% (A) and acetonitrile/formic acid 0.1% (B), as the mobile phases. The following binary gradient was used: start with 100% A, kept isocratic for 1 min, linearly increased to 100% B in 15 min. It was maintained at 100% B for 5.5 min, and then linearly decreased back to 100 % A in 5 min, which was maintained for an additional 1 min. The mobile phase flow rate was kept constant at 400 μL min^{-1} during the analysis. The tandem mass spectrometer (API 4000 QTrap with turbo/electrospray ionization and nitrogen as the collision gas; Applied Biosystem, Foster city, CA) was operated with atmospheric-pressure chemical ionization (APCI) in positive ionic mode using multiple reaction monitoring for all measurements. The source temperature was 450 ºC and the entrance potential, 10 V. The ionspray voltage was adjusted to 5 kV. The MS analysis was carried out in a hybrid triple quadrupole/linear ion trap mass spectrometer LC-MS/MS system. The spectra mass/charge range used was 120 to 500 amu, with Q-MS (Q1). Data acquisition was carried out by Analyst 1.4 Software.
The LC-MS/MS (EPAL, Lisbon, Portugal) analysis of atenolol, ketoprofen and diclofenac metabolites generated in MP UV lamp was performed using a Waters UPLC Acquity system from Waters equipped with a binary pump, an automatic injector and a thermostatic column compartment coupled to a Mass Spectrometer Quattro micro API triple quadrupole and Acquity TDQ equipped with a Z-spray electrospray interface (Micromass). Chromatographic separation was achieved with an Acquity BEH C18 (2.1 x 50 mm, 1.7 μm) from Waters. The tandem mass spectrometer was operated with electrospray ionization (ESI) in positive and negative ionization modes using Full Scan, Product Ion Scan and Multiple Reaction Monitoring modes with the same mobile phase of the other LC-MS/MS system.

7.2.3.3 GC-MS analysis

Derivatization was used to achieve a chemical modification of the acid compounds that would not otherwise be suitable for GC analysis. The fragmentation pattern characteristic of the

chemical derivatives formed can then be used as a mass spectral fingerprint to confirm the identity of the original compounds.

Derivatization reaction: 200 μL of sample was evaporated in an N_2 stream to remove all the water. 20 μL of the derivatization reagent *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was added to the vial and the mixture was incubated at 60 ºC for 60 min.

GC-MS analysis: the gas chromatograph was an Agilent 6850 fitted with a 5975 VL MSD (Triple Axis Detector) mass spectrometric detector (Agilent Technologies, Waldbronn, Germany). The injection port was operated in splitless mode, during 5 min. A DB-5MS, 5% phenyl and 95% dimethylpolysiloxane capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness) from Agilent was used, with helium as carrier gas at a flow rate of 1 mL min^{-1}. The injection port temperature was 250 ºC. The ion source, the quadrupole and the transference line were kept at 230, 150 and 280 ºC, respectively. The oven temperature was maintained at 60 ºC for 3 min, programmed to 250 ºC at 10 ºC min^{-1}, then programmed to 310 ºC at 20 ºC min^{-1}, and held 13 min. The MS spectrum was obtained with electron energy 70 eV, mass range *m/z* 40-650 amu and using MSD ChemStation software. The identification of the photolysis intermediates was possible through a mass spectra database library of NIST (2005) and Wiley (2005) that suggest possible chemical structures, followed by the injection of standards derivatized with the same procedure as the samples.

7.3 Results and Discussion

7.3.1 Low pressure and medium pressure UV photoliability

Direct photolysis can only take place when a compound is able to absorb light at the wavelengths to which it is exposed. A measure of this absorption capacity, or photoliability, is given by the decadic molar absorption coefficient (ε), defined as the probability that a compound will absorb light at a certain wavelength (λ). In this study, the decadic molar absorption coefficient determined for the LP/UV lamp (λ=254 nm) for ketoprofen was in the same order of magnitude than the value obtained by Pereira et al. (2007), and was 1 or 2 orders of magnitude higher than for diclofenac and atenolol, respectively (Table 7.1), showing that ketoprofen has a higher probability to absorb monochromatic light at λ=254 nm than the other compounds. Therefore, LP/UV photolysis was only assessed for ketoprofen in this study, while MP/UV photolysis efficiency was investigated for the three target compounds.

Table 7.1 - Decadic molar absorption coefficient at 254 nm of the studied PhACs, and specific rate of light absorption and quantum yield for the interval 200-450 nm.

Compound	ε (254 nm) (M^{-1} cm^{-1})	Σ k_s (200-450 nm) (einstein mol^{-1} s^{-1})	ϕ (mol $einstein^{-1}$)
Ketoprofen	37155	0.00231	0.759
Atenolol	527	0.00372	0.036
Diclofenac	5929	0.07121	0.066

The decadic molar absorption coefficient was also determined for a wavelength range including that covered by the MP/UV lamp (200-450 nm). The profile obtained for ketoprofen was overall higher than the other two compounds (Figure 7.1), which shows the stronger photoliability of ketoprofen for MP/UV irradiation, followed by diclofenac and finally, atenolol. The latter displayed low ε values for wavelengths >200nm, thus it was expected to be degraded to a lesser extent than the other compounds in the conditions investigated in this study.

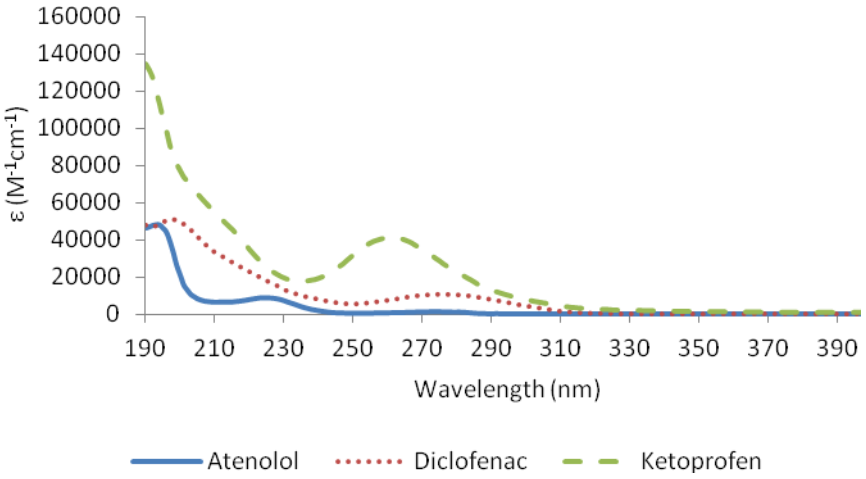


Figure 7.1 - Decadic molar absorption coefficient (ε) for atenolol, diclofenac and ketoprofen. MP/UV lamp used in this study covered λ=200-450 nm.

Another important parameter to assess the direct photolysis of a compound is the quantum yield (ϕ), which gives the number of molecules degraded per photon absorbed by the solution due to the compound's presence. This implies that the degradation rate of a compound is proportional to the compound's quantum yield (Pereira et al., 2007a). Thus, the quantum yield (mol $einstein^{-1}$) for each compound in the wavelength interval used in this study (200-450 nm) was obtained from:

$$\phi = \frac{k}{\sum_{200nm}^{450nm} k_s} \qquad \text{(Eq. 7.2)}$$

where $k_s(\lambda)$ is the specific rate of light absorption by the compound, determined by equation 7.3 (Pereira *et al.*, 2007a).

$$k_s(\lambda) = \frac{E_p^o.\varepsilon.(1-10^{-Abs(\lambda).z})}{Abs(\lambda).z} \qquad \text{(Eq. 7.3)}$$

where $E_p^{\,0}$ (einstein cm^{-2} s^{-1}) is the photon fluence rate at 200-450 nm determined according to equation 7.1, ε (M^{-1} cm^{-1}) is the decadic molar absorption coefficient, Abs(λ) is the compound absorbance at wavelength λ, and z is the solution depth (2.75 cm for the reactor used in this study).

The quantum yield obtained for ketoprofen (Table 7.1) was substantially higher than the two other compounds. This result, combined with the higher decadic molar absorption coefficient profile (Figure 7.1) shows that light absorbed by ketoprofen results in high degradation of this compound. Despite the intermediate ε profile observed for diclofenac, its quantum yield value had the same order of magnitude as atenolol. The low quantum yield observed for the atenolol is consistent with its low decadic molar absorption coefficient and indicates its low photodegradation by direct photolysis.

7.3.2. Photolysis kinetics in pure water

The photodegradation of the three studied PhACs in pure water followed a pseudo-first-order kinetics (Figure 7.2), and the time-based pseudo-first-order rate constants (*k*) were determined according to equation 7.5 (Lopez *et al.*, 2003, Kim *et al.*, 2009):

$$\ln\left[\frac{C}{C_o}\right] = -k.t \qquad \text{(Eq. 7.4)}$$

where C is the concentration of the PhAC and C_0 is the initial concentration of the PhAC.
The time-based rate constants, *k* (s^{-1}), were converted to photon fluence-based rate constants, $k_{E_p^o}$ (m^2 $einstein^{-1}$), using the following equation:

$$k_{E_p^o} = \frac{k}{E_p^o} \qquad \text{(Eq. 7.5)}$$

where the photon fluence rates ($E_p^{\,0}$) were determined through actinometry (equation 7.1). The $k_{E_p^o}$ is a measure of the sensitivity of the photochemical transformation, it is independent of the photon fluence

rate variations and allows to directly compare constant rates obtained with different equipments (Canonica *et al.*, 2008).

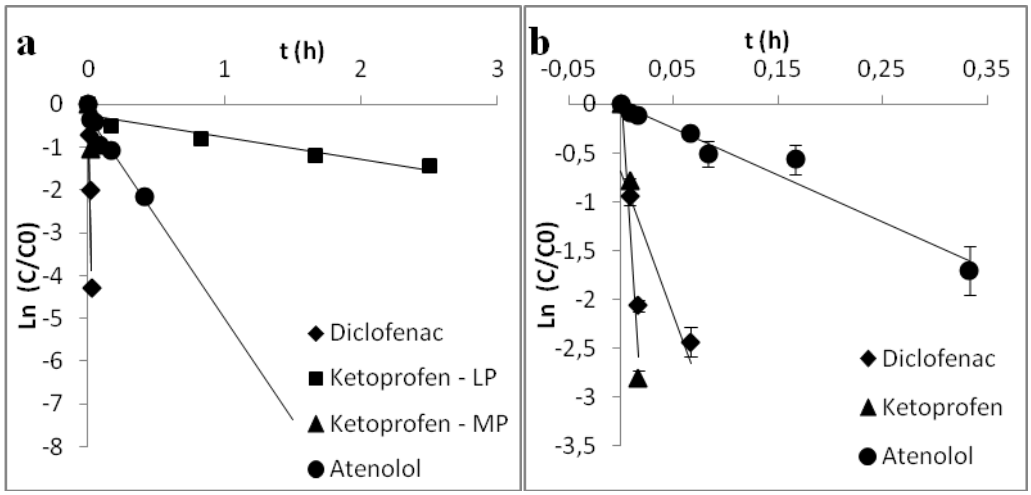


Figure 7.2 - Degradation kinetics of ketoprofen (LP and MP), diclofenac and atenolol in pure water as single compound (a) or combined in a mixture with the three PhACs (b).

MP/UV phtolysis of ketoprofen and diclofenac had comparable time-based degradation rate constants and one order of magnitude higher than atenolol (Table 7.2). Both compounds were removed below detection levels in only a few minutes, whereas atenolol took over 40 minutes of irradiation. The time-based degradation rate constant for LP/UV photolysis of ketoprofen was 0.000143 s^{-1}, and it took 150 minutes for 78% removal when subjected to a dose of 1500 mJ cm^{-2}. Thus, ketoprofen was degraded at a higher rate with a MP lamp than a LP lamp under the conditions used in this study (Figure 7.1).

The low photon fluence-based rate constant obtained for atenolol (Table 7.2) confirmed the low photoliability of this compound for MP direct photolysis, which was expected from its low decadic molar absorption coefficient and quantum yield values (Table 7.1). Overall, these parameters reflect the probability of a compound to be degraded at the wavelength range used, and in this study indicated a higher degradation potential for ketoprofen, followed by diclofenac and atenolol (Table 7.1 and Figure 7.1), which was confirmed by the fluence-based rate constants obtained (Table 7.2).

Table 7.2 - Time- and fluence-based degradation rate constants of the studied PhACs under MP/UV irradiation in pure water as single compounds and combined in a mixture; photon fluence rate in the 200-450 nm interval for each experiment, and irradiation time to total removal for each condition.

	Single compound			Multiple compounds		
	E_p^0 (einstein m^{-2} s^{-1})	k (s^{-1})	k_{Ep0} (m^2 $einstein^{-1}$)	E_p^0 (einstein m^{-2} s^{-1})	k (s^{-1})	k_{Ep0} (m^2 $einstein^{-1}$)
Ketoprofen	0.000070	0.01753	250.2	0.00069	0.04669	67.4
Atenolol	0.000513	0.00132	2.6		0.00134	1.9
Diclofenac	0.002602	0.04721	18.1		0.00824	11.9

When the three compounds were combined in a mixture and subjected to MP/UV photolysis, the pseudo-first-order photon fluence-based rate constants decreased, as can be expected from higher competition for light absorption in the presence of other substances. The degradation kinetics followed the same order (higher for ketoprofen, then diclofenac and finally atenolol), but ketoprofen seemed to be substantially slower than the other two compounds despite its larger absorbance spectrum (Figure 7.1), suggesting that its photoliability is dependent on light absorption at wavelengths where the other two compounds also have strong absorbance.

7.3.3 Wastewater matrix effect

The MP/UV photolysis of ketoprofen, atenolol and diclofenac was investigated in a real matrix, i.e., the secondary effluent of a biological WWTP. The treated effluent was characterised by 37 mg COD L^{-1}, 218 NTU turbidity, pH 6.91, 28 mg T-N L^{-1}, 5 mg N-NO_2^- L^{-1}, 15 mg N- NO_3^- L^{-1}, 2.1 mg P L^{-1} (total P) and 13 mg SS L^{-1} before filtration.

Table 7.3 - Time- and fluence-based degradation rate constants of a mixture of the studied PhACs under MP/UV irradiation in filtered and unfiltered wastewater; photon fluence rate in the 200-450 nm interval for each experiment, and irradiation time to total removal for each compound.

	FILTERED EFFLUENT			UNFILTERED EFFLUENT		
	E_p^0 (einstein m^{-2} s^{-1})	k (s^{-1})	kE_p^0 (m^2 $einstein^{-1}$)	E_p^0 (einstein m^{-2} s^{-1})	k (s^{-1})	$k\,E_p^0$ (m^2 $einstein^{-1}$)
Ketoprofen	0.00072	0.00801	11.1	0.00041	0.01347	33.1
Atenolol		0.00017	0.2		0.00342	8.4
Diclofenac		0.01284	17.8		0.00829	20.4

The time- and fluence-based degradation rate constants obtained for ketoprofen in a treated wastewater matrix (Table 7.3) were lower than in a pure water matrix (Table 7.2). This result is not surprising since the wastewater effluent likely comprised many compounds that competed for light absorption within the broad absorption spectrum of ketoprofen. The fluence-based degradation rate constant of atenolol also decreased in filtered effluent as compared to pure water, but it increased in unfiltered effluent. An increase was also observed in both cases for diclofenac.

The higher fluence-based degradation rate constants in unfiltered effluent for all compounds as compared to filtered effluent could be explained by indirect photolysis arising from free radicals generated from the incidence of UV radiation on the dissolved organic matter present in the effluent. This effect was stronger for unfiltered effluent since the particles present in secondary effluent of a WWTP are mostly comprised of biomass, thus the difference between the two matrices is a higher content of organic matter in the unfiltered effluent, which probably increased the amount of free radicals generated from UV irradiation.

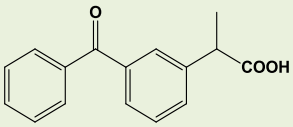
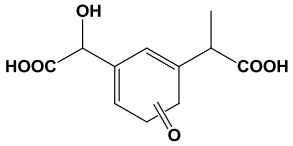
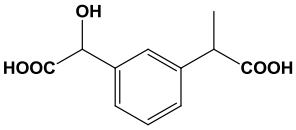
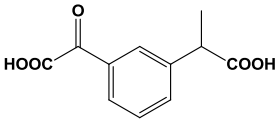
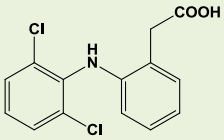
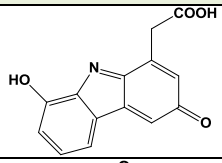
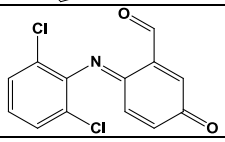
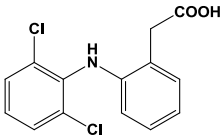
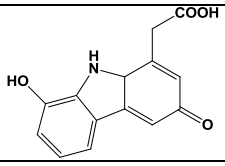
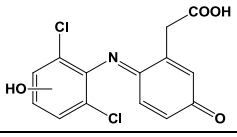
Overall, longer irradiation times were necessary to degrade the PhACs in a wastewater matrix than in pure water. The degradation of atenolol was particularly inefficient in a real matrix and it was not possible to remove the compound beyond 53% and 85% in filtered and unfiltered effluent, respectively, within the 75 min experiments. This result could be anticipated from the low decadic molar absorption coefficient profile for atenolol in the wavelength range used in this study (Figure 7.1), suggesting that MP/UV irradiation is not an appropriate methodology for polishing atenolol in WWTP tertiary treatment. Advanced oxidation processes, such as indirect photolysis, may improve atenolol degradation, as suggested by the enhanced removal obtained in unfiltered wastewater.

7.3.4 Phototransformation products of diclofenac, ketoprofen and atenolol

7.3.4.1 Phototransformation products identification

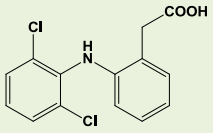
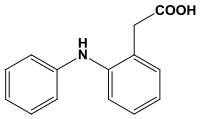
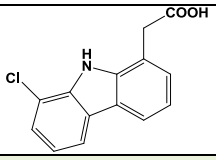
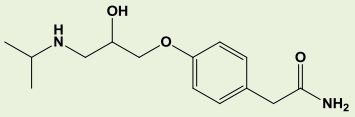
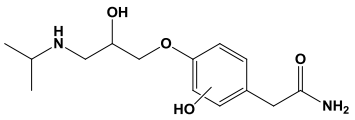
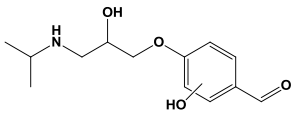
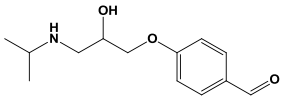
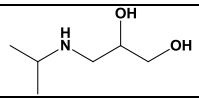
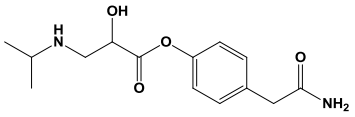
UV irradiation of the target compounds resulted in the formation of new chromatographic peaks corresponding to transformation products generated during the photolysis process. Most of these products (Table 7.4) were identified through LC-MS/MS and/or GC-MS after derivatization.

Table 7.4 - Identification of the transformation products generated by MP/UV photolysis (unless indicated) of ketoprofen, diclofenac and atenolol.

Compound	Structure	Formula and M_W	t_r (min)	*m/z* (relative abundance)	Analytical Mode
Ketoprofen 2-(3-benzoylphenyl) propanoic acid		$C_{16}H_{14}O_3$ (MW 254)	16.4	105(100), 124(16), 131(30), 147(6), 209(12), 255(1)	LC-MS/MS (APCI+)
Product **K1*** 2-(3-(carboxy(hydroxy)-methyl)-?-oxocyclohexa-1,3-dien-1-yl)propanoic acid[a]		$C_{11}H_{12}O_6$ (MW 240)	15.3	105(90), 124(52), 147(79), 225(44) 241(52)	LC-MS/MS (APCI+)
Product **K2** 2-(3-(carboxy(hydroxy)-methyl)phenyl)propanoic acid		$C_{11}H_{12}O_5$ (MW 224)	17.5	105 (61), 119 (19), 124 (60), 147(96), 183 (10), 225(53)	LC-MS/MS (APCI+)
Product **K3** 2-(3-(carboxyoxomethyl)-phenyl)propanoic acid		$C_{11}H_{10}O_5$ (MW 222)	16.6	105(16), 124(60), 147(96), 223(39)	LC-MS/MS (APCI+)
Diclofenac 2-[2-(2,6-dichlorophenyl amino)phenyl]acetic acid		$C_{14}H_{11}Cl_2NO_2$ (MW 296)	18.1	107(25), 124(64), 214(10), 278(82), 297(1)	LC-MS/MS (APCI+)
Product **D1** 2-(8-hydroxy-3-oxo-3*H*-carbazol-1-yl)acetic acid		$C_{14}H_9NO_4$ (MW 255)	14.2	124(19), 170(9), 210(93), 226(8), 238(11), 256(8)	LC-MS/MS (APCI+)
Product **D2** (*E*)-6-[2,6-dichlorophenyl)-imino]-3-oxocyclohexa-1,4-dienecarbaldehyde		$C_{13}H_7Cl_2NO_2$ (MW 279)	16.4	124(19), 149(5), 168(18), 180(86), 279(1)	LC-MS/MS (APCI+)
Diclofenac 2-[2-(2,6-dichlorophenyl amino)phenyl]acetic acid		$C_{14}H_{11}Cl_2NO_2$ (MW 296)	17.4	297(100)	LC-DAD-MS (ESI+)
Product **D3** 2-(8-hydroxy-3-oxo-9,9a-dihydro-3*H*-carbazol-1-yl)acetic acid		$C_{14}H_{10}NO_4$ (MW 256)	7.8	257(100)	LC-DAD-MS (ESI+)
Product **D4** (*E*)-2-[3-(2,6-dichloro-?-hydroxyphenylimino)-6-oxocyclohexa-1,4-dienyl] acetic acid[b]		$C_{14}H_9Cl_2NO_4$ (MW 325)	15.5	326(100)	LC-DAD-MS (ESI+)

*Generated by LP/UV photolysis; [a] - the carbonyl position in the cyclic ring can be C5 or C6; ** The *m/z* 73 correspond to the fragment generated by derivatizing reagent (MSTFA) but the group trimethylsilyl, TMSi (-Si(CH_3)$_3$) is not shown in the chemical structure presented

Table 7.4 - (cont.) Identification of the transformation products generated by MP/UV photolysis (unless indicated) of ketoprofen, diclofenac and atenolol.

Compound	Structure	Formula and M_W	t_r (min)	*m/z* (relative abundance)	Analytical Mode
Diclofenac 2-[2-(2,6-dichlorophenyl amino)phenyl]acetic acid**		$C_{14}H_{11}Cl_2NO_2$ (MW 296)	22.3	73(75), 179(25), 214(100), 242(50), 277(25) 367(30)	GC-MS (EI+)
Product **D5** 2-[2-(phenylamino]phenyl) acetic acid**		$C_{14}H_{13}NO_2$ (MW 227)	21.7	73(100), 152(25), 179(75), 207(20), 300(75)	GC-MS (EI+)
Product **D6** 2-(8-chloro-9*H*-carbazol-1-yl)acetic acid**		$C_{14}H_{10}ClNO_2$ (MW 260)	22.7	73(100), 151(18) 178(20), 213(40), 241(15) 333(10)	GC-MS (EI+)
Atenolol 2-[4-[2-hydroxy-3-(isopropylamino)propoxy] phenyl]acetamide		$C_{14}H_{22}N_2O_3$ (MW 266)	13.3	116(2), 145(100), 190 (60), 208(12), 225 (2), 267(35)	LC-MS/MS (ESI+)
Product **A1** 2-(?-hydroxy-4-(2-hydroxy-3-isopropylamino)propoxy) phenyl)acetamide[c]		$C_{14}H_{22}N_2O_4$ (MW 283)	10.0	61(5) 118(20), 197(8), 215(5), 283(100)	LC-MS/MS (ESI+)
Product **A2** ?-hydroxy-4-[2-hydroxy-3-(isopropylamino)propoxy] benzaldehyde[c]		$C_{13}H_{19}NO_4$ (MW 254)	15.6	61(5), 118(15), 197(5), 254(100)	LC-MS/MS (ESI+)
Product **A3** 4-[2-hydroxy-3-(isopropylamino)propoxy] benzaldehyde		$C_{14}H_{19}NO_3$ (MW 238)	16.2	61(5), 87(3), 118(5), 238(100)	LC-MS/MS (ESI+)
Product **A4** 3-(isopropylamino)-propane-1,2-diol		$C_6H_{15}NO_2$ (MW 134)	0.93	92 (10), 118 (12), 134(100)	LC-MS/MS (ESI+)
Product **A5** 4-[(2-amino-2-oxoethyl)phenyl- 2-hydroxy-3-(isopropylamino)] propanoate		$C_{14}H_{20}N_2O_4$ (MW 281)	4.68	61(15), 118(15), 197(3), 281(100)	LC-MS/MS (ESI+)

*Generated by LP/UV photolysis; [b] - the phenolic hydroxyl group position can be C3, C4 or C5; [c] - the phenolic hydroxyl group position can be C2 or C3;** The *m/z* 73 correspond to the fragment generated by derivatizing reagent (MSTFA) but the group trimethylsilyl, TMSi (-$Si(CH_3)_3$) is not shown in the chemical structure presented

Two products were generated from MP/UV photolysis of ketoprofen (K2 and K3, Table 7.4), which were also the principal products obtained from LP/UV photolysis. Intermediate K1 was identified from LP/UV photolysis, but only with low peak area and it was not detected from MP/UV photolysis. The chemical structures of K2 and K3 were identified through LC-MS/MS(APCI+) as 2-(3-(carboxycarbonyl)phenyl) propanoic acid and 2-(3-(carboxy(hydroxy)methyl) phenyl) propanoic acid, respectively. These transformation products involved the oxidation of one of the rings of ketoprofen, which had not been observed by Kosjek *et al.* (2010) in a study reporting 22 ketoprofen transformation

products using LP and MP photolysis. Interestingly, these structures had previously been identified as ketoprofen biodegradation metabolites using activated sludge (Quintana *et al.*, 2005), suggesting that both photolysis and biodegradation of ketoprofen can proceed through a similar pathway involving oxidative ring opening.

The diclofenac photoproducts were analysed through liquid and gas chromatography coupled to mass spectrometry. The target compound appears in the chromatogram at 18.1 min. In the mass spectrum, it is possible to identify the protonated molecular ion $[M+H]^+$ of diclofenac, and the fragment ions could confirm the structure of the compound. The base peak corresponds to the fragment ion *m/z* 278 $[M-18]^+$. The ion fragment with *m/z* 214 corresponds to decarboxylation of the compound followed by the loss of one chlorine atom, $[Cl\text{-}M\text{-}COOH]^+$. All of the photoproducts detected by HPLC (D1, D2, D3 and D4) have lower retention times than diclofenac, suggesting that these transformation products are more polar than the parent compound. Pérez-Estrada *et al.* (2005) studied the photo-Fenton degradation of diclofenac and determined the intermediates as well as the mechanistic pathway of transformation. Two of the compounds identified in that study had the same chemical structure as products D2 and D4 proposed in the present work.

The identification of transformation products obtained in the photodegradation of diclofenac was complemented with GC-MS analysis of the irradiated sample after derivatisation with the reagent MSTFA. The chromatogram obtained for the standard of diclofenac has a peak at 22.3 min corresponding to the target compound. The identification of the peak of diclofenac was performed by the interpretation of mass spectra obtained by GC-MS. The base peak at *m/z* 214(100%) corresponds to the fragment ion $[M\text{-}COOTMSi\text{-}Cl]^+$. The molecular ion of the silylated compound $[M]^+$ is present in the mass spectrum *m/z* 367(30%). The chromatogram obtained with the irradiated sample showed two products with retention times of 21.7 and 22.7 min. The first photoproduct identified by GC/MS (D5, Table 7.4) presents a molecular ion $[M]^+$ *m/z* 298, compatible with the formula $C_{14}H_{13}NO_2$ after silylation. This metabolite corresponds to the loss of the two chlorine atoms and does not match any of the previously proposed structures in literature. In the mass spectrum of the second photoproduct, product D6, it is possible to identify the silylated form of the molecular ion $[M]^+$ at *m/z* 333, which corresponds to a molecular formula of $C_{14}H_{10}ClNO_2$. The formation of this photoproduct could be due to the loss of a chlorine atom followed by cyclisation and formation of carbazole (2-(8-chloro-9H-carbazol-2-yl) acetic acid). This structure was previously proposed to occur in solar photodegradation of diclofenac by Agüera *et al.* (2005), who detected its acetone form.

The analysis of an atenolol standard by LC-MS/MS(ESI+) gave a peak at the retention time of 13.3 min, with a MS base peak with *m/z* 145 corresponding to $[M+H\text{-}NH_2CH(CH_3)_2\text{-}H_2O\text{-}CO\text{-}NH_3]^+$, and molecular ion peak with *m/z* 265. Analysis of the irradiated atenolol sample allowed the identification of five photoproducts with base peaks in the mass spectra of *m/z* 283, *m/z* 254, *m/z* 238, *m/z* 134 and

m/z 281 (A1-A5, Table 7.4). The chemical structure of product A3 resulted from the loss of the formamide group (-NH_2CO), followed by an oxidation reaction, while product A2 may have resulted from the hydroxylation of the structure of A3, probably at the benzene ring. Product A4, resulting from the loss of the aromatic ring of atenolol, lost the chromophore capacity to be detected by HPLC-DAD and could only be measured in association to a MS system. All atenolol UV photolysis products identified in this work, except for A4, are consistent with those obtained by Radjenovic *et al.* (2009) with TiO_2 and photon-Fenton solar photocatalysis.

7.3.4.2 Dynamics of the transformation products generated by UV photolysis

The phototransformation of ketoprofen in the LP/UV photolysis experiment in pure water by HPLC-DAD-MS resulted in two main products (K2 and K3, Table 7.4). Product K1 was also identified but only with very low HPLC peak areas.

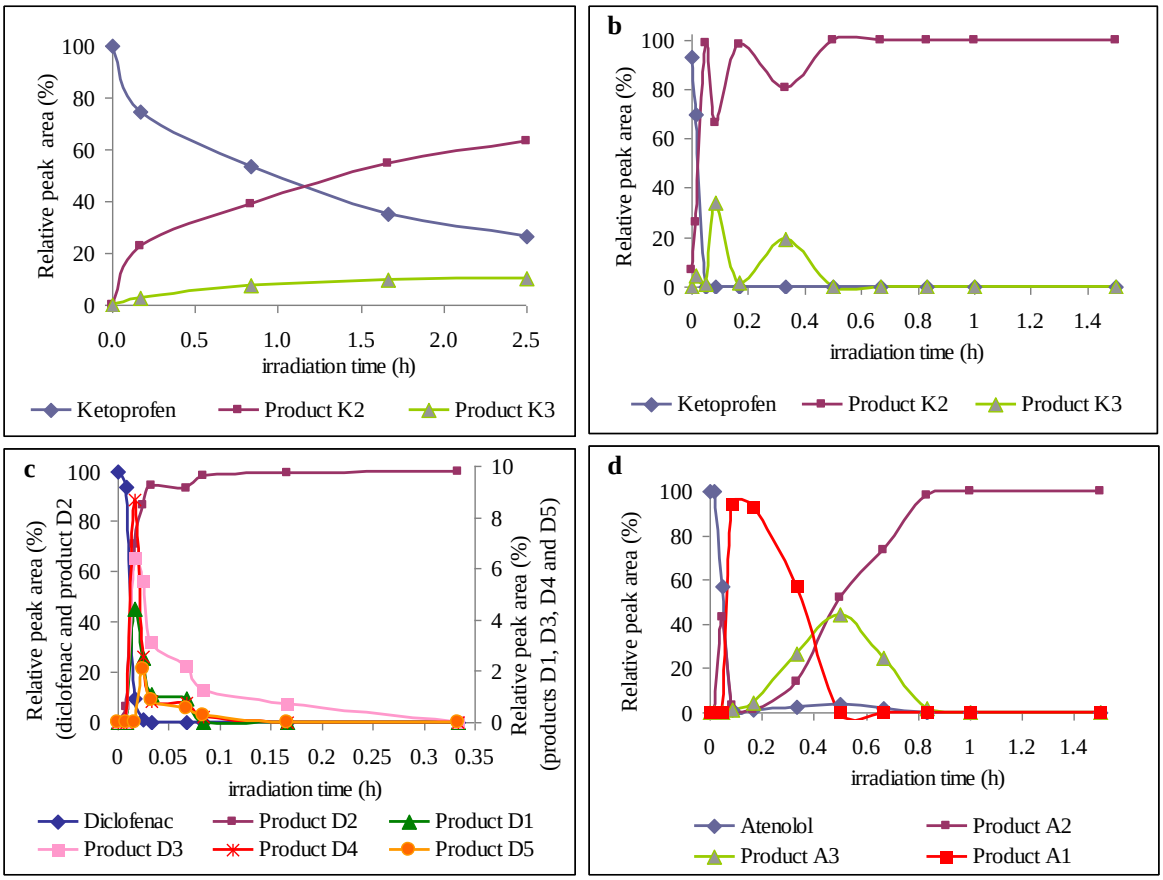


Figure 7.3 - Relative peak area of the PhACs and corresponding transformation products in the UV photolysis experiments carried out in pure water: dynamics of LP/UV photolysis of ketoprofen (a) and MP/UV photolysis of ketoprofen (b), diclofenac (c) and atenolol (d).

UV photolysis of ketoprofen with MP lamp irradiation produced the same two main products. Product K2 (2-(3-(carboxy(hydroxy)methyl) phenyl) propanoic acid) was the most persistent in both cases (Figure 3). In the MP photolysis experiment, products K3 was unstable and it seemed to be converted

into product K2 and back to K3 during the first 30 minutes of irradiation, until it was totally converted into K2, which accumulated in the reactor.

MP/UV photolysis of diclofenac generated the persistent product D2 or (E)-6-[2,6-dichlorophenyl)-imino]-3-oxocyclohexa-1,4-dienecarbaldehyde). Some other intermediates were also detected, most of which disappeared after 10 minutes of irradiation (D1, D4 and D5, see Table 7.3), and one of which prevailed for approx. 20 minutes (D3), but none of them were detected after that irradiation time.

Photolysis of atenolol generated three main products. Product A1, which consisted of the addition of a hydroxyl group to the aromatic ring, was observed immediately after the beginning of the irradiation. Shortly after, this product gave place to products A2 and A3, where product A2 (3-hydroxy-4-(2-hydroxy-3-(isopropylamino)propoxy) benzaldehyde) was the most persistent, remaining unaltered after approx. 50 min of irradiation when the other intermediates were no longer detected. Medana et al. (2008) also observed the appearance and disappearance of product A1 within 30 minutes of solar irradiation with TiO_2 photocatalysis, although products A2 and A3 were not identified as possible subsequent intermediates. Furthermore, all products seemed to be fully mineralized in that study, which suggests that the use of a catalyst could be a solution to enhance the extension of atenolol photolysis.

Several persistent transformation products from the MP/UV photolysis of ketoprofen, diclofenac and atenolol were identified in this study. This work suggests that it is important to assess the occurrence of these intermediates in effluents of WWTP using direct photolysis for disinfection purposes. Furthermore, future research should be conducted to evaluate their ecotoxicity potential.

7.4 Conclusions

The UV photolysis of ketoprofen, diclofenac and atenolol was investigated and the following conclusions are taken from this study:

- Decadic molar absorption coefficient and quantum yield values indicated high photodegradation for ketoprofen, followed by diclofenac, and low photolysis for atenolol;
- Time- and fluence-based rate constants determined in pure water confirmed these kinetics, suggesting that ketoprofen, and to a certain extent diclofenac, can be adequately removed in MP/UV disinfection systems. LP/UV photolysis of ketoprofen at 1500 mJ cm^{-2} showed a lower extent of degradation.
- The presence of other PhACs in pure water or of other organic compounds in filtered treated wastewater seemed to reduce the photodegradation kinetics of the studied PhACs. However, photodegradation was higher in unfiltered than in filtered wastewater, suggesting increased indirect photolysis from free radicals arising from particulate organic matter.

- MP/UV photolysis products of ketoprofen, diclofenac and atenolol were identified and monitored throughout the irradiation time. This enabled the identification of the most persistent transformation products, which are possibly discharged from WWTP using direct photolysis for disinfection. In the future, it is important to assess the toxicity and environmental persistence of these intermediates, and if necessary, investigate complementary solutions for their mitigation.

Chapter 8

CONCLUSIONS

8.1 Main Achievements

8.2 Contributions to the advancement of PPCP monitoring and removal studies in WWTPs

8.3 Future work

8. Conclusions

8.1 Main Achievements

Five important conclusions were obtained with this work:

1) It was possible to identify many of the pharmaceutical active compounds and musks as target compounds of this work in the five different WWTP in Portugal at the influent, effluent and sludges in different periods of the year. With these results it was possible to conclude that some compounds are very frequently detected but no seasonal pattern was observed.

The total PhACs and musks concentrations found in this work were in a similar range as previously reported studies. The most abundant PhACs were the NSAIDs (particularly ibuprofen), while the antihypertensives (particularly enalapril), caffeine, and clofibric acid were also present in relatively high concentrations in the influent and effluent. Clofibric acid represented one of the few compounds present at a similar range of concentrations in the influent and effluent of the plants. The analytical methodology employed in this work was effective for monitoring pharmaceutical and personal care products in wastewater treatment plants. This method reduced the analytical effort necessary to cover a wide range of compounds with different natures, and still achieved good LOD and LOQ levels (with the exception of the estrogens), with high recoveries in influent wastewater.

2) The dynamics of PPCPs in a WWTP was evaluated through an intensive sampling campaign covering a large number of pharmaceuticals and musks. It was found that the PhACs concentrations in the influent were subject to a wider variability than the musks, which were more repeatable. The typical diurnal pattern for macropollutants (i.e. higher loading during the day as compared to the night) was observed for the musks and some PhACs, while other frequently detected PhACs (e.g. ketoprofen) displayed the opposite trend or no trend. In general, the mean PhAC loadings varied between 1-3 orders of magnitude from one sampling day or week to the next, whereas the mean musk loadings were far less than one order of magnitude apart. This information is relevant to the design of sampling campaigns for modelling purposes.

3) The combination of biodegradation, adsorption and removal by UV are each important mechanism that lead to the transformation of PPCPs in WWTPs. Biodegradation was found to be the principal degradation mechanism, followed by adsorption and UV, which was found to be an effective effluent polishing step for PhAC removal. More than 75% removal was usually found for 17 of the 18 most commonly detected PPCPs, with the sole exception being diclofenac, which often showed negative removal rates and was mostly degraded by UV.

4) Clofibric acid (CLF) which is considered to be environmentally persistent and refractory was biotransformed by microbial consortia in two sequencing batch reactors (SBR). CLF biodegradation was observed to a greater extent in a SBR fed with CLF and propanil as compared to CLF feeding alone. Nevertheless, the presence of propanil was confirmed not to be essential towards co-metabolism

of the clofibric acid. The heterotrophic populations within the bioreactor were likely more responsible for CLF biodegradation than the autotrophic populations, after testing with and without inhibition of nitrification. Combining the information from GC-MS and HPLC-DAD, it was possible to identify and quantify three main metabolites produced in the reactors, including α-hydroxyisobutyric acid, lactic acid (LA) and 4-chlorophenol (4-CP). The α-hydroxyisobutyric acid was the first important metabolite produced, and accumulates in the reactor, getting transformed into LA over time. The 4-chlorophenol was detected only in small concentrations, due either to fast biodegradation when compared to the other metabolites or due to only a small amount of CLF being channeled through the 4-CP pathway. Based on the identification of CLF metabolites, a metabolic pathway for its biodegradation was proposed

5) The UV photolysis of ketoprofen, diclofenac and atenolol was investigated. Decadic molar absorption coefficient and quantum yield values indicated high photodegradation for ketoprofen, followed by diclofenac, and low photolysis for atenolol; Ketoprofen, and to a certain extent diclofenac, were quite efficiently removed in MP/UV, while LP/UV photolysis of ketoprofen showed lower degradation extent. The presence of particulate organic matter present in unfiltered wastewater had a negative impact in the photodegradation of PhACs. The most persistent transformation products from MP/UV photolysis of ketoprofen, diclofenac and atenolol were identified and monitored throughout irradiation time.

8.2 Contributions to the advancement of PPCP monitoring and removal studies in WWTPs

This work gives an important contribution to the knowledge of the occurrence and fate of PhAC and musks in WWTPs as well as to the best available analytical techniques for monitoring this kind of compounds by the WWTP operators and engineers. The simplified analytical methodology should facilitate PPCP monitoring, which is needed throughout the year because seasonal patterns were not observed. The influent variability and repeatability was also another important contribution of this study, since it was determined that PhAC loading is indeed highly variable, while musks can be monitored much more reliably. The WWTP study of biotransformation, adsorption and UV highlighted the importance of each individual mechanism and illustrated that a WWTP has the potential to be operated successfully for efficient removal of PPCPs (>75%). Nevertheless, it was also shown that the removal of PPCP compounds via biotransformation or UV radiation can produce by-products that have the potential to be highly toxic to aquatic organisms. The biodegradation of the reported refractory compound, clofibric acid was studied in a SBR reactor and the biotransformation mechanism that was observed was characterized through a metabolic pathway by analyzing the metabolites generated.. It is of high importance to understand the biodegradation pathway and the side-products generated in order to evaluate effects on ecotoxicity. This was of likewise importance for the UV radiation studies, where the most persistent by-products were identified and monitored, in

combination with the study of the degradation kinetics of atenolol, ketoprofen and diclofenac by this disinfection technology.

8.3 Future work

Recommendations for future work in this field are outlined below:

(1) Investigate new strategies for the biological removal of musks and other PhACs that are not very easily biodegradable. The study of other PhAC and musk compounds in lab scale reactors should be performed in order to understand the best operational conditions that achieve the maximum removal of these specific compounds with the purpose of helping environmental engineers in the operation of WWTPs. Identification of the microbial communities responsible for this removal would be highly beneficial.

(2) Test new technologies for sludge treatment and measure the potential impact of the treatment on the application of the sludges in agriculture. Some PhACs and musks can be removed by adsorption to the sludges and additional studies should be done to investigate the ultimate fate of PPCPs in different sludge treatment technologies (e.g. stabilization, aerobic and anaerobic digestion, composting). The sludge application in agriculture can produce problems of soil, surface and groundwater contamination by desorption of the PhACs and musks from the sludges.

(3) Comparing advanced oxidation technologies (e.g. ozone, hydrogen peroxide) with UV radiation and combining these technologies with the biological wastewater treatment process through recycling the effluent from the tertiary treatment stage back to the secondary stage. Other oxidation technologies should be tested in the future (e.g. sonolysis) in order to compare efficiencies and treatment costs for the removal of PhACs and musks.

(4) Study the ecotoxicological effects of the final wastewater discharges on fish and other living organisms. The effect of the discharges of PhAC and musks is not well studied with and without the use of tertiary treatment technologies (e.g. UV radiation used for disinfection). The effect of UV radiation on atenolol, ketoprofen and diclofenac and their by-products are already being carried out with fish (*Zebra fish*) for testing the biochemical reactions in the liver and other organs.

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This work was published in the scientific journals and presented in the following conferences:

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1. Salgado, R., Noronha, J.P., Oehmen, A., Carvalho, G., Reis M.A.M. (2010). Analysis of 65 pharmaceuticals and personal care products in five wastewater Treatment plants in Portugal using a simplified Analytical Methodology, Water Science and Technology, 62.12, 2862-2871.

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3. Salgado, R., Marques, R., Noronha, JP, Oehmen, A., Carvalho, G., Reis, MAM (2011), Assessing the Removal of Pharmaceutical Compounds and musks in Full-Scale Activated Sludge Plant, Environ. Sci. Pollut. Res., DOI 10.1007/s11356-011-0693-z

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1. Salgado, R., Carvalho, G., Noronha, J.P., Oehmen, A., Reis, M.A., Oliveira, R., Detection of Pharmaceutical and Musks Compounds in Wastewater Treatment Plants in Lisbon, Portugal, SPQ, Portuguese Chemical Society , Analytical 07th, IST, Lisbon, Portugal, March, 2007.

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1. Salgado, R., Noronha, J.P., Oehmen, A., Carvalho, G., Oliveira, R., Reis, MA, Occurrence and Fate of Pharmaceutical Active Compounds, Musks and Wastewater Treatment Plants in Steroids in Lisbon, Portugal, Dechema / Micropole and Ecohazard, Frankfurt, June, 2007.

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APPENDIX I OF CHAPTER 4

The probability that the frequency of occurrence of each compound was due to chance was determined through chi-square tests, as follows:

$$\chi^2 = \sum_{i=1}^{4}\sum_{j=1}^{2}\frac{(\theta-\varepsilon)^2}{\varepsilon}$$

where θ is the observed number of samples where the compound was detected and ξ is the expected (average) value; i and j are the entries of a contingency matrix where the rows (i) are the analysed days and the columns (j) are the number of samples per day where the compound was either detected or not detected (see table S.1 for example).

Table S1. Example of contingency table for etofenamate: number of samples per day (a total of 12 samples were analysed per day) where the compound was detected or not-detected.

Day	Detected	Not detected	Total
1	0	12	12
2	8	4	12
3	11	1	12
4	10	2	12
Total	29	19	

The chi-square values obtained for each compound were used to determine the p-values, or probability that the observed frequency of occurrence was due to chance alone. The number of degrees of freedom (*df*) was calculated as:

$df = (i - 1)\cdot(j - 1)$

P-values <0.05 were considered significant, $p<0.01$ was considered very significant and $p<0.001$ was considered highly significant.

APPENDIX II OF CHAPTER 5

Daily influent, effluents loads before and after UV and secondary sludge loads for the 6 days of the campaign

	Influent load ($g\ d^{-1}$)						Effluent load before UV ($g\ d^{-1}$)						Effluent load after UV ($g\ d^{-1}$)						Secondary sludge load (gd^{-1})			
Compound	**Day 0**	**Day 1**	**Day 2**	**Day 7**	**Day 8**	**Day 10**	**Day 1**	**Day 2**	**Day 3**	**Day 8**	**Day 9**	**Day 10**	**Day 1**	**Day 2**	**Day 3**	**Day 8**	**Day 9**	**Day 10**	**Day 1**	**Day 3**	**Day 9**	**Day 10**
Diclofenac	35.05	131.61	1.29	12.98	13.56	14.19	55.41	13.19	3.25	0.00	28.23	10.33	45.24	4.31	2.22	0.00	0.00	3.78	1.41	1.11	17.89	15.29
Etofenamate	0.00	25.33	1.69	4.82	3.74	18.01	0.00	0.00	0.88	0.00	0.00	0.00	0.00	0.00	0.65	0.00	0.00	0.00	0.16	1.03	3.44	3.09
Ibuprofen	46.00	15.23	2.96	3.03	1.40	2.48	0.78	1.81	1.97	0.00	0.62	0.30	0.00	0.55	0.88	0.00	0.44	0.19	0.04	0.28	0.20	1.35
Ketoprofen	83.34	31.50	0.20	0.20	0.21	0.00	0.00	2.67	0.40	0.00	0.09	0.00	0.00	0.41	0.33	0.00	0.00	0.00	0.02	0.00	0.04	0.14
Fluoxetine	0.00	3.67	0.00	0.63	2.35	0.00	0.00	2.66	0.00	0.00	0.24	0.00	0.00	0.70	0.00	0.00	0.23	0.00	0.03	0.03	0.00	0.00
Clorazepate	0.00	1.42	0.82	0.71	0.79	1.83	0.00	1.62	0.61	0.00	0.62	0.69	0.00	0.38	0.00	0.00	0.00	0.52	0.00	0.00	0.78	0.12
Hydroxazine	0.00	0.00	0.00	1.35	0.54	7.38	0.00	0.00	0.00	0.00	0.19	0.00	0.00	0.00	0.00	0.00	0.13	0.00	1.20	0.79	0.00	3.25
Indapamide	0.00	0.00	0.00	7.64	8.64	3.81	0.00	0.00	0.00	0.00	15.87	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.45	0.03	8.50	3.70
Enalapril	0.00	0.00	0.73	1.99	3.81	1.16	0.00	0.00	0.87	0.00	0.75	0.44	0.00	0.00	0.00	0.00	0.00	0.38	0.01	0.08	0.00	0.36
Captopril	2.88	1.31	0.36	2.25	2.52	2.83	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.40	0.50	0.42	0.79
Atenolol	12.80	1.45	3.53	1.36	2.03	0.85	0.00	0.00	0.70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0,00
Clofibric acid	49.88	2.05	0.23	0.79	1.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.77	0.00
Ampicillin	0.00	0.43	0.00	0.45	0.25	0.52	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00
Paroxetine	0.00	32.93	0.00	0.72	0.00	0.00	15.82	0.00	0.00	0.00	0.00	0.00	22.84	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Galaxolide	1.41	1.35	0.15	1.33	0.51	0.40	0.03	0.05	0.05	0.08	0.08	0.08	0.04	0.00	0.00	0.05	0.07	0.06	0.95	2.02	17.73	16.12
Tonalide	0.37	0.61	0.05	0.40	0.18	0.13	0.03	0.02	0.03	0.05	0.02	0.02	0.03	0.00	0.00	0.05	0.02	0.02	0.08	0.17	2.15	2.20
Cashmeran	1.77	4.63	0.06	4.14	1.73	0.39	0.07	0.02	0.03	0.32	0.06	0.04	0.00	0.00	0,02	0.18	0.03	0.03	1.34	2.04	36.05	22.98
Celestolide	0.64	1.60	0.09	0.43	0.25	0.14	0.02	0.04	0.06	0.25	0.04	0.04	0.02	0.00	0.00	0.16	0.03	0.04	0.08	0.19	2.22	0.18
Traseolide	0.26	0.71	0.04	0.15	0.09	0.06	0.01	0.02	0.02	0.08	0.02	0.03	0.01	0.00	0.00	0.07	0.02	0.02	0.00	0.06	0.64	0.07