Universitat Autònoma de Barcelona

Fungal treatment for the elimination of pharmaceutical compounds from wastewater: chemical, microbiological and ecotoxicological evaluation

Daniel Lucas Fernández

Universitat Autònoma de Barcelona

Institut Català de Recerca de l'Aigua

Doctoral Thesis

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Work by: Daniel Lucas Fernández

Directors: Damià Barceló Cullerès and Sara Rodríguez-Mozaz

Tutor: Montserrat Sarrà Adroguer

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Certifican

Que el trabajo titulado "*Fungal treatment for the elimination of emerging contaminants from wastewater: chemical, microbiological and ecotoxicological approach*." que presenta Daniel Lucas Fernández para la obtención del título de doctor se ha realizado bajo nuestra dirección.

Damià Barceló Cullerès Sara Rodríguez Mozaz

Dedicado a mis padres

Table of contents

Summary

During the past years the presence of pharmaceuticals in the environment has attracted the attention of scientific community as well as raised public concern, as they could pose a risk for the environment. Their presence in natural aquatic environment points out to the inability of conventional wastewater treatment plants to eliminate these compounds efficiently. Many research efforts have been devoted in the last years to explore and develop advance and alternative wastewater treatment strategies, able to eliminate pharmaceuticals from wastewater in an efficient and cheap manner. Among all the new technologies studied, fungal treatment of wastewater has been investigated due to the ability of some fungal species to degrade chemical contaminants, including pharmaceuticals. The elimination of these compounds have recently been studied in urban and hospital wastewater as well as in the waste generated in reverse osmosis treatment of wastewater effluents. These studies have been performed at lab-scale but some aspects of the fungal wastewater treatment still need to be addressed before its implementation at full-scale. The ultimate goal of this thesis is to study some of these aspects in order to generate more information about these wastewater technologies concerning removal of emerging pollutants. Therefore, the work presented aims to address the following specific objectives: i) to evaluate pharmaceuticals removal with the fungal treatment from an ecotoxicological point of view, ii) to evaluate the efficiency of the fungal treatment in the elimination of antibiotics, iii) to assess the spread of antibiotic resistance genes during the fungal treatment and iv) to study the relevance of absorption and/or adsorption processes contributing to pharmaceutical removal during fungal treatment. Based on these goals, the work of the thesis is presented in three parts:

The **first part** is dedicated to the general evaluation of removal of pharmaceuticals in different wastewaters and to the ecotoxicological evaluation of those removals. An "Environmental Risk Assessment" is performed for the holistic evaluation of fungal treatment concerning pharmaceutical elimination and the hazard associated.

In the **second part**, antibiotics removal and antibiotic resistance genes spread is studied jointly in a lab-scale fungal experiment performed with hospital wastewater. Both chemical and microbiological analytical methodologies were used for quantification of antibiotic residues and resistance genes respectively. A possible relationship between antibiotics and antibiotic resistance gene associated was also studied.

The **third part** deals with the sorption processes taking place during the pharmaceuticals removal: first, in experiments performed with six different fungi for the elimination of selected pharmaceuticals in spiked water, and second in the experiments performed with *Trametes versicolor* for the elimination of pharmaceuticals present in a veterinary hospital wastewater. Pharmaceutical concentrations in the fungal biomass were also compared with those concentrations commonly measured in sludge from conventional wastewater treatment plants.

The results from this thesis provide valuable information about several aspects of fungal treatment and point it out as a very promising technology for the removal of pharmaceuticals from wastewater effluents.

Resumen

Durante los últimos años la presencia de productos farmacéuticos en el medio ambiente ha atraído la atención de la comunidad científica, así como ha causado la preocupación pública, ya que estos podrían suponer un riesgo para el medio ambiente. Su presencia en el medio acuático hace patente la incapacidad de las plantas de tratamiento de aguas residuales convencionales para eliminar estos compuestos de manera eficiente. En los últimos años se han realizado muchos esfuerzos en explorar y desarrollar tratamientos alternativos de las aguas residuales, capaces de eliminar los fármacos de una manera efectiva y económica. Entre todas las nuevas tecnologías consideradas, en los últimos años se ha estudiado el tratamiento fúngico de las aguas residuales debido a la capacidad de algunas especies de hongos para degradar contaminantes químicos, incluidos los productos farmacéuticos. La eliminación de estos compuestos se ha estudiado aguas residuales urbanas y de hospitales, así como en los residuos generados en el tratamiento de ósmosis inversa de efluentes de aguas residuales. Estos estudios se han realizado a escala de laboratorio, pero algunos aspectos del tratamiento fúngico todavía necesitan ser tratados antes de su implementación en plantas depuradoras a tamaño real. El objetivo general de esta tesis es estudiar algunos de estos aspectos sobre esta tecnología en relación a la eliminación de contaminantes emergentes en aguas residuales. En concreto, el trabajo presentado aborda los siguientes objetivos: i) evaluar la eliminación de los fármacos con el tratamiento fúngico desde el punto de vista ecotoxicológico, ii) determinar la eficacia del tratamiento fúngico en la eliminación de antibióticos, iii) valorar la dispersión de genes de resistencia a los antibióticos durante el tratamiento fúngico y iv) estudiar la relevancia de los procesos de absorción y/ adsorción implicados en la eliminación de los fármacos durante el tratamiento fúngico. En base a estos objetivos, el trabajo de la tesis se presenta estructurado en tres bloques:

El **primer bloque** se centra en la eliminación de fármacos en distintas aguas residuales y el efecto ecotoxicológico que esta eliminación lleva asociada. Para ello se realizó una "evaluación del riesgo ambiental" para valorar la eliminación de los fármacos y de los respectivos índices de peligrosidad asociados.

En el **segundo bloque**, se estudian conjuntamente la eliminación de antibióticos y la propagación de genes de resistencia a antibióticos en un tratamiento fúngico a escala de laboratorio realizado con aguas residuales de hospitales. Se utilizaron métodos analíticos tanto químicos como microbiológicos para la cuantificación de antibióticos y genes de resistencia, respectivamente. También se estudió la posible relación entre la cantidad de antibióticos y la evolución de los genes de resistencia.

El **tercer bloque** se focaliza en los procesos de sorción que tienen lugar durante la eliminación de los fármacos: En primer lugar, en experimentos realizados con seis hongos diferentes con aguas dopadas con cuatro fármacos de especial interés, y en segundo lugar en experimentos realizados con el hongo *Trametes versicolor* para la eliminación de fármacos presentes en las aguas residuales de un hospital veterinario. Las concentraciones farmacéuticas en la biomasa de hongos también se compararon con las concentraciones comúnmente medidas en lodos de plantas de tratamiento de aguas residuales convencionales.

Los resultados de esta tesis proporcionan información valiosa sobre varios aspectos del tratamiento fúngico y confirman dicho tratamiento como una tecnología muy prometedora para la eliminación de fármacos de las aguas residuales.

Resum

Al llarg dels últims anys la presència de productes farmacèutics al medi ambient ha captat l'atenció de la comunitat científica, així com la preocupació pública, ja que aquests podrien suposar un risc per al medi ambient.

La seva presència en el medi aquàtic fa patent l'incapacitat de les plantes de tractament d'aigües residuals convencionals per eliminar aquests compostos de manera eficient. Durant els últims anys s'han dedicat molts esforços a explorar i desenvolupar tractament alternatius de les aigües residuals capaços d'eliminar fàrmacs d'aigües residuals de manera efectiva i econòmica.

D'entre totes les noves tecnologies estudiades, el tractament fúngic de les aigües residuals ha estat considerat degut a la capacitat d'algunes espècies de fongs per degradar contamintats químics, inclosos els productes farmacèutics.

L'eliminació d'aquests compostos s'ha estudiat en aigües residuals urbanes i d'hospitals, així com en residus generats en el tractament d'osmosis inversa d'afluents d'aigües residuals. Aquests estudis s'han realizat a escala de laboratori però alguns aspectes del tractament fúngic encara necessiten ser tractats abans de la seva implementació a un nivell superior.

L'objectiu d'aquesta tesis és estudiar alguns d'aquests aspectes sobre aquesta tecnologia d'aigües residuals en relació a l'eliminació de contaminants emergents. En concret, el treball presentat abarca els següents objectius: i) evaluar l'elminació dels fàrmacs amb el tractament fúngic des del punt de vista ecotoxicològic; ii) determinar l'eficàcia del tractament fúngic en l'eliminació d'antibiòtics; iii) valorar la dispersió de gens de resistència als antibiòtics durant el tractament fúngic i iv) estudiar la rellevància dels processos d'absorció i/o adsorció implicats en l'eliminació dels fàrmacs durant el tractament fúngic. Sobre aquests objectius, el treball de la tesis es presenta en tres capítols:

El **primer bloc** es centra en l'eliminació de fàrmacs en diferents aigües residuals i l'efecte ecotoxicològic que aquesta eliminació porta associada. Es realitza una

"Evaluació del risc ambiental" per a evaluar l'eliminació dels fàrmacs i dels respectius índexs de perillositat associats.

En el **segon bloc**, l'eliminació d'antibiòtics i la propagació de gens de resistència a antibiòtics s'estudien conjuntament en un tractament fúngic a escala de laboratori realitzat en aigües residuals d'hospitals. Es van utilitzar mètodes analítics tant químics com microbiològics per a la quantificació d'antibiòtics i gens de resistència, respectivament. També es va estudiar la possible relació entre la quantitat d'antibiòtics i l'evolució dels gens de resistència.

El **tercer bloc** es focalitza en els processos de sorció que tènen lloc durant l'eliminació dels fàrmacs: En primer lloc, en experiments realitzats amb sis fongs diferents amb aigües dopades amb quatre fàrmacs d'especial interès, i en segon lloc en experiments realitzats amb el fong *Trametes versicolor* per a l'eliminació de fàrmacs presents en les aigües residuals d'un hospital veterinari. Les concentracions farmacèutiques a la biomassa de fongs també es van comparar amb les concentracions mesurades comunament en llots de plantes de tractament d'aigües residuals convencionals.

Els resultats d'aquesta tesis proporcionen informació valuosa sobre varis aspectes del tractament fúngic i confirmen el tractament fúngic com a una tecnologia molt prometedora per a l'eliminació de fàrmacs de les aigües residuals.

Abbreviations and acronyms

AOP	Advanced oxidation process
ARB	Antibiotic resistance bacteria
ARG	Antibiotic resistance gene
ATCC	American type culture
BDL	Below detection limit
BFR	Brominated flame retardant
BQL	Below quantification limit
BRF	Brown rot fungi
CAS	Conventional activated sludge
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
EC50	Half maximal effective concentration
ECOSAR	Ecological Structure Activity Relationships
EDC	Endocrine disruptor
EDTA	Ethylene diamine tetra acetic acid
EMEA	European Medicines Evaluation Agency
ERA	Environmental risk assessment
ESI	Electrospray ionization
FDA	Food and Drug Administration
HGT	Horizontal gene transfer
HPLC	High performance liquid chromatography
HQ	Hazard quotient
HRT	Hydraulic retention time
HWW	Hospital wastewater
KC	Killed control
K_d	Solid-water distribution coefficient
LC	Liquid chromatography
LC50	Half maximal lethal concentration

LDF	Litter decomposing fungi
LiP	Lignin peroxidase
LME	Lignin-modifying enzyme
LMP	Lignin-modifying peroxidase
LOD	Limit of detection
LOQ	Limit of quantification
MBR	Membrane bioreactor
MnP	Manganese peroxidase
MRM	Multiple reaction monitoring
ND	Not detected
PAH	Polyaromatic hydrocarbon
PCB	Polychlorinated biphenyl
PCP	Personal care product
PEC	Predicted environmental concentrations
PhAC	Pharmaceutically active compound
PNEC	Predicted non effect concentration
PPCP	Pharmaceutical and personal care product
qPCR	Real-time polymerase chain reaction
QSAR	Quantitative structure-activity relationships
RNA	Ribonucleic acid
RO	Reverse osmosis
ROC	Reverse osmosis concentrate
rRNA	Ribosomal ribonucleic acid
SPE	Solid phase extraction
SRF	Soft rot fungi
SRT	Solid retention time
SS	Suspended solids
TP	Transformation product
tRNA	Transfer ribonucleic acid
UPLC	Ultra performance liquid chromatography

UV	Ultraviolet
VGT	Vertical gene transfer
VHW	Veterinary hospital wastewater
VP	Versatile peroxidase
WHO	World Health Organization
WRF	White rot fungi
WWTP	Wastewater treatment plant

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Después de muchos esfuerzos parece que esta etapa del doctorado va tocando a su fin. Una etapa que siempre recordare y de la que me llevo un montón de cosas más allá de conocimientos científicos y un título de Doctor. Durante esta "travesía" me he encontrado con una gran cantidad de personas que de una u otra manera han contribuido a que hoy este aquí, escribiendo estas líneas de agradecimiento en las que espero no olvidarme a nadie.

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Tampoco me podré olvidar de gente como Diana, Meri, Marta, Saulo y Jose Luis. Sois muy muy grandes; trabajar a vuestro lado ha sido extremadamente fácil. Leyendo vuestros trabajos uno se imagina que sois obsesos del trabajo que solo vivís para eso, y luego cuando se os conoce uno alucina al ver que también tenéis vida fuera del laboratorio. Eso sí, si me permitís un último consejo, que más de una vez ya os he dicho… ¡Tenéis que echarle más morro a la vida! Trabajáis como mulas y luego no os hacéis valorar.

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siempre hemos sido Zipi y Zape y aunque nuestros caminos a veces se separen, siempre encontramos cualquier motivo para que se vuelvan a cruzar, y lo seguirán haciendo.

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¿Qué es un científico, al fin y al cabo? Es un hombre curioso, mirando por una cerradura, la cerradura de la naturaleza, intentando descubrir lo que está pasando

Jacques-Yves Cousteau

Chapter 1

Chapter 1 - Background and introduction

1. Emerging contaminants in the environment

Since the mid-20th century the study of environmental contamination has mainly focused on the presence and impact of chemical pollutants released (intentionally or unintentionally) into the environment in great quantities, such as pesticides, and contaminants derived from industrial and mining activities, etc. As all these compounds have been studied for a long time, many of them are regulated and maximum levels set up in the environment [1, 2]. During the last decades, favored by technological improvements (especially in analytical chemistry field), the environmental contamination studies have focused on the so-called "emerging contaminants". They are defined as "synthetic or naturally occurring chemicals that are not commonly monitored in the environment but that have the potential to enter in the environment and cause known or suspected adverse ecological and (or) human health effects" [3]. Within emerging contaminants, there is a huge variety of compounds: plant protection products, biocides, metals, polyaromatic hydrocarbons (PAHs) that are mainly incineration by-products, pharmaceutically active compounds (PhACs), endocrine disruptors (EDCs), personal care products (PCPs), brominated flame retardants (BFRs), UV filters, nano materials and even antibiotic resistance genes (ARGs).

1.1. Pharmaceuticals (PhACs)

Among the emerging contaminants, PhACs comprise diverse chemical groups of compounds designed to be highly active and interact with humans, animals or infectious organisms, playing a key role in the treatment of diseases in both human and animals. In recent years, the consumption of PhACs has increased due to the discovery of novel compounds, the expanding population, the inverting age structure in the population and the increased sales of generic drugs [4, 5].

After the intake, part of the PhAC is assimilated whereas other part is excreted (modified or as the parent compound) via urine or faeces into raw sewage and further

into wastewater treatment systems [6]. Several studies have proved that unfortunately, treatment technologies used in conventional wastewater treatment plants (WWTPs) are not enough effective to eliminate PhACs because they are not originally designed to remove complex compounds such as PhACs [7, 8]. Due to this low removal efficiency, WWTPs are considered the primary source of PhACs into the environment. Other possible pathways include disposal of PhACs leftovers to sewage and trash, release of PhACs used in livestock farms, aquaculture, veterinary facilities, surface runoff, etc. (Figure 1.1).

Figure 1.1 - Origins and fate of pharmaceuticals and personal care products (PPCPs) in the environment, adapted from http://epa.gov/nerlesd1/chemistry/pharma

Even though at relatively low levels, PhACs can be found in the natural ecosystems; their generally swift degradation rates, are exceeded by their introduction rates into the aquatic environment and thus, they are considered pseudo-persistent contaminants [9];a wide range of these compounds have been found in several environmental compartments and matrices comprising the hydrosphere (surface water [10, 11], marine water [12], groundwater [13-15] and drinking water [16-18]), the geosphere [19, 20] and even the biosphere [21-23].

In accordance with their environmental relevance based on the studies performed in the last years (environmental impact of PhACs are reviewed in section 1.3.), some PhACs are currently being considered for environmental legislation in different countries. In the

case of the European Union, the anti-inflammatory drug diclofenac and three macrolides antibiotics (erythromycin, clarithromycin and azythromycin) have been included in the so called "watch list" of priority substances under the Water Framework Directive (WFD) for the "specific purpose of facilitating the determination of appropriate measures to address the risk posed by these substances" [1]. In United States, the Environmental Protection Agency (EPA) has included the antibiotic erythromycin and 5 synthetic hormones to a list of contaminants that must be controlled [2]. Finally, in 2008 the Global Water Research Coalition (GWRC) published a report in which a large number of PhACs were classified in several classes: high, medium and low priority compounds (Table 1.1). This report identifies compounds that are most likely found in water supplies and that may have significant impacts on human and environmental health [24].

Table 1.1 - PhACs priority list by GWRC[24]. a- Psychiatric drugs; b- Antibiotics; c-Analgesics/Anti-inflamatories; d- Lipid lowering agents; e- β-blocking agents; f- Chemotherapy agents; g- Diuretics; h- X-ray contrast agents; i- Histamine receptors antagonists; j- Antidiabetics; k- Antiepileptics; l- Antihypertensive; m-Antiasthma; n- Antifungal; o- Calcium channel blocker

High	**Medium**	**Low**
Carbamazepine[a]	Acetaminophen [c]	Iomeprol [h]
Sulfamethoxazole [b]	Acetylsalicylic acid [c]	Iopamidol [h]
Diclofenac [c]	Clofibric acid [a]	Metformin [j]
Ibuprofen [c]	Cyclophosphamide [f]	Dilantin [k]
Naproxen [c]	Furosemide [g]	Doxycycline [b]
Bezafibrate [d]	Iopromide [h]	Enalapril [l]
Atenolol [e]	Amidotrizoic acid [h]	Fluoxetine [a]
Ciprofloxacin [b]	Diazepam [a]	Norfluoxetin [a]
Erythromycin [b]	Lincomycin [b]	Oxazepam [a]
Gemfibrozil [d]	Amoxicilin [b]	Salbutamol
	Hydrochlorothiazide [g]	Simvastatin [a]
	Metoprolol [e]	Cefalexin [b]
	Ranitidine [i]	Cimetidine [i]
	Trimethoprim [b]	Clotrimazole [n]
	Sotalol [e]	Diltiazem [m]
	Codeine [c]	Valproic acid [a]
	Ofloxacin [b]	
	Clarithromycin [b]	

1.1.1. Antibiotics

Antibiotics are one of the PhACs types with higher usage and consumption worldwide. By definition, an antibiotic is a chemotherapeutic agent that inhibits or abolishes the growth of microorganisms, such as bacteria, fungi, or protozoa [25]. There are a large number of antibiotics in the market; in 2009 Kümmerer reported over 250 different chemical entities registered as antibiotics for human and/or animal health treatments. Antibiotics can be classified by their chemical structure into different sub-groups such as ß-lactams, quinolones, tetracyclines, macrolides, sulphonamides,etc. [25]. They can be also classified according their targets, which can be summarized as: i) bacterial cell wall, ii) cytoplasmic membrane, iii) protein synthesis systems, iv) nucleic acid synthesis systems, v) bacterial metabolic pathways and vi) resistance mechanisms [26].

According to a report released in 2015 by the Center for Disease Dynamics, Economics and Policy (CDDEP)[27], based on the analysis of data from scientific literature and national and regional surveillance systems from 71 countries over the past 10 years, antibiotic use is growing steadily worldwide (30%), driven mainly by rising demand in low- and middle-income countries (Figure 1.2). This increase in the use of antibiotics and the concern about their side effects in the environment, has led to the increase in the number of studies performed on these compounds in the last years [28-31].

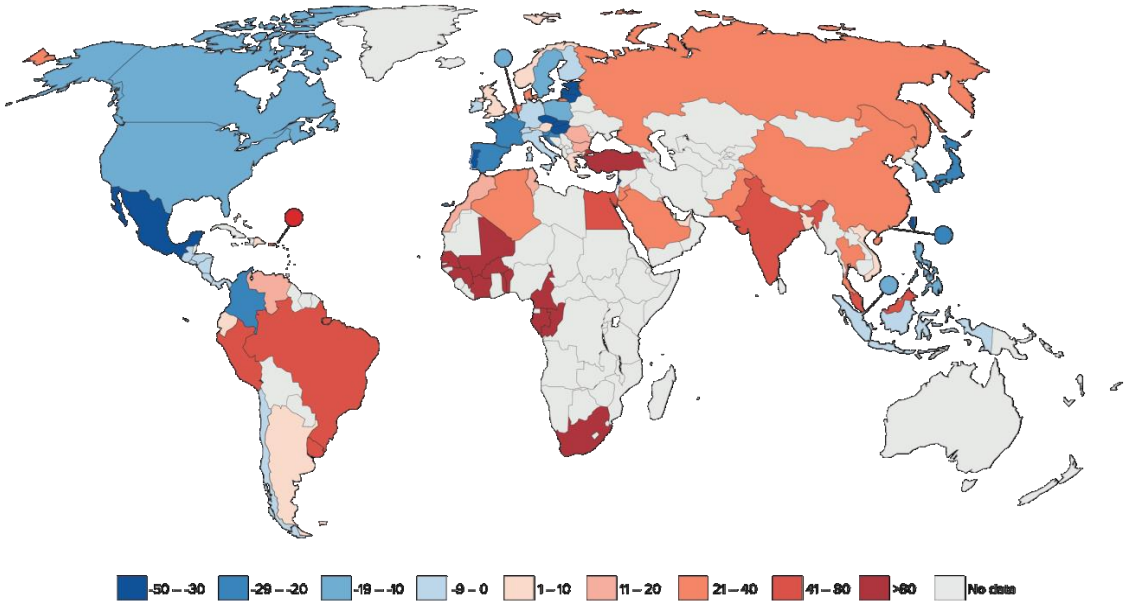


Figure 1.2 - Percentage change in antibiotic consumption per capita 2000 - 2010, by country.

1.2. Antibiotic resistance

Antibiotic resistance represents a serious and growing global health threat. According to recent data from the European Centre for Disease Prevention and Control and the European Medicines Agency, every year approximately 25000 european citizens (5.1 per 100000 inhabitants) die from infections caused by bacteria that have developed resistance towards antimicrobials (http://www.ecdc.europa.eu/). In the USA, nosocomial infections are responsible for 12000 deaths each year [32], and it is estimated that more than 70% of bacteria that cause these infections are resistant to at least one of the antibiotics commonly used to treat them [33]. Although this phenomenon occurs in nature and has an ancient origin, several studies [34-37] have supported the idea that the overuse and misuse of antibiotics has led to the emergence and spread of antibiotic-resistant bacteria. Susceptible bacteria can acquire resistance to antibiotics by either genetic mutation or by horizontal transfer of antibiotic resistance genes (ARGs). These ARGs encode diverse mechanisms (Figure 1.3) that allow microorganisms to survive in the presence of one or several antibiotic compounds.

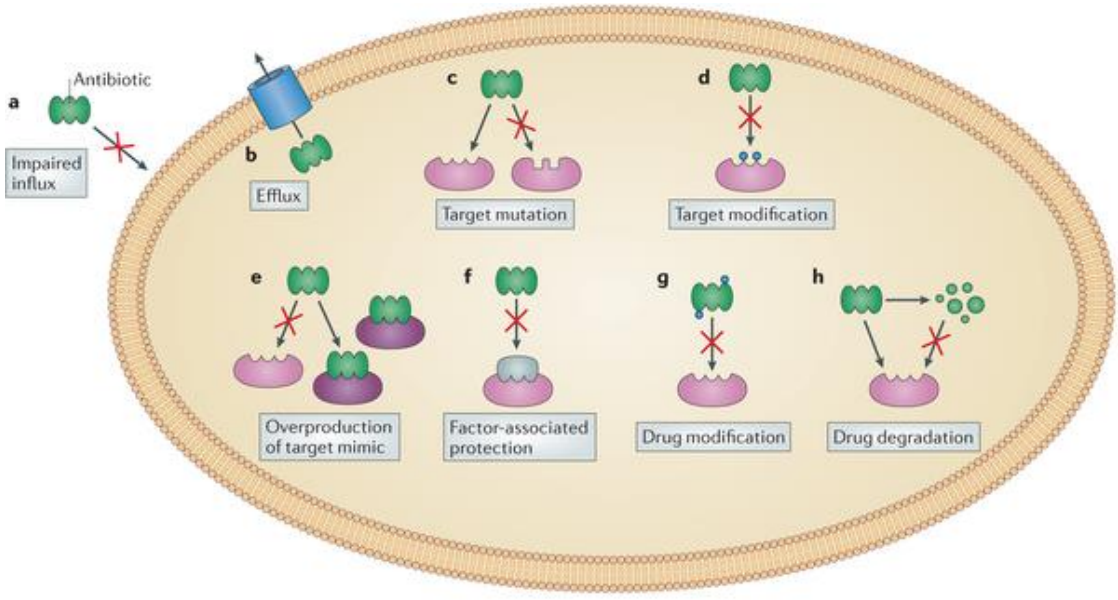


Figure 1.3 - Typical resistance mechanisms: (a) low membrane permeability;(b) active efflux of the antibiotic from the cell; (c) target mutation; (d)target modification; (e) overproduction of a molecule that mimics the target; (f) recruitment of a specialized protein factor to actively remove the drug from the target; (g,h) modification or degradation of the antibiotic [38].

Once the resistance genes have developed, they can be transferred directly to all the bacteria's progeny during DNA replication. This is known as vertical gene transfer (VGT) or vertical evolution. The process is strictly a matter of Darwinian

evolution driven by principles of natural selection. For instance, antibiotics may exert selective pressure, where the wild types (non-mutants) are killed and the resistant mutants are allowed to survive and multiply, increasing the prevalence of the gene in the environment (Figure 1.4).

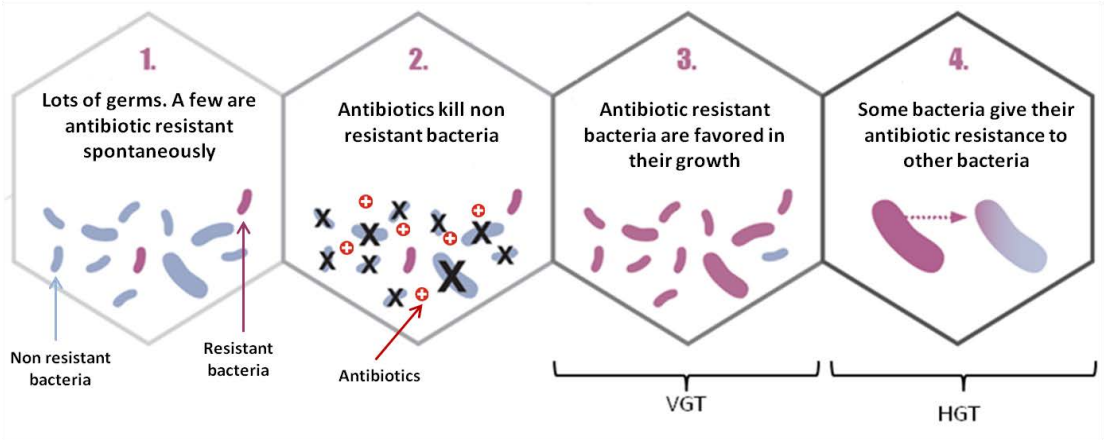


Figure 1.4 - Brief scheme about antibiotics selective pressure mechanism [37]

Horizontal gene transfer (HGT) is another mechanism responsible for the acquisition of antibiotic resistance (Figures 1.4 and 1.5), a process where genetic material contained in small packets of DNA can be transferred between individual bacteria of the same species or even between different species. There are three mechanisms of HGT: transformation, transduction or conjugation (Figure 1.5). Transformation occurs when naked DNA is released on lysis of an organism and is taken up by another organism; then the ARG can be integrated into the chromosome or plasmid of the recipient cell. In transduction, ARGs are transferred from one bacterium to another by means of bacteriophages and can be integrated into the genetic pool of the recipient cell. Conjugation occurs by direct contact between two bacteria: plasmids form a mating bridge across the bacteria and DNA is exchanged, which can result in acquisition of ARGs by the recipient cell [39].

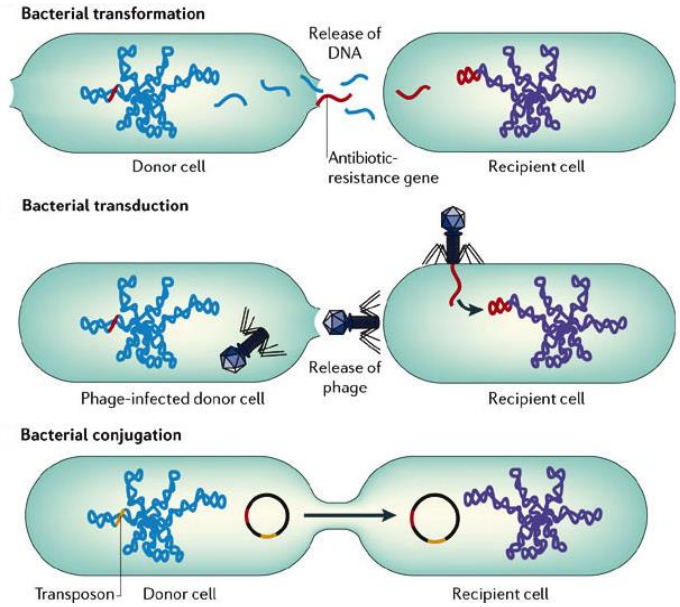


Figure 1.5 - Mechanisms of HGT in bacteria

Despite ARGs have an environmental origin [40], the introduction and accumulation of antimicrobials in both the environment and hospital facilities (Figure 1.6) facilitates an increase in the amount and spread of the ARGs [41]. As a consequence, ARGs can be found in almost all environments (including wastewater and WWTPs, surface water, lagoon water of animal production areas, aquaculture water, sediments, soils, groundwater and drinking water) and they are currently considered as emerging pollutants [42-44]. As can be seen in figure 1.6, antibiotics can reach aquatic environments via WWTP effluents,where, by selective pressure in the bacterial population, can favor the spread of ARGs already present either in the WWTP effluent or in the natural microbiota. The main problem arises when ARGs are acquired by pathogenic bacteria, compromising the effectiveness of antimicrobial therapies [45, 46]. The infectious agents responsible for illnesses such as acute respiratory infections, diarrheal diseases, measles, acquired immune deficiency syndrome (AIDS), malaria and tuberculosis, are becoming resistant to first-line drugs and, in some instances to second- and third-line treatments [47]. Moreover, after the great development of antibiotics in the mid-20th century (the "golden age" of antibiotics), the number of new licensed antibiotics has progressively declined due to the development of new antibiotics has become expensive and time-consuming, and the pharmaceutical industry has shifted its investment to the development of chronic disease therapies and life style drugs, that are more lucrative [48]. The emergence and spread of ARB are thus considered by the

World Health Organization (WHO) as one of themajor threats to public health in the 21st century, and withouturgent actions we are heading to a post-antibiotic era, in whichcommon infections and minor injuries could eventually causedeaths [49].

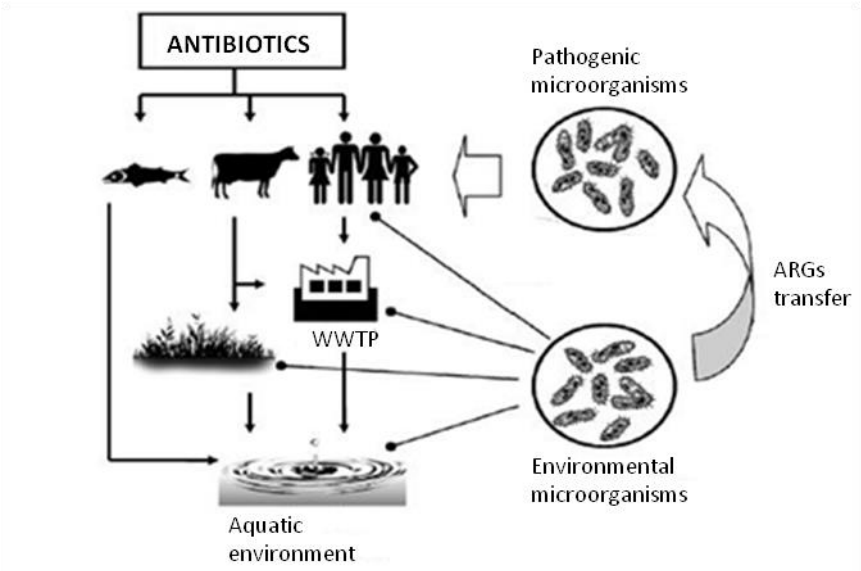


Figure 1.6 - Possible fates of antibiotics residues and ARGs in the environment. Adapted from Baran et al. (2011) [50]

Some effort would need to be done in order to reduce the possibility of ARGs entering and spreading in the environments; the most effective and direct approach is thought to be the reasonable use of antibiotics in health protection and agriculture production. As WWTPs are among the main sources of antibiotics release into the environment, many studies have evaluated the fate of ARGs in conventional WWTPs [31, 51-55]. As it happens with PhACs, conventional WWTPs are not effective in eliminating all kinds of ARGs (Table 1.2), and therefore the efficiency of different non-conventional wastewater technologies in the removal and inactivation of ARGs has been studied by several authors (see section 1.5) and recently reviewed by Sharma et al. (2016) [56].

Table 1.2 - ARGs trends in conventional WWTPs. ↓ - Decrease of the copy number of the gene. ↑ - Increase in the copy number of the gene. ↔ - No changes detected. * - Unknown value

Gen	Related antibiotics	Trends in absolute values		Trends in relative values		Ref
aacA4, aacA-aphD	Amikacin, dibekacin, isepamicin, netilmicin, sisomicin and tobramycin	↓	52-100%			[57]
aadA	Spectinomycin and streptomycin	↑	98%			
aadB	Dibekacin, gentamicin, kanamycin, sisomicin and tobramycin	↑	23%			
acrB	Acriflavin, aminoglycoside, β-lactams, glycylcycline and macrolides	↓	≈100%			
ampC	Cephalosporines	↑	Appearance			
amrB	Acriflavine, aminoglycoside and macrolides	↓	≈100%			
arnA	Polymyxins	↑	Appearance			
bacA	Bacitracin	↓	≈100%			
bla_{IMP13}, $blaI_{MP18}$	Carbapenem, cephalosporin, cephamycin and penicillin	↓	≈100%			
blaLCR1, blaNPS1 , blaOXA, blaOXA3	Cloxacillin and penicillin	↓	≈100%			
bla_{OXA53}	Cloxacillin and penicillin	↑	Appearance			
catB3, ceoB, cmlA, cmx, floR	Chloramphenicol	↓	50-100%			
ereA, ereB	Erythromycin	↑	Appereance 800%			
ermA, ermB,ermF, ermG, ermT, ermX	Lincosamide, macrolide and streptogramin B	↑	Appearance 73%			
lnuB	Lincomycin	↑	Appearance			
macB, mefA	Macrolides	↓	≈100%			
mdtF	Doxorubicin and erythromycin	↓	≈100%			
mexF	Chloramphenicol and fluoroquinolone	↓	≈100%			
mexI, mexW	Aminoglycoside, β-lactams, fluoroquinolones, tetracycline and tigecycline	↓	≈100%			
rosA, rosB	Fosmidomycin	↓	≈100%			
smeB, smeD, smeE	Flouroquinolones	↑	Appearance 33%			
strA, strB	Streptomycin	↓	3-15%			
tet32, tet33, tet36, tet39, tetC, tetM, tetO, tetT, tetW, tetY	Tetracyclines	↑	Appearance			
tetA, tetG, tetV, tetX	Tetracyclines	↓	67-100%			
vanRB, vanRA	Vancomycin	↑	Appearance			
vatB	Streptogramin A	↑	Appearance			
gryA	Nalidixic acid			↓	*	[58]
qnrC	Quinolones			↑	*	
parC, qnrD	Quinolones			↓	*	
sul I, sul II	Sulfonamides			↑	*	
sul III	Sulfonamides			↓	*	

tetA, tetE, tetM, tetZ	Tetracyclines			↑	*	
tetB, tetW	Tetracyclines			↓	*	
blaCTX32, blaSHV34	β-lactams	↓	96-98%	↔	0%	[59]
bla_{OXA58}	Cloxacillin and penicillin	↓	93%	↔	0%	
sul I, sul II	Sulfonamides	↓	97-98%	↔	0%	
tetC, tetM	Tetracyclines	↓	93-97%	↔	0%	
tetW	Tetracyclines	↓	≈100%	↓	88%	[60]
bla_{TEM}	β-lactams	↓	99%	↑	76%	
ermB	Lincosamide, macrolide and streptogramin B	↓	≈100%	↓	82%	
sul I	Sulfonamides	↓	99%	↑	135%	
qnrS	Quinolones	↓	98%	↑	302%	
bla_{TEM}	β-lactams	↓	≈100%	↓	87%	[61]
qnr family$^{\Delta}$	Quinolones	↓	76%	↓	*	[62]
erm family$^{\Delta}$	Macrolides	↓	73%	↓	*	
sul family$^{\Delta}$	Sulfonamides	↓	79%	↓	*	
tet family$^{\Delta}$	Tetracyclines	↓	80%	↓	*	
mecA	Methicillin, penicillin and other penicillin-like antibiotics	↓	*	↓	*	[63]
sul I	Sulfonamides	↑	*	↑	*	[64]
sul II	Sulfonamides	↔	*	↔	0%	
sul I	Sulfonamides	↓	≈100%	↔	0%	[51]
tetO, tetW	Tetracyclines	↓	≈100%	↔	0%	
sul I	Sulfonamides	↓	≈100%	↔	0%	[65]
tetO, tetW	Tetracyclines	↓	≈100%	↓	*	
sul I, sul II	Sulfonamides			↓	*	[66]
tetM, tetO, tetQ, tetW	Tetracyclines			↓	*	
tetA, tetC	Tetracyclines	↓	≈100%	↓	≈100%	[67]
tetG, tetQ	Tetracyclines	↓	≈100%	↓	*	[68]
tetH, tetO	Tetracyclines	↓	≈100%	↓	70-100%	[69]
tetQ	Tetracyclines	↑	83%	↑	76%	
tetW	Tetracyclines	↔	0%	↑	16%	
tetZ	Tetracyclines	↔	0%	↓	93%	

$^{\Delta}$*erm* family includes *ermB* and *ermC*; *qnr* family includes *qnrA, qnrB, qnrD* and *qnrS*; *sul* family includes *sul* I, *sul* II, *sul* III and *sulA; tet* family includes *tetA, tetB, tetC, tetD, tetE, tetG, tetH,tetJ, tetK, tetL, tetM, tetO, tetQ, tetT, tetW,tetX, tetZ, tetA/P* and *tetB/P*

1.3. Environmental effects of PhACs (including antibiotics) and ARGs in aquatic ecosystems

PhACs

Although PhACs are generally present in the environment at trace levels (ng L^{-1} to low µg L^{-1}), they have raised scientist and public concernsince these compounds are biologically active (developed to have an effect on organisms). Even though PhACs are designed to target humans, aquatic organisms exhibiting the same target receptors could also experience some side effects. In addition, secondary effects not considered important in the treatment of humans may have major implications for non-mammalian aquatic organisms [70]. Some examples of unintended side effects of PhACs have been reported in the last years. In 2004 a relationship between the death of a huge number of oriental white-backed vultures (*Gyps bengalensis*) in the Indian subcontinent and the use of diclofenac in cattle raising was established [71]. Renal dysfunction in rainbow trouts (*Onchorhynchus mykiss*) caused also by diclofenac exposure to environmentally relevant concentration range has also been identified [72]. Other reported effects of exposure to PhACs in organisms under laboratory controlled condition range from behavioral modifications [73], reproductive alterations [74] and modifications of gene expression and proteome [75-78]. The evidence that environmental implications related to PhACs exposure can be similar to those related to regulated priority organic pollutants have led to their consideration in comprehensive risk assessment studies [79]. General principles and guidelines for environmental risk assessment (ERA) of new PhACs undergoing their registration process have been introduced by European Medicines Evaluation Agency (EMEA) and the Food and Drug Administration (FDA). Both organizations use similar tiered system of evaluation [80, 81] and are based in the comparison between the predicted environmental concentrations (PECs) and the worst-case predicted non effect concentrations (PNECs) estimated from standard toxicity assays [81, 82]. Despite all the effort made to date, there is still a great lack of information regarding the environmental effects of PhACs. First of all, the normative approved by both EMEA (2003) and FDA (1998) has only been applied to newly released PhACs; however, most of the PhACs prescribed and detected in the environment nowadays were approved long before environmental toxicity testing

paradigms were established [83]. Therefore, in the last years huge efforts have been made in order to apply ERAs to those PhACs commonly detected in the environment and approved before environmental normative were established [84-117]. However, due to the large number of PhACs currently used, approximately 3500, performing full testing batteries for all compounds is not logistically or scientifically practical. In fact, prioritizing PhACs for further studies was recently identified as a major need by a balanced group of scientists from governments, industry and academia, in order to optimize the economic investments [83].

Due to the lack of experimentally derived toxicity data, the use of Quantitative structure-activity relationships (QSARs) models for predicting ecotoxicity (besides other chemical properties and environmental fate) has been recommended as an alternative by several international organisms [118-120]. A QSAR is a model describing the mathematical relationship between a property of the chemical, in this case toxicity, and one or more descriptors of the chemical contaminant. The descriptors are chemical and physical characteristics obtained from knowledge of the structure of the chemical or experimentally. Some of the descriptors that have been used in QSARs to estimate chemical toxicity include the octanol–water partition coefficient [121-123]; various stereo-electronic characteristics [124-128]; molecular size, volume, and shape [129-131]; presence/absence of functional groups [132-134] and many others [135].

Antibiotics and ARGs

Antibiotics may pose a risk to the ecosystems even at very low concentrations and persistence rates, as they are designed to have a biochemical effect in the microorganisms, and thus have a significant impact in the processes controlled by native biological communities [136-138]. To date most research on the impact of antibiotic discharges into the environment are based on the effects on benthic invertebrates and algae [139, 140]. However, little is known about the potential effects on the diversity and functioning of bacterial communities in ecosystems, despite its fundamental role as regulators of the processes that define the majority of ecosystem services provided by these freshwater bodies, such as the capacity for self-purification [141]. Antibiotics might act as signaling agents (a kind of hormones) in microbial environments [142-144]. Common receptors have been identified in plants for a number of antibiotics affecting chloroplast replication (fluoroquinolones), transcription–translation

(tetracyclines, macrolides, lincosamides, aminoglycosides, pleuromutilins), folate biosynthesis (sulfonamides, and probably trimetoprim), and fatty acid synthesis (triclosan) [145].

However, the most concerning effect of the antibiotics in the environment is the selective pressure they exert in aquatic microbes, favoring the spread of ARGs, and ARB [34-37]. As explained in section 1.2, this spread can lead to serious health problems, compromising the effectiveness of antimicrobial therapy.

1.4. PhACs removal mechanisms

Due to the relatively low efficiency of conventional WWTPs degrading PhACs, some studies have tried to examine the mechanisms involved in the PhACs removal [146-148]. These mechanisms have been studied in order to achieve a better understanding about the differences in the elimination rates of the PhACs. The main removal mechanisms for PhACs in conventional WWTPs include photodegradation, volatilization, sorption to solids and biological transformation [149].

Photodegradation

Various photodegradation mechanisms can contribute to the significant removal of PhACs from surface waters [150, 151]. Some PhACs such as diclofenac or triclosan are photodegraded when they are exposed to sunlight either by direct or indirect mechanisms [152]. Direct photodegradation consists of absorption of solar light by the aquatic contaminants, followed by a chemical reaction. In the case of indirect photodegradation, pollutants are degraded by strong oxidant species such as hydroxyl radicals [152, 153] generated by natural photosensitizers (e.g., nitrate and humic acids) under solar radiation [154]. Photodegradation processes are largely influenced either by the dissolved or suspended solids contained in the wastewater. The results of the competition between direct and indirect photodegradation reactions will determine the rate at which this degradation takes place and the photoproducts that are generated [153]. However, photodegradation is unlikely significant during conventional wastewater treatment because of the low light exposure relative to the volume of the system and the high concentration of solids blocking light [31].

Volatilization

The fraction of compound volatilized (ϕ) depends on the solid-water distribution coefficient (Kd, L Kg-1), defined as the ratio between the concentrations in the solid and liquid phases at equilibrium, the suspended solids (SS, Kg L^{-1}), the flow of air getting in contact with wastewater (Q_{air}, in m^3 air m^{-3} wastewater), type of aeration and the Henry coefficient (H, in µg m^{-3}air/µg m^{-3} wastewater), as shown in the following equation.

$$\phi = \frac{H \cdot Q_{air}}{1 + H \cdot Q_{air} + K_d \cdot SS}$$

Taking into account the typical air flow rates used in a conventional activated sludge (CAS) system (5– 15 m^3 air m^{-3} wastewater[7]) and the Henry coefficient of the PhACs (ranging from $3{,}6 \cdot 10^{-6}$ (fluoxetine) to $2{,}2 \cdot 10^{-27}$ (iopromide)), losses due to stripping are completely negligible [146].

Biological transformation

Biodegradation has been one of the most studied removal processes and has been highlighted as the main degradative mechanism for many PhACs in WWTP [155-170]. Biological removal of each PhAC is conditioned by several factors, such as PhAC concentration [7], chemical structure of the PhAC [146], solid and hydraulic retention times (SRT and HRT respectively) [171], temperature [172, 173], source and concentration of the inoculum [169, 170], wastewater characteristics [174], etc. The large number of factor involved makes it very difficult to establish a general degradation model for PhACs.

Sorption

One of the most important elimination processes in WWTPs is sorption to suspended solids in the wastewater and subsequent removal by sedimentation as primary and secondary sludge. Sorption mainly occurs by i) absorption, involving hydrophobic interactions of the aliphatic and aromatic groups of a compound with the lipophilic cell

membrane of the microorganisms and the fat fractions of the sludge, and by ii) adsorption, where electrostatic interactions of positively charged groups (e.g., amino groups) with the negatively charged surfaces of the microorganisms are of importance [175]. A common approach to determine the fraction of PhACs sorbed onto sludge is the use of K_d. This coefficient takes into account both absorption and adsorption.

$$K_d = \frac{C_{sorbed}}{C_{soluble}}$$

Where C_{sorbed} is the sorbed PhACs concentration onto sludge (µg Kg^{-1}) and $C_{soluble}$ the dissolved concentration of the compound (µg L^{-1}).

Unfortunately, sorption processes with PhACs are still an issue to be studied more deeply. Despite many studies have been performed so far, very few ideas can extracted from them. Both sorption values (ranging from 1µg kg^{-1} to 3g kg^{-1} [176]) and K_d coefficients (e.g. in the case of diclofenac ranging from 0.12 [177] to 501.2 [178]) of each compound differ widely from one study to the other, depending on several treatment parameters such as temperature, pH, aeration, reactor design, sludge characteristics, etc [171, 175, 179-185].

Sorption processes are very important in the PhACs elimination in WWTPs due to their adsorption/absorption into the suspended solids. Sludge is the residue generated during the biological treatment process ofwastewaters, and its handling is one of the most important challenges in wastewater management [186]. Depending on the concentration of different pollutants, sludge needs to be treated before being released into the environment whereas in some other cases the sludge the treatment is not necessary as the pollutants concentration in not considered dangerous. In the EU's Working Document on Sludge, contaminants such as metals, pathogens and organic pollutants are regulated. However, the concern on organic pollutants is limited to halogenated compounds, alkylbenzene sulfonates, phthalates, nonylphenols, polychlorinated biphenyls, polycyclic aromatic hydrocarbons and dioxins. Consequently, legislation for most of the groups of emerging pollutants, including PhACs is still lacking [187].

Nowadays there are several ways by which the final sludge from WWTPs is disposed in the environment. In this sense, the fate of the sludge (treated or untreated depending on the local requirements) includes application in agricultural lands (37%), incineration

(11%), landfilling (40%) and minor areas such as forestry and land reclamation (12%) [187]. Depending on the handling of this sludge, PhACs adsorbed/absorbed into the sludge can also reach to the environment [176, 187]. During the last years, different type of sludge treatments (anaerobic digestion [188] and fungal treatment with biopiles [187, 189] have been tested in order to reduce the quantity of PhACs present in the sludge before releasing it to the environment.

1.5. Advanced technologies for the removal of emerging contaminants in hospital wastewater

Different types of advanced technologies for treating emerging contaminants have been tested, including physico-chemical and biological treatments [179, 190-194], in order to find a proper alternative to conventional activated sludge (CAS) or even to be added as a tertiary treatment after CAS treatment, the classic treatment in conventional WWTPs. The use of this kind of technologies could be especially important in the treatment of wastewaters with a high PhACs concentration, such as hospital and to a lesser degree, veterinary hospital wastewaters [195]. In Europe there is not a specific directive or guideline for the management of hospital effluents, however some ideas may be highlighted according to different European directives published in the last years. The European Directive n. 98 of 19th November 2008 (EU, 2008/98/EC) about the management of hazardous waste and the list of hazardous waste of the European Decision n. 532 of 3 May 2000 (EU, 2000/532/CEE) stated that some hospital liquid waste (PhACs, solvents, soaps, no-halogenated organic substance, etc.) must not be discharged into a foul sewer but treated as a waste and collected and disposed as such. For the effluents from the hospital foul sewer, there is not a specific disposition, so member states of the EuropeanUnion have their own legislation, evaluation and selection criteriafor HWW quality and its management. However, quite rarely, national (or regional) legal regulations define how to manage and treat hospital wastewaters before its disposal (discharge in public sewage for treatment at a municipal WWTP or discharge into a surface water body) [196, 197]. Therefore, if a hospital facility is considered by the legislation of the state, to be industrial or a facility that discharges not only domestic wastewater (as it is the case of Spain and France), specific characteristics

of the wastewater will be required for the permission to discharge it in the municipal WWTP; usually a pretreatment is required. On the contrary, in the countries where the hospital wastewater is considered to be domestic or communal, neither authorization nor specific characteristics are required (as in Germany). In other countries, if HWW complies with the specific characteristics established by the WWTP authority, the wastewater may be considered to be domestic effluent and discharged in WWTPs without any pretreatment. If the indicator parameters exceeded the limits imposed, the wastewater may be pretreated (as in Italy) [198]. Indicators required are usually physico-chemical indicator, macropollutants (NH4, NOx, oil and grease, tensioactives, phosphorous, chlorines and others) and in some rare cases, microbiological indicators (typically *E. coli*), but none have a specific PhAC residue and pathogen limitation before discharge in WWTPs or in surface water[198].

The fact of considering hospital wastewater equal to urban wastewater in terms of pollutant nature or pollutants loads could be ungrounded. Hospital wastewaters very often show higher levels of PhACs than urban wastewaters [199]; and veterinary hospital wastewater can be positioned between both urban and hospital wastewaters [195]. This common practice of co-treating hospitals and urban wastewaters jointly at a municipal WWTP is considered asan inadequate solution by many authors [195, 200-203] because it is based on dilution of the effluents and it has been demonstrated that wastewater dilution, and hence, dilution of PhACs concentration is detrimental for their biological removal by CAS [7, 195]. Therefore the use of alternative wastewater treatments at the source point for this kind of effluents has been highly recommended by many authors [7, 195, 199, 200, 202-205].

Alternative treatments for PhACs

The alternative treatments investigated for the elimination of PhACs in wastewaters can be physico-chemical and/or biological treatments. Physico-chemical treatments include technologies, typically used as tertiary treatment, such as advanced oxidation processes (AOPs), membrane filtration and activated carbon adsorption. Among oxidation technologies, ozonation is the most studied and one of the most promising technologies, with a very high removal values for selected PhACs [193, 194, 206]. In addition, it has the advantage of providing simultaneous oxidation of pollutants and disinfection of the wastewater. Other AOPs has been also tested for PhACs removal including the use of

other oxidants like chlorine and hydroxyl radical [207], photolysis [192, 208] and electro and photo-Fenton related reactions [209, 210]. Degradation values obtained for this other AOPs tested are quite variable but in none of the cases exceed the removal efficiency shown by the ozonation. Nevertheless, all of them are expensive treatments with high consumption of energy and/or chemicals which implies a high economic investments [211, 212]. Moreover, the generation of toxic transformation byproducts in these type of treatments is a likely side effect [213].

Filtration technologies, such as nanofiltration, ultrafiltration and reverse osmosis, have been also extensively tested [179, 214, 215] with different retention values for the PhACs. However, as a mainly physical process, the majority of the pollutants are not degraded and remain in the concentrates or brines (the liquid fraction with lower volume and higher pollutants concentration). This concentrates need to be treated as a hazardous waste of the wastewater treatment, which is a major disadvantage for this kind of technologies.

Adsorption to activated carbon is another alternative technology evaluated. It gives quite good results in terms of retention of the PhACs, although the most polar ones usually achieve lower removal percentages [216, 217]. Its main drawback is the need for the regeneration of the activated carbon, causing an increase of the economic costs of the whole treatment.

Biological treatments are usually less expensive and present lower energy consumption than the physico-chemical ones [211]. This kind of treatments includes technologies such as membrane bioreactors (MBRs), artificial wetlands, algal ponds and fungal treatments (the latter is explained in section 2). The most popular methodology is the use of MBRs, which combines a physical process like microfiltration or ultrafiltration with a suspended growth biological reactor. This methodology is already implemented in some full-scale WWTPs with quite good removal values [179, 218]. However, MBRs presents some drawbacks that limit their widespread application. The main inconvenientis its high cost; while overall costs, and especially the costs of membrane modules, have declined dramatically over the last years, the energy demand to cope with membrane fouling is still expensive [219, 220].

Artificial wetlands have also been suggested as a biological alternative. Despite being a method with few energy requirements, the degradation percentages (similar to those

obtained at CAS) do not worth the high retention time and land-use necessary for this type of procedure [221-223]. They are therefore recommended for sites without space restrictions, in rural areas or in developing countries that cannot invest in conventional WWTPs [224, 225].

Algal ponds are another biological alternative which has been studied during the last years [226]. According to the experiments performed, some ideas can be highlighted regarding this kind of treatment. Removal efficiencies were found to vary depending on the pollutants [227, 228] or system tested, potentially due to differences in system design, operation, and environmental conditions [227, 229]. The PhACs removals measured are similar to those observed in conventional systems [230] and even, for some compounds such as clarithromycin, roxithromycin, sulfamethazine and sulfapyridine, the removal obtained with the algal tests is better than in the activated sludge tests [231]. These results suggest that the unique conditions occurring in algae ponds indeed specifically impact EC removal. In light of the large inter-study variability seen in the literature, more large-scale studies are needed to identify overall trends based on design, operation and climate. Further batch studies are also needed to quantify kinetics, identify mechanisms of removal, and confirm algal degradation potential under more relevant conditions [226].

Alternative treatments for ARGs

Three different alternative approaches have been investigated concerning ARGs fate and removal: i) treatment with disinfectants including chlorination processes [232-234], UV disinfection [235-237], Fenton and ozone oxidation [232, 238-240] and photocatalytical processes [241-243], ii) treatment in constructed wetlands [244-246] and finally iii) nanotechnology [247-249].

Physico-chemical disinfectants have demonstrated quite good inactivationrates for ARGs. The inactivation values of selected ARGs were 99%, 75%, and 99% for chlorination, ozonation, and Fenton oxidation, respectively [56]. However, much more research is needed to improve the understanding of the elimination of ARGs from treated water using chemical disinfection processes, particularly chlorination which is widely used all over the world [56]. Few studies using only UV irradiation has demonstrated its effectiveness; low UV doses were not effective in decreasing the

frequency of conjugative transfer [68]. Nevertheless, with high UV doses the frequency of ARG transfer was largely suppressed [235, 237]. Photocatalytical processes have shown efficiency inactivating ARGs, but the time required for treatment is a very long period of time. The advancement in photocatalys is under visible light may improve the efficiency of photocatalyticaltreatment [56]. In spite of the promising results, all these disinfection mechanisms have one main drawback, which is the economic investment necessary to work with this kind of technologies (see section 1.5).

Due to the environmentally friendly nature of constructed wetlands, more researchers and engineers are investigating their suitability for removing antibiotics from wastewater. The results obtained are very promising, reaching a 99% reduction of tetracycline resistance genes [244]. However, it is true that some question marks remain over the implementation constructed wetlands; the way in which ARGs are developed or reduced in a wetland has not been sufficiently explored; the operating conditions under which ARGs are developed or reduced needs to be studied further. In particular, the relationship between the flow scheme of a wetland and the abundance of ARGs should beexplored [56].

The application of nanoparticles to treat antibiotic resistant bacteria (ARB) and ARGs is mainly restricted to medical research [250, 251]. A combination of nanoparticles encapsulating antibiotics has been tested to combat multidrug resistant bacteria with very promising results [250, 252]; nanoparticles are then used as antibiotic carriers to avoid bacterial resistant mechanisms. Only when the nanoparticle is inside the bacteria the antibiotics will be released. In another approach, nanoparticles were designed to interact with specific chemical receptors present on the surface of the target bacteria [253]. Until now the use of nanoparticles for wastewater treatment focused on their great adsorption capacity for chemical contaminants and only in one study their performance in terms of ARGs removal was tested. However, nanoalumina particles showed an enhancement in the conjugation processes from *E. coli* to *Salmonella spp.* during lab scale experiments [247] which would promote the spread of ARGs.

Another alternative treatment, studied during the last years, especially for the elimination PhACs is the use of ligninolytic fungi or wood degrading fungi. This is the alternative biological treatment evaluated in the present thesis and therefore, it is extensively explained in the next section.

2. White-Rot fungi (WRF)

White rot fungi (WRF) belongs to a specific group of fungi named lignolytic fungi; they are a heterogeneous group of fungi that have the ability to degrade lignin from decaying wood to take profit of the cellulose and hemicelluloses present inside [254, 255]. Lignin is one of the most abundant compounds on Earth but at the same time is one of the most difficult compounds to degrade due to its heterogeneity and lack of a defined primary structure (Figure 1.7) [211].

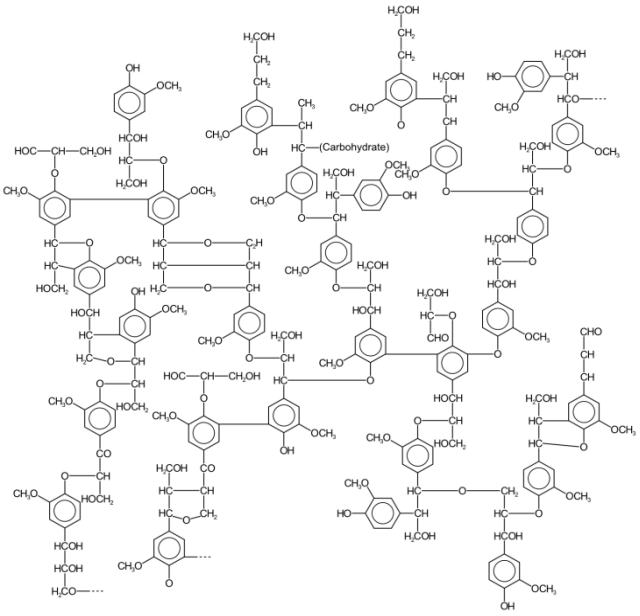


Figure 1.7 - Example of a possible lignin structure

Ligninolytic fungi are classified into three major categories based on the type of wood decay caused: brown rot fungi (BRF), white rot fungi (WRF) and soft rot fungi (SRF). The wood decayed by BRF is typically brown and crumbly and it is degraded via both non-enzymatic and enzymatic systems. A series of cellulolytic enzymes are employed in the degradation process by BRF, but no lignin degrading enzymes are typically involved. WRF are typically associated with hardwood decay and their wood decay patterns can take on different forms. White rotted wood normally has a bleached appearance and this may either occur uniformly, leaving the wood a spongy or stringy mass, or it may appear as a selective decay or a pocket rot. WRF possess both cellulolytic and lignin degrading enzymes and these fungi therefore have the potential to

degrade the entirety of the wood structure under the correct environmental conditions. SRF typically attack higher moisture and lower lignin content wood, and can create unique cavities in the wood cell wall [256]. Among these three groups, WRF are the most effective lignin degraders and have been the most extensively studied group for water treatment applications [257]. Taxonomically WRF comprise a heterogeneous collection of several hundreds of species of basidiomycetes and some ascomycetes [257]. WRF present a powerfuland unspecific extracellular oxidative enzymatic system mainly employed in the primary attack of lignin and its posterior mineralization [258]. Due to the fact that lignin is an insoluble polymer and the presence of a rigid cell wall on the fungi, some enzymes are excreted to break down the complex insoluble polymers so that simpler compounds can be further absorbed by fungi (Figure 1.8) [259]. The presence of these extracellular enzymes also confers them a high tolerance to toxic compounds [211]. In addition, the expression of the enzymes involved in the degradation is not triggered by the presence of specific contaminants, thus reducing their need to adaptation at polluted sites or matrices [187]. All these features makes the WRF a very interesting agent for potential use in bioremediation [187, 211].

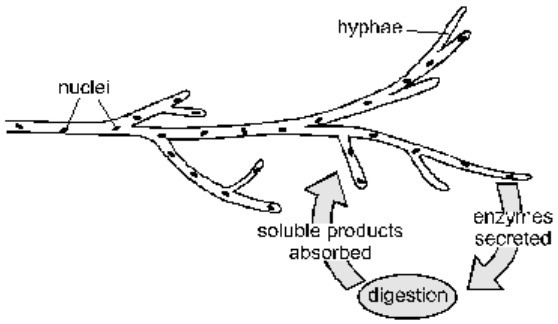


Figure 1.8 - Digestion of a typical white-rot fungi

2.1. Enzymatic system of WRF

WRF secrete mainly two different groups of lignin-modifying enzymes (LMEs), laccases and lignin-modifyingperoxidases (LMPs), in particular lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidases (VP), which act synergistically during lignin degradation [260]; the main difference between these two types of enzymes is the electron acceptor, O_2 for laccases and H_2O_2 for peroxidases. WRF also possess an intracellular enzymatic system, the cytochrome P450 system, involved in the degradation of lignin and several organic pollutants [261-263]. The cytochromes P450

constitute a large family of enzymes present in all forms of life (plants, bacteria and animals, even humans), and play a key role in the oxidative transformation of endogenous and exogenous molecules [264, 265]. In the case of WRF, cytochrome P450 plays an important role in the mineralization of ligninolytic metabolites produced during lignin despolymerization by LMPs [266].

Use of WRF in bioremediation

For many years, most of the studies in bioremediation have focused on bacteria as degraders because of their rapid growth, and their usual ability to use the pollutants as carbon and energy sources [187]. However during the last decades new options have been tested in the bioremediation field including the use of plants (phytoremediation) [267-269], archaea [270-272], genetically engineered microorganisms [273-275] and fungi (mycoremediation).

The concept of developing a technology for the environmental application of fungi, particularly WRF, appeared in the 80`s [276]. Since then, many studies have been performed using different WRF species, e.g. *Bjerkandera adusta* [277-279], *Phanerochaetes chrysosporium* [280-282], *Pleorotus ostreatus* [283-285], *Irpex lacteus* [286-288], *Trametes versicolor* [289-291], etc. The degradation of a great variety of contaminants has been studied with these fungi, including pollutants like PAHs [292-294], pesticides [295-297], polychlorinated biphenyls (PCBs) [298-300], ammunition wastes [301-303], textile dyes [304-306], etc.

Specific industrial effluents have also been subjected to WRF-mediated treatment studies. Decoloration, dechlorination and detoxification of highly toxic bleach plant effluents derived from pulp and paper industry have been reported [307-309]. Also degradation and decoloration of synthetic dyes from textile industry have been widely documented [286, 305, 306]. Likewise, treatment of the acidic, phenolic-rich olive oil mill wastewater has shown chemical oxygen demand reduction, decoloration and dephenolization [310-313].

Based on the demonstrated ability of WRF in the biodegradation of such amount of pollutants, research has lately focused on their application in the removal of emerging contaminants from contaminated wastes. Therefore the number of publications related with fungal degradation of emerging pollutants have increased dramatically in the last

years, and lots of studies have been reported about the degradation of BFRs [314-316], UV filters [317, 318], EDCs [319-321] and a large variety of PhACs [187, 211, 257] in all type of wastewater (urban and industrial) as well as in sludge.

2.2. Degradation of PhACs with *Trametes versicolor*

Among WRF, *Trametes versicolor* was selected in this thesis to study the elimination of PhACs in the fungal treatment. Several studies support the suitability of the choice; Valášková et al. (2006) [322] studied the enzyme production conditions of diverse WRF; *T. versicolor* showed the best stability in enzyme production, with enzyme activities above 75% [322]. Mougin et al. (2002) found that laccase production in *T. versicolor* was enhanced by the presence of several xenobiotics and their transformation products [323]. Marco-Urrea et al. (2009) performed an experiment in which 4 different WRF where tested in order to analyze their PhACs degradative efficiency. Finally they concluded that T. versicolor was the most efficient fungus in terms of PhACs degradation [263].

Biodegradation capabilities of T. versicolor have been explored both, using the entire fungal mycelia, and using the enzymes isolated from the fungi [324]. The latter present two main drawbacks for their application for PhACs removal: the ligninolytic enzymes need cofactors or mediators (usually expensive and even toxic) for a good performance; on the other hand, degradation of lignin is a cooperative process of multiple enzymes and, therefore, whole cell applications are usually more efficient than single enzyme use. Moreover, due to the deactivation of enzymes over time, fungal culture is still needed for the continuous production of enzymes [325]. Additionally, a decrease in the range of treatable contaminants occurs when using purified enzymes, even with the addition of mediators, in comparison with the use of the entire fungus [211].

Several studies have been carried out al lab scale in order to evaluate the degradative ability of *T. versicolor* with specific compounds (spiked in water samples) (Table 1.3).

Table 1.3 - Summary of PhACs degradation by *T. versicolor* in individual degradation experiments

Therapeutic family	Pharmaceutical	Reference
Anti-inflammatories/ analgesic drugs	Diclofenac	[326]
	Fenoprofen	[327]
	Ibuprofen	[263]
	Indomethacin	[327]
	Ketoprofen	[328]
	Naproxen	[329]
	Propyphenazone	[327]
Antibiotics	Ciprofloxacin	[330]
	Norfloxacin	[330]
	Sulfamethazine	[189, 331]
	Sulfathiazole	[331]
	Sulfapyridine	[331]
Antimicrobial agents	Triclosan	[332]
β-blockers	Atenolol	[333]
	Propranolol	[333]
Lipid regulator	Clofibric acid	[263, 333]
	Gemfibrozil	[327]
Psychiatric drugs	Carbamazepine	[263, 327, 333, 334]
X-ray contrast media	Diatrizoate	[335]
	Iodipamide	[335]
	Aminotrizoate	[335]
	Acetrizoate	[335]
	Aminotriiodoisophthalic acid	[335, 336]
	Iopromide	[336]

Recently, and based on the satisfactory results, further studies were performed, jointly with the Chemical Engineering department from Universitat Autònoma de Barcelona and our research group, to evaluate the efficiency of *T. versicolor* in PhACs removal from real effluents such as urban wastewater [191], hospital wastewater [205], veterinary hospital wastewater [195] and reverse osmosis concentrate (ROC) from a tertiary treatment in a urban WWTP [204, 211]. Removal values achieved with the fungal treatment in these studies are very promising, especially with some specific compounds that are usually recalcitrant in CAS such as diclofenac, phenazone, propranolol, ciprofloxacin, ofloxacin, furosemide, venlafaxine, iopromide, metronidazole, etc. Besides PhACs degradation, different operational parameters were also tested in order to achieve the optimal ones before scaling up the fungal treatment to a pilot plant (Table 1.4).

Table 1.4 - Operational parameters tested for the fungal treatment with *T. versicolor* of real wastewater effluents.

Samples	Reactor type	Influent sterilization	Nutrients input	Treatment time	Other parameters tested	Ref.
Urban wastewater	Batch	**Sterile/ Non sterile**	Yes	8 days	-	[191]
Hospital wastewater	Batch	**Sterile/ Non sterile**	Yes	8 days	-	[205]
Veterinary hospital wastewater	**Batch/ Continuous**	Non sterile	Yes	**Variable**	-	[195]
ROC	**Batch/ Continuous**	**Sterile/ Non sterile**	**Variable addition**	**Variable**	**Sampling mode Aeration rate**	[204, 211]

Chapter 2

Chapter 2 – Objectives

The main goal of this thesis was to evaluate the fungal treatment of wastewaters with regards the presence of PhACs. Removal efficiency and removal mechanisms of these compounds during treatment as well as their environmental impact are the aspects addressed in detail along research performed. In order to achieve this, the following specific objectives were defined:

1. To quantify the PhACs removal achieved with the fungal treatment of different wastewaters effluents (urban, hospital, veterinary hospital wastewaters and ROC)

2. To analyze from an ecotoxicological point of view, the effectiveness of the fungal treatment, in terms of reduction of the hazard indexes of the wastewater treated

3. To measure the efficiency of the fungal treatment of wastewater in terms of antibiotics elimination

4. To evaluate the effect of the fungal treatment on the spread of ARG during the wastewater treatment

5. To study the sorption processes involved in the PhACs elimination during the fungal treatment of wastewater

Chapter 3

Chapter 3 – Materials and methods

1. Fungus

Six different species of fungi were used in this thesis, three WRF: *Trametes versicolor* (ATCC #42530 strain), *Irpex lacteus* (AX1 strain) and *Ganoderma lucidum* (FP-58537-Sp strain); and three litter decomposing fungi (LDF): *Stropharia rugosoannulata* (FBCC 475 strain), *Gymnopilus luteofolius* (FBCC 466 strain) and *Agrocybe erebia* (FBCC 476 strain).

All these fungi were subcultured on 2% malt extract agar petri plates (pH 4.5) at 25 °C. Pellet production was achieved for all the fungi, and it was done following the same procedure as previously described by Font et al. (2003)[337] and Blanquez et al. (2004) [338].

2. Chemicals and reagents

All PhACs standards and isotopically labelled compounds, used as internal standards, were of high purity grade (>90%). Compounds were purchased from Sigma Aldrich (Steinheim, Germany), US Pharmacopeia (Rockville, MD, USA), European Pharmacopeia EP (Strasbourg, France), Toronto Research Chemicals TRC (Ontario, Canada) and CDN isotopes (Quebec, Canada).

The solvents, HPLC grade methanol, acetonitrile, water (LiChrosolv®) and formic acid (98%), were provided by Merck (Darmstadt, Germany). Glucose, ammonium tartrate dibasic, malt extract, ethylene diamine tetra acetic acid disodium salt solution (Na_2EDTA) at 0.1 mol L^{-1} and other chemicals were purchased from Sigma Aldrich (Steinheim, Germany). Fiberglass GF/A filters (1 μm) and nylon membrane filters (0.45 μm) were purchased from Whatman (London, UK) and Millex Millipore (Barcelona, Spain) respectively.

Individual stock standard, isotopically labelled internal standard and surrogate solutions were prepared on a weight basis in methanol (at a concentration of 1000 mg L^{-1}), except ofloxacin and ciprofloxacin, which were dissolved in methanol adding 100 μL of NaOH 1 M, and cefalexin, which was solved in HPLC grade water, as described in the

literature [339, 340], since these substances are barely soluble or insoluble in pure methanol. After preparation, standards were stored at −20 °C.

Working standard solutions, containing all compounds, were also prepared in methanol/water (10:90, v/v). Separate mixtures of isotopically labelled internal standards, used for internal standard calibration, and surrogates, were prepared in methanol and further dilutions were also prepared in a methanol/water (10:90, v/v) mixture [341].

3. Water samples

In this thesis, different types of water samples have been analyzed covering different origins.

3.1. University village wastewaters

These wastewater samples were collected from the student's village of Universitat Autónoma de Barcelona (Bellaterra, Barcelona, Spain). Samples for sterile batch treatments were sterilized the same day of sampling by autoclaving 30 min at 121ºC and then they were stored in the freezer at -20ºC until the experiments were set up. The day before starting the treatment, the water samples were thawed at room temperature and autoclaved again. Samples for non-sterile batch treatment were just stored at 4ºC until using them [191].

3.2. Hospital wastewater

Hospital samples were collected from the main sewer of Girona University Hospital of Girona Dr. Josep Trueta (Girona, Spain) with a maximum capacity of 364 beds for the inpatients and 11 operation rooms. Two samples of 20 L were collected directly from the sewer manifold of the hospital. As with the universitary village wastewaters, samples for sterile batch treatments were sterilised the same day of sampling by autoclaving 30 min at 121ºC and then they were stored in the freezer at -20ºC until the experiments were set up. The day before starting the treatment, water sample was thawed at room temperature and autoclaved again. Sample for non-sterile batch treatment was just stored at 4ºC until using it [205].

3.3. Reverse Osmosis Concentrate (ROC)

The ROC effluent was obtained from a pilot plant located in Castell-Platja d'Aro WWTP (Girona, Spain). The pilot plant is described in Dolar et al. (2012)[342] and it consists first of a membrane bioreactor (MBR), which treats urban wastewater (96%), followed by a reverse osmosis unit (Figure 3.1). The volume treated in the pilot plant is 200 L h^{-1}, with a recovery rate of 66%.

ROC samples for sterile batch treatments were sterilised the same day of sampling by autoclaving 30 min at 121ºC and then they were stored in the freezer at -20ºC until the experiments were set up. The day before starting the treatment, ROC was thawed at room temperature and autoclaved again. ROC for non-sterile continuous treatment was just stored at 4ºC until using it. Wastewater in the feed storage tank of the continuous bioreactor was replaced by fresh one stored at 4ºC every 3-5 days [204, 211].

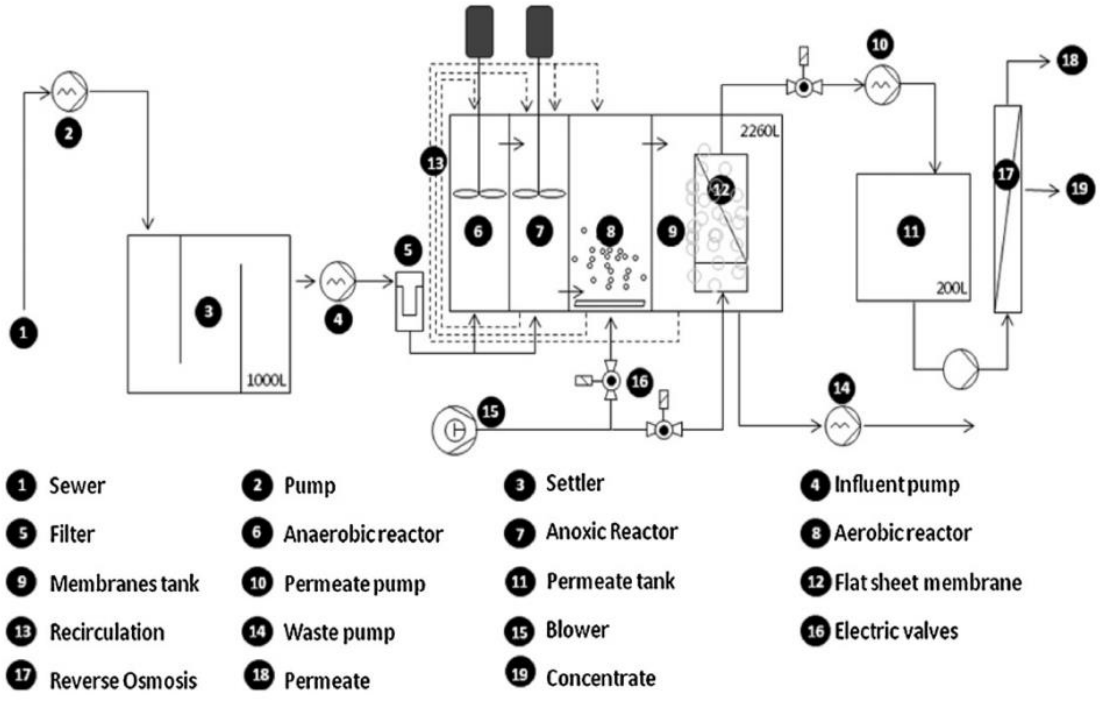


Figure 3.1 - Pilot plant of Castell-Platja d'Aro, treating urban wastewater with an MBR followed by a RO. Scheme taken from Dolar et al. (2012) [342].

3.4. Veterinary hospital wastewater (VHW)

These samples were obtained from a veterinary hospital located in the Universitat Autònoma de Barcelona campus (Bellatera, Barcelona, Spain) the same day that each

bioreactor (both batch and continuous) was set up and also once a week during continuous bioreactors operation; this veterinary hospital receives 15,455 visitors per year: 12,435 dogs, 859 cats, 300 horses and 1861 exotic animals.

As all the experiments with VHW were plannend with non-sterile water, samples from the hospital were taken from the veterinary hospital main sewer and then they were just stored at 4ºC until its usage. Wastewater in the feed storage tank of the continuous bioreactor was replaced by fresh one stored at 4ºC every 3-5 days [195].

3.5. Conventional urban wastewater

It was obtained from a municipal WWTP from a town located in Sarrià de Ter, Girona, Spain (20,000 equivalent inhabitants, 2.100 m^3 d^{-1} volume treated; with a HRT of 48 h and a SRT of 20– 22 days; [10]). Conventional wastewater from the monitoring study by Collado et al. (2014) [10] was used as reference values of CAS treatment since both, their study and our fungal treatment experiments, targeted the same set of PhACs using the same analytical methodology [341]. Levels of these compounds along the treatment in the WWTP were measured in three different seasons of the year in dry weather conditions: in May 2011, January 2012 and August 2012. Every seasonal campaign 48-h composite and flow proportional samples were collected both, at the WWTP inlet (before the primary treatments) and at the outlet of the secondary treatment, by means of an auto-sampler. Each sample was analyzed in triplicate. PhAC concentrations and removal values obtained in this study were consistent with the values found in the literature for other urban WWTPs [8] and thus considered representative of conventional WWTP.

3.6. Spiked synthetic medium

Four selected PhACs were added to the defined medium where the fungi were growing. The medium was spiked with some PhACs at slightly higher concentrations than those commonly found in wastewater treatment plants, reaching a final concentration between 47 and 184 μg L^{-1}. However, concentrations were low enough to avoid any possible toxic effect of the PhACs on the fungi [343]. The pollutants present in the stock solution included diclofenac (anti-inflammatory), carbamazepine (anti-convulsant), venlafaxine (antidepressant) and iopromide (media contrast agent). PhAcs were selected based on its

ubiquity in hospital wastewater effluents and low biodegradability exhibited in previous fungal treatments performed with *T. versicolor* [257, 344]

4. PhACs quantification

Quantification of the PhACs was performed according Gros et al. (2012)[341] procedure. Briefly, successive filtration of water samples was done through 2.7, 1.0 and 0.45 mm pore-size membranes (Millipore; Billerica, MA, USA) to remove big particles that could cause problems in the analysis. After filtration, water samples of 25 mL each were pH-adjusted to 3 with HCl 1.0 M and Na2EDTA 3%, v/) and loaded into Solid Phase Extraction (SPE)-HLB cartridges (60 mg, 3 mL) (Waters Corp.; Mildford, MA, USA) for analytes preconcentration. According to the method previously mentioned, cartridges were eluted passing 6 ml of pure methanol at a flow rate of 2 ml min-1 through the cartridges. The extracts were then evaporated under nitrogen stream using a Reacti-Therm 18824 system (Thermo Scientific, Whaltman, MA, USA) and reconstituted with 1 mL of methanol-water (10:90 v/v). Lastly, 10 mL of standard of internal standard mix at 10 ng mL^{-1} were added in the extracts for internal standard calibration and to compensate, if it was necessary, a possible matrix effect.

Chromatographic separation of the extracted samples was carried out with a ultraperformance liquid chromatography system (Waters Corp. Mildford, MA, USA), using an Acquity HSS T3 column (50 × 2.1 mm i.d. 1.7 μm particle size) for the compounds analyzed under positive electrospray ionization (PI) and an Acquity BEH C18 column (50 × 2.1 mm i.d., 1.7 μm particle size) for the ones analyzed under negative electrospray ionization (NI), both from Waters Corporation. The UPLC instrument was coupled to 5500 QqLit, triple quadrupole linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA) with a Turbo V ion spray source. Two MRM transitions per compound were recorded by using the Scheduled MRM™ algorithm, and the data were acquired and processed using the Analyst 2.1 software.

5. Statistical analysis

Mean values were compared using Student's t-test, in which $p < 0.05$ was considered significant (IBM SPSS Statistics 21.0 software; IBM, Chicago, IL, USA).

Chapter 4

Chapter 4 – Ecotoxicological approach

Based on the publication:
Removal of pharmaceuticals from wastewater by fungal treatment andreduction of hazard quotients
D. Lucas, D. Barceló, S. Rodriguez-Mozaz.
Science of the Total Environment (2016) 571: pp. 909-915.
DOI: 10.1016/j.scitotenv.2016.07.074

Abstract

The elimination of 81 pharmaceuticals (PhACs) by means of a biological treatment based on the fungus *Trametes versicolor* was evaluated in this work. PhACs removal studied in different types of wastewaters (urban, reverseosmosis concentrate, hospital, and veterinary hospital wastewaters) were reviewed and compared with conventional activated sludge (CAS) treatment. In addition, hazard indexes were calculated based on the exposure levelsand ecotoxicity for each compound and used for the evaluation of the contaminants removal. PhACs elimination achieved with the fungal treatment (mean value 76%) was similar or slightly worse than the elimination achieved in the CAS treatment (85%). However, the fungal reactor was superior in removing more hazardous compounds (antibiotics and psychiatric drugs) than the conventional activated sludge in terms of environmental risk reduction (93% and 53% of reduction respectively). Fungal treatment can thus be considered as a good alternative toconventional treatment technologies for the elimination of PhACs from wastewaters.

Keywords

Fungal treatment, Wastewater treatment, *Trametes versicolor*, Pharmaceuticals, Environmental risk assessment, Hazard quotients

1. Introduction

As explained previously over the last years a wide range of PhACs residues have been found in several environmental matrices [360-364] due to their extensive consumption and pseudo-persistence in the environment [8, 365-368]. Several studies suggest that conventional activated sludge (CAS) technologies used in urban wastewater treatment plants (WWTPs) are not effective enough to eliminate PhACs, since they are not

designed to remove such complex compounds [233]. As a consequence, some innovative wastewater treatment technologies have been developed in order to achieve higher removal efficiency of this type of pollutants [369-373]. Among them, the fungal treatment of wastewaters has been highlighted as a promising technology because of the unspecific enzymatic system of lignolytic fungi, which is able to degrade a wide range of PhACs, even though they are present at very low concentrations [263, 331, 374, 375].

At his point, we know that the fungal treatment manages to remove a large variety of drugs, but it would be important to determine the environmental impact of this PhACs removal. This could be achieved by environmental risk assessment (ERA), which is a standardized procedure to estimate the probability of a compound to cause undesired environmental effects [376] based on both, concentration and ecotoxicity of each particular compound. Many studies have assessed the environmental risk of the PhACs in several treated wastewater effluents [10, 84, 238, 365, 377-379], but only one has considered the efficiency of CAS treatment in ecotoxicological terms [380]; and none has used this approach to evaluate alternative wastewater technologies, such as those based on fungal treatment. In this chapter we present the use of ERA as a complementary tool to evaluate effectiveness of fungal treatment in comparison to a CAS treatment. Four different types of wastewaters treated with the fungal treatment were considered [191, 195, 204, 205, 211]. As shown in Table 4.1 different operational parameters for the fungal treatment were tested (batch and continuous operation, nutrients addition, treatment time, etc.); in order to maximize the degradative capacity of PhACs. Removal data achieved for PhACs with the fungal was then compared with removal values from CAS treatment [10]. Even though the fungal treatments were performed at lab-scale and the operational parameters varied from one treatment to the other, a comparison with a full-scale CAS can provide a preliminary idea about the efficiency and potential of the fungal treatment.

Table 4.1 - Wastewater samples considered in the present study

Samples	Treatment	Reactor type	Sterile influent	Nutrients input	Treatment time	Reference
Urban wastewater	CAS	Continuous	No	No	2 days	[10]
University village wastewater I	Fungal treatment	Batch	Yes	Yes	8 days	[191]
University village wastewater II	Fungal treatment	Batch	No	Yes	8 days	[191]
Reverse osmosis concentrate I	Fungal treatment	Batch	Yes	Yes	6 days	[204]
Reverse osmosis concentrate II	Fungal treatment	Continuous	No	Yes	6 days	[211]
Hospital wastewater I	Fungal treatment	Batch	Yes	Yes	8 days	[205]
Hospital wastewater II	Fungal treatment	Batch	No	Yes	8 days	[205]
Veterinary hospital I	Fungal treatment	Batch	No	Yes	14 days	[195]
Veterinary hospital II	Fungal treatment	Continuous	No	Yes	8 days	[195]

2. Environmental risk assessment

In order to analyze the environmental risk of the water samples, a hazard quotient (HQ) was calculated for each compound according to the European Community (EC) guidelines [82]. HQs values for each compound were calculated before and after each treatment according the following equation:

HQ = PhACs concentration / Predicted No Effect Concentration (PNEC)

PhAC concentration is the value for each compound in the water sample obtained before and after the corresponding wastewater treatment. According to the European Committee [82] each of the reported PNECs is 1000 times lower [2] than the toxicity concentration value found for the most sensitive species assayed, so as to take into account the effect on other, potentially more sensitive, aquatic species to those used in toxicity studies [8].

$$\text{PNEC} = \frac{\text{EC}_{50} \text{ or } \text{LC}_{50}}{1000}$$

EC_{50} or LC_{50} values from each compound (Table 4.2) were obtained from experimental data, international databases and also from the literature. When data from some PhACs were not available, they were calculated according to modeled ecotoxicological data using the ECOSAR software [351].

To calculate a total HQ value for each sample, a sum of all HQ values for each compound detected was calculated, as has been done in similar studies [352, 353].

Table 4.2 - PNEC for the PhACs analyzed in this study

	Compounds	Species assayed	Test (endpoint)	Value (µg/L)	References	PNEC (µg/L)
Analgesics/anti-inflamatories	Acetaminophen	Fish	LC 50	258000	[354]	**6,92**
		Daphnia	**EC 50**	**6920**	**[367]**	
		Algae	EC 50	134000	[367]	
	Codeine	Fish	LC 50	238000	[105]	**16**
		Daphnia	**EC 50**	**16000**	**[105]**	
		Algae	EC 50	24000	[105]	
	Diclofenac	**Fish**	**LOEC**	**20**	**[108]**	**0,02**
		Daphnia	EC 50 (24h)	22700	[93]	
		Algae	EC 50 (53h)	480	[112]	
	Ibuprofen	**Fish**	**LC 50 (24h)**	**200**	**[108]**	**0,2**
		Daphnia	EC 50	9020	[94]	
		Algae	EC 50 (96h)	2300	[109]	
	Indomethacine	Fish	LC 50 (96h)	2930	[367]	**16,14**
		Daphnia	**LC 50 (48h)**	**16140**	**[367]**	
		Algae	EC 50 (96h)	3573	[367]	
	Ketoprofen	Fish	LC 50 (96h)	264000	[367]	**2**
		Daphnia	EC 50 (48h)	2300	[109]	
		Algae	**EC 50 (96h)**	**2000**	**[109]**	
	Meloxicam	Fish	LC 50 (96h)	12910	[367]	**8,42**
		Daphnia	**LC 50 (48h)**	**8420**	**[367]**	
		Algae	EC 50 (96h)	11140	[367]	
	Naproxen	Fish	LC 50 (96h)	52000	[114]	**3,7**
		Daphnia	EC 50 (48h)	43640	[100]	
		Algae	**EC 50 (96h)**	**3700**	**[109]**	
	Oxycodone	Fish	LC 50 (96h)	4121750	[367]	**300**
		Daphnia	**LC 50 (48h)**	**300000**	**[105]**	
		Algae	EC 50 (96h)	924460	[367]	
	Phenazone	Fish	LC 50 (96h)	2842080	[367]	**616,7**
		Daphnia	LC 50 (48h)	1417040	[367]	
		Algae	**EC 50 (96h)**	**616700**	**[367]**	
	Piroxicam	Fish	LC 50 (96h)	82490	[367]	**45,86**
		Daphnia	LC 50 (48h)	49380	[367]	
		Algae	**EC 50 (96h)**	**45860**	**[367]**	
	Propyphenazone	Fish	LC 50 (96h)	171250	[367]	**74,11**
		Daphnia	LC 50 (48h)	97670	[367]	
		Algae	**EC 50 (96h)**	**74110**	**[367]**	
	Salicylic acid	Fish	EC 50 (48h)	37000	[355]	**36**
		Daphnia	**LOEC**	**36000**	**[108]**	
		Algae	EC 50 (96h)	32490	[367]	
	Tenoxicam	Fish	LC 50 (96h)	121770	[367]	**62,19**
		Daphnia	LC 50 (48h)	71730	[367]	
		Algae	**EC 50 (96h)**	**62190**	**[367]**	
Antibiotics	Azithromycin	Fish	LC 50 (96h)	47070	[367]	**0,019**
		Daphnia	LC 50 (48h)	29980	[367]	
		Algae	**EC 50 (96h)**	**19**	**[108]**	
	Cefalexin	Fish	LC 50 (96h)	7731210	[367]	**1534,81**
		Daphnia	LC 50 (48h)	3788540	[367]	
		Algae	**EC 50 (96h)**	**1534810**	**[367]**	
	Ciprofloxacin	Fish	LC 50 (96h)	17053154	[367]	**0,005**
		Daphnia	EC 50 (48h)	65300	[107]	
		Algae	**EC 50**	**5**	**[95]**	
	Clarithromycin	Fish	LC 50 (96h)	53813	[367]	**0,002**
		Daphnia	EC 50 (24h)	25720	[101]	
		Algae	**EC 50 (72h)**	**2**	**[101]**	
	Dimetridazole	Fish	LC 50 (96h)	973770	[367]	**252,85**
		Daphnia	LC 50 (48h)	502790	[367]	
		Algae	**EC 50 (96h)**	**252850**	**[367]**	
	Erythromycin	Fish	LC 50 (96h)	410000	[106]	**0,02**
		Daphnia	EC 50 (24h)	22450	[101]	
		Algae	**EC 50 (72h)**	**20**	**[101]**	
	Metronidazole	Fish	LC 50 (96h)	8845230	[367]	**38,8**
		Daphnia	LC 50 (48h)	4174426	[367]	
		Algae	**EC 50 (72h)**	**38800**	**[108]**	
	Metronidazole-OH	Fish	LC 50 (96h)	86311420	[367]	**8551,99**
		Daphnia	LC 50 (48h)	36941450	[367]	

		Algae	**EC 50 (96h)**	**8551990**	**[367]**	
	Ofloxacin	Fish	LC 50 (96h)	28067230	[367]	**0,016**
		Daphnia	LC 50 (48h)	17410	[101]	
		Algae	**EC 50 (96h)**	**16**	**[93]**	
	Ronidazole	Fish	LC 50 (96h)	22180050	[367]	**3048,08**
		Daphnia	LC 50 (48h)	10119770	[367]	
		Algae	**EC 50 (96h)**	**3048080**	**[367]**	
	Sulfamethoxazole	Fish	LC 50 (96h)	27350	[104]	**0,027**
		Daphnia	EC 50 (48h)	15510	[101]	
		Algae	**EC 50 (96h)**	**27**	**[93]**	
	Tetracyclin	Fish	EC 50	220000	[94]	**1**
		Daphnia	LC 50 (48h)	149000000	[367]	
		Algae	**EC 50 (96h)**	**1000**	**[117]**	
	Trimethoprim	Fish	LC 50 (96h)	3304100	[367]	**0,0058**
		Daphnia	EC 50 (48h)	92000	[88]	
		Algae	EC 50	16000	[94]	
		Mussel	**LOEC**	**5,8**	**[85]**	
Anticoagulant	Warfarin	**Fish**	**LC 50 (96h)**	**12000**	**[108]**	**12**
		Daphnia	EC 50 (24h)	88800	[108]	
		Algae	EC 50 (96h)	73960	[367]	
Antidiabetic	Glibenclamide	Fish	LC 50 (96h)	1270	[367]	**0,93**
		Daphnia	**LC 50 (48h)**	**930**	**[367]**	
		Algae	EC 50 (96h)	2010	[367]	
Antihelmintics	Albendazole	Fish	LC 50 (96h)	20800	[367]	**13,12**
		Daphnia	**LC 50 (48h)**	**13120**	**[367]**	
		Algae	EC 50 (96h)	15100	[367]	
	Levamisol	Fish	LC 50 (96h)	27960	[367]	**17,2**
		Daphnia	**LC 50 (48h)**	**17200**	**[367]**	
		Algae	EC 50 (96h)	17830	[367]	
	Thiabendazole	Fish	LC 50	14000	[367]	**0,31**
		Daphnia	**EC 50**	**310**	**[367]**	
		Algae	EC 50 (96h)	70080	[367]	
Anti-hypertensives	Amlodipine	Fish	LC 50 (96h)	290000	[367]	**126,9**
		Daphnia	LC 50 (48h)	165800	[367]	
		Algae	**EC 50 (96h)**	**126900**	**[367]**	
	Irbesartan	Fish	LC 50 (96h)	380	[367]	**0,29**
		Daphnia	**LC 50 (48h)**	**290**	**[367]**	
		Algae	EC 50 (96h)	770	[367]	
	Losartan	Fish	LC 50 (96h)	5390	[367]	**3,69**
		Daphnia	**LC 50 (48h)**	**3690**	**[367]**	
		Algae	EC 50 (96h)	5930	[367]	
	Valsartan	Fish	LC 50 (96h)	11816	[367]	**7,813**
		Daphnia	**LC 50 (48h)**	**7813**	**[367]**	
		Algae	EC 50 (96h)	10923	[367]	
Antiplatelet agent	Clopidogrel	Fish	LC 50 (96h)	6120	[367]	**4,11**
		Daphnia	**LC 50 (48h)**	**4110**	**[367]**	
		Algae	EC 50 (96h)	6130	[367]	
Calcium channel blockers	Diltiazem	Fish	LC 50 (96h)	15000	[103]	**8,2**
		Daphnia	**EC 50 (96h)**	**8200**	**[103]**	
		Algae	EC 50 (96h)	40590	[367]	
	Norverapamil	Fish	LC 50 (96h)	1724	[367]	**1,24**
		Daphnia	**LC 50 (48h)**	**1240**	**[367]**	
		Algae	EC 50 (96h)	2490	[367]	
	Verapamil	**Fish**	**LC 50 (96h)**	**600**	**[108]**	**0,6**
		Daphnia	LC 50 (48h)	7000	[116]	
		Algae	EC 50 (96h)	1830	[367]	
Diuretic	Furosemide	Fish	LC 50 (96h)	166000	[367]	**1**
		Daphnia	EC 50 (48h)	2354	[356]	
		Algae	EC 50 (96h)	103000	[367]	
		Bacteria	**LOEC**	**1000**	**[108]**	
	Hydrochlorothiazide	Fish	LC 50 (96h)	18684110	[367]	**2924,57**
		Daphnia	LC 50 (48h)	8741680	[367]	
		Algae	**EC 50 (96h)**	**2924570**	**[367]**	
	Torasemide	Fish	LC 50 (96h)	316682	[367]	**130,87**
		Daphnia	LC 50 (48h)	179000	[367]	
		Algae	**EC 50 (96h)**	**130870**	**[367]**	
Histamine H_1 and H_2 receptor	Cimetidine	Fish	LC 50 (96h)	3956174	[367]	**271,3**
		Daphnia	**EC 50 (48h)**	**271300**	**[108]**	

antagonists		Algae	EC 50 (96h)	831063	[367]	
	Desloratadine	Fish	LC 50 (96h)	1110	[367]	**0,81**
		Daphnia	**LC 50 (48h)**	**810**	**[367]**	
		Algae	EC 50 (96h)	1640	[367]	
	Famotidine	Fish	LC 50 (96h)	67097480	[367]	**22**
		Daphnia	EC 50	398000	[357]	
		Algae	**EC 50 (96h)**	**22000**	**[91]**	
	Loratadine	Fish	LC 50 (96h)	163	[367]	**0,13**
		Daphnia	**LC 50 (48h)**	**130**	**[367]**	
		Algae	EC 50 (96h)	391	[367]	
	Ranitidine	Fish	LC 50 (96h)	8798900	[367]	**0,04**
		Daphnia	**EC 50 (48h)**	**40**	**[108]**	
		Algae	EC 50 (96h)	1657380	[367]	
Lipid regulators and cholesterol lowering statin drugs	Atorvastatin	Fish	LC 50 (96h)	56	[367]	**0,19**
		Daphnia	LC 50 (48h)	48	[367]	
		Algae	**EC 50 (96h)**	**187**	**[367]**	
	Bezafibrate	Fish	EC 50	6000	[94]	**0,46**
		Daphnia	**EC 50**	**460**	**[108]**	
		Algae	EC 50	18000	[94]	
	Fluvastatin	Fish	LC 50 (96h)	937	[367]	**0,692**
		Daphnia	**LC 50 (48h)**	**692**	**[367]**	
		Algae	EC 50 (96h)	1529	[367]	
	Gemfibrozil	**Fish**	**EC 50**	**300**	**[108]**	**0,3**
		Daphnia	EC 50	10400	[94]	
		Algae	EC 50	4000	[94]	
	Pravastatin	**Fish**	**EC 50**	**1800**	**[94]**	**1,8**
		Daphnia	LC 50 (48h)	22560	[367]	
		Algae	EC 50 (96h)	25570	[367]	
Prostatic hyperplasia	Tamsulosin	Fish	LC 50 (96h)	127710	[367]	**67,41**
		Daphnia	LC 50 (48h)	75710	[367]	
		Algae	**EC 50 (96h)**	**67410**	**[367]**	
Psychiatric drugs	10,11- EpoxyCBZ	Fish	LC 50 (96h)	1827320	[367]	**469,23**
		Daphnia	LC 50 (48h)	941470	[367]	
		Algae	**EC 50 (96h)**	**469230**	**[367]**	
	2-HydroxyCBZ	Fish	LC 50 (96h)	691650	[367]	**221,92**
		Daphnia	LC 50 (48h)	372140	[367]	
		Algae	**EC 50 (96h)**	**221920**	**[367]**	
	Acridone	Fish	LC 50 (96h)	307600	[367]	**112,1**
		Daphnia	LC 50 (48h)	169600	[367]	
		Algae	**EC 50 (96h)**	**112100**	**[367]**	
	Alprazolam	Fish	LC 50 (96h)	5406	[367]	**3,6**
		Daphnia	**LC 50 (48h)**	**3600**	**[367]**	
		Algae	EC 50 (96h)	5400	[367]	
	Carbamazepine	Fish	EC 50	35400	[94]	**2**
		Daphnia	EC 50	76300	[94]	
		Algae	EC 50	85000	[94]	
		Bacteria	**LOEC**	**2000**	**[108]**	
	Citalopram	**Fish**	**LC 50 (96h)**	**6,35**	**[108]**	**0,00635**
		Daphnia	LC 50 (48h)	3900	[96]	
		Algae	EC 50 (48h)	1600	[89]	
	Diazepam	Fish	LC 50 (96h)	12700	[110]	**0,01**
		Daphnia	**EC 50 (24h)**	**10**	**[108]**	
		Algae	IC 50 (96h)	16500	[110]	
	Fluoxetine	Fish	LC 50 (48h)	198	[113]	**0,0029**
		Daphnia	LC 50 (48h)	230	[86]	
		Algae	EC 50 (48h)	24	[86]	
		Frog	**LOEC**	**2,9**	**[108]**	
	Lorazepam	Fish	LC 50 (96h)	113470	[367]	**58,24**
		Daphnia	LC 50 (48h)	66910	[367]	
		Algae	**EC 50 (96h)**	**58240**	**[367]**	
	Norfluoxetine	Fish	LC 50 (96h)	2660	[367]	**1,847**
		Daphnia	**LC 50 (48h)**	**1847**	**[367]**	
		Algae	EC 50 (96h)	3166	[367]	
	Olanzapine	Fish	LC 50 (96h)	80420	[367]	**44,38**
		Daphnia	LC 50 (48h)	48090	[367]	
		Algae	**EC 50 (96h)**	**44380**	**[367]**	
	Paroxetine	Fish	LC 50 (96h)	4460	[367]	**0,58**
		Daphnia	**LC 50 (48h)**	**580**	**[96]**	

		Algae	EC 50 (96h)	5080	[367]	
	Sertraline	Fish	LC 50 (96h)	1900	[115]	**0,0121**
		Daphnia	LC 50 (48h)	120	[96]	
		Algae	**IC 50 (96h)**	**12,1**	**[102]**	
	Trazodone	Fish	LC 50 (96h)	24900	[367]	**15,81**
		Daphnia	**LC 50 (48h)**	**15810**	**[367]**	
		Algae	EC 50 (96h)	18713	[367]	
	Venlafaxine	Fish	LC 50 (96h)	16130	[367]	**10,31**
		Daphnia	**LC 50 (48h)**	**10310**	**[367]**	
		Algae	EC 50 (96h)	12520	[367]	
Sedation and muscle relaxation	Xylazine	Fish	LC 50 (96h)	997	[367]	**0,714**
		Daphnia	**LC 50 (48h)**	**714**	**[367]**	
		Algae	EC 50 (96h)	1390	[367]	
ß-blocking agents	Atenolol	**Fish**	**LC 50 (96h)**	**20000**	**[108]**	**20**
		Daphnia	EC 50 (48h)	200000	[98]	
		Algae	EC 50 (48h)	620000	[90]	
	Carazolol	Fish	LC 50 (96h)	62710	[367]	**36,256**
		Daphnia	LC 50 (48h)	37843	[367]	
		Algae	**EC 50 (96h)**	**36256**	**[367]**	
	Metoprolol	**Fish**	**EC 50 (72H)**	**100**	**[108]**	**0,1**
		Daphnia	LC 50 (48h)	8800	[99]	
		Algae	EC 50 (48h)	7300	[140]	
	Nadolol	Fish	LC 50 (96h)	290000	[367]	**5,021**
		Daphnia	LC 50 (48h)	19000	[367]	
		Algae	**EC 50 (96h)**	**5021**	**[367]**	
	Propranolol	**Fish**	**EC 50 (48h)**	**50**	**[108]**	**0,05**
		Daphnia	LC 50 (48h)	460	[87]	
		Algae	EC 50 (96h)	668	[93]	
	Sotalol	Fish	LC 50 (96h)	243000	[367]	**13**
		Daphnia	LC 50 (48h)	42000	[367]	
		Algae	**EC 50 (96h)**	**13000**	**[367]**	
Synthetic glucocorticoid	Dexamethasone	**Fish**	**LC 50**	**254**	**[111]**	**0,254**
		Daphnia	EC 50 (24h)	48300	[92]	
		Algae	EC 50 (96h)	41000	[367]	
To treat asthma	Salbutamol	Fish	LC 50 (96h)	3298473	[367]	**730,91**
		Daphnia	LC 50 (48h)	1651310	[367]	
		Algae	**EC 50 (96h)**	**730910**	**[367]**	
Tranquilizer	Azaperol	Fish	LC 50 (96h)	30700	[367]	**19,2**
		Daphnia	**LC 50 (48h)**	**19200**	**[367]**	
		Algae	EC 50 (96h)	21400	[367]	
	Azaperone	Fish	LC 50 (96h)	21020	[367]	**13,37**
		Daphnia	**LC 50 (48h)**	**13370**	**[367]**	
		Algae	EC 50 (96h)	15950	[367]	
X-ray contrast agents	Iopromide	Fish	LC 50 (96h)	609000000	[367]	**256**
		Daphnia	LC 50 (48h)	55086	[367]	
		Algae	**EC 50 (96h)**	**256000**	**[367]**	

3. Results and discussion

PhACs in raw wastewater

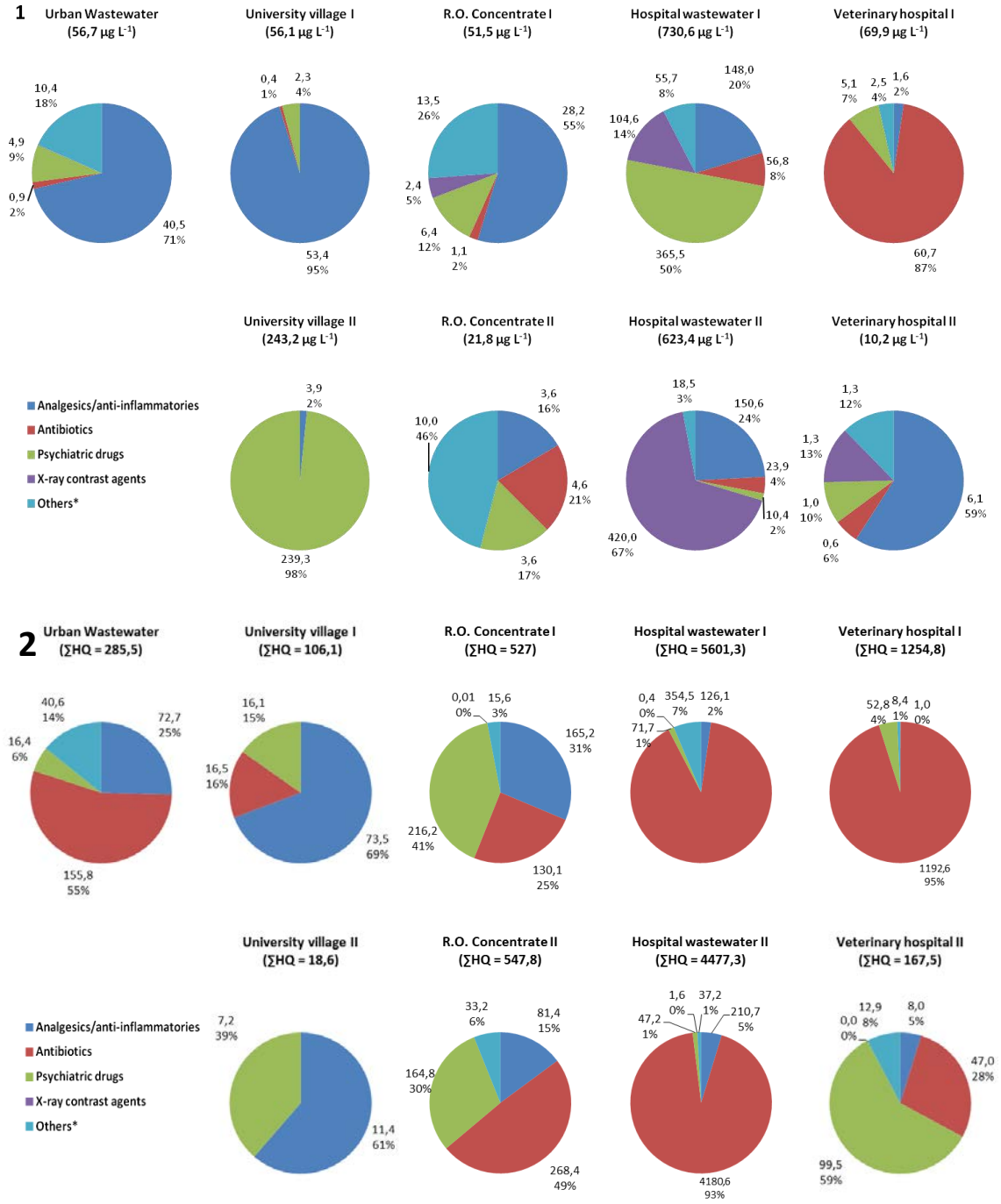
As expectedthe amount of PhACs in raw water samples varied a lot between the samplesdepending on their origin (Table 4.3)

Table 4.3 - Sum of PhAC concentrations (μg L^{-1}) in each water sample analyzed and their respectiveremovals.

		t = 0	t = final	
		Conc.	Conc.	Removal
CAS treatment	Urban wastewater	56.7	8.2	85%
Fungal treatment	University village I	56.1	2.8	95%
	University village II	243.2	16.9	93%
	R.O. concentrate I	51.5	27.7	46%
	R.O. concentrate II	21.8	9.4	57%
	Hospital wastewater I	730.6	65.3	91%
	Hospital wastewater II	623.4	283.8	54%
	Veterinary hospital I	69.9	17.4	75%
	Veterinary hospital II	10.2	5.2	50%

Unsurprisingly the highest concentration were detected wastewater samples from the hospital (730.6 and 623.4 μg L^{-1}), where more PhACs are used during the medical treatment of the inpatients. PhACs concentrations were lower in veterinary hospital (69.9 and 10.2 μg L^{-1}) as was also reported in previous studies [199, 381], probably due to the higher use of water (and therefore dilution of the PhACs) for cleaning the veterinary facilities, and the fact that urine from big animals (e.g., horses) was collected with straw and disposed separately [195]. Concentrations in the other raw wastewaters (urban wastewater, university village and R.O. concentrate) ranged between 21.8 and 243.2 μg L^{-1} (Table 4.3). Besides contaminants levels, PhACs ratios were also different depending on the raw wastewater considered (Figure 4.1); for instance, recalcitrant compounds, such as diclofenac and losartan, appear in R.O. concentrate at higher concentration than other more biodegradable compounds (Table 4.4). The concentrate is the fraction containing the contaminants rejected from the R.O. filtration of effluent wastewater, namely those contaminants that have not been degraded during the wastewater treatment [204]. For the rest of wastewaters considered in this study, the differences in PhACs profile could be attributed to the different consumption patterns [197, 380, 382] and seasonal variations in PhACs consumption [10, 383, 384]. For

example, seven compounds (dimetridazole, norfluoxetine, verapamil, cimetidine, famotidine, carazolol and salbutamol) were solely detected in hospital effluents (Table 4.4). Another example is the X-ray contrast agent iopromide; although found in other effluents, was present at much higher concentration in hospital wastewater, in accordance with its more intensive use [199, 379].



Figures 4.1 and 4.2 - 1) Percentages of the main therapeutic groups present in the water samples during the characterization expressed in terms of concentration. In brackets the total concentration measured in each sample is shown. 2) Percentages of the main therapeutic groups present in the water samples in the characterization expressed in terms of hazard quotients. In brackets sum of HQ values in each sample is shown.
*"Others" includes the following therapeutic groups: Anti-asthma drugs, anticoagulant, antidiabetic, antihelmintics, antihypertensives, antiplatelet agent, β-blocking agents, calcium channel blockers, diuretics, histamine H1 and H2 receptor antagonists, lipid regulators and cholesterol lowering statin drugs, prostatic hyperplasia, sedatives and muscle relaxation, synthetic glucocorticoid and tranquilizers.

Table 4.4 - Concentrations and removals of the PhACs analyzed in each wastewater sample.

Concentrations (μg L^{-1})

	CAS treatment			Fungal reactor																							
	Urban wastewater			University village I			University village II			R.O. Concentrate I			R.O. Concentrate II			Hospital wastewater I			Hospital wastewater II			Veterinay hospital I			Veterinay hospital II		
Analgesics/anti-inflamatories	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**
Acetaminophen	12,96	0,04	**100%**	3,87	0,00	**100%**	1,56	0,00	**100%**	1,33	0,07	**95%**	0,13	0,00	**100%**	114,46	0,57	**99%**	109,30	0,26	**100%**	0,07	0,00	**100%**	0,02		**100%**
Codeine	0,08	0,06	**26%**	0,02	0,00	**100%**				0,12	0,04	**63%**	0,21	0,09	**54%**	0,13	0,01	**94%**	0,61	0,00	**100%**						
Diclofenac	0,29	0,31	**-7%**							1,18	0,09	**93%**	1,61	0,01	**99%**	1,01	0,04	**97%**	0,32	0,00	**100%**				0,12	0,34	**-189%**
Ibuprofen	10,75	0,03	**100%**	12,61	0,04	**100%**	2,23	0,00	**100%**	21,17	6,96	**67%**				10,30		**100%**	35,50	0,00	**100%**	0,08	0,00	**100%**	0,34	0,15	**56%**
Indomethacine	0,05	0,06	**-36%**										0,32	0,08	**76%**										0,01	0,06	**-781%**
Ketoprofen	0,51	0,15	**71%**	0,48	0,31	**35%**	0,08	0,00	**100%**	0,48	1,22	**-152%**	1,14	0,51	**56%**	8,23	0,30	**96%**	2,17	0,10	**95%**	1,23	4,49	**-264%**	0,47	0,50	**-8%**
Meloxicam	0,92	0,33	**65%**																								
Naproxen	7,66	0,25	**97%**	35,58	0,00	**100%**							0,07	0,00	**100%**	13,68		**100%**	1,62	0,00	**100%**				0,06		**100%**
Oxycodone										0,22	0,09	**57%**															
Phenazone	0,03	0,01	**62%**							0,15	0,00	**100%**	0,06	0,00	**100%**	0,12	0,04	**71%**	0,50	0,02	**96%**						
Piroxicam	0,33	0,23	**31%**										0,07	0,00	**100%**					0,15		0,17	0,22	**-28%**	0,14	0,22	**-56%**
Propyphenazone																											
Salicylic acid	6,59	0,07	**99%**	0,85	1,24	**-46%**				3,52	8,49	**-141%**				0,05		**100%**	0,61	0,84	**-38%**				4,89	0,70	**86%**
Tenoxicam	0,33	0,24	**27%**																			0,02	0,00	**100%**			
SUBTOTAL	40,47	1,75	**96%**	53,41	1,59	**97%**	3,87	0,00	**100%**	28,16	16,96	**40%**	3,61	0,69	**81%**	147,98	0,95	**99%**	150,64	1,37	**99%**	1,58	4,71	**-198%**	6,05	1,97	**67%**
Antibiotics	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**
Azithromycin	0,13	0,14	**-11%**										1,72	1,23	**29%**				1,37	1,02	**26%**	0,07	0,06	**6%**			
Cefalexin																						45,22	0,00	**100%**			
Ciprofloxacin	0,39	0,18	**55%**							0,63	0,00	**100%**	0,63	0,45	**29%**	12,05	3,77	**69%**	13,00	0,17	**99%**	5,66	0,00	**100%**	0,24		**100%**
Clarithromycin	0,10	0,10	**1%**										0,05	0,01	**89%**				2,20	0,44	**80%**	0,01	0,00	**100%**			
Dimetridazole																0,22	0,22	**0%**	0,07	0,00	**100%**						
Erythromycin	0,02	0,02	**-20%**	0,33	0,00	**100%**							0,15	0,00	**100%**				0,01	0,00	**100%**						
Metronidazole				0,05	0,00	**100%**							0,13	0,00	**100%**	2,13	1,47	**31%**	0,91	0,17	**81%**	4,65	3,58	**23%**	0,26	0,23	**11%**
Metronidazole-OH										0,20	0,21	**-9%**	0,45	1,33	**-193%**	2,54	1,56	**39%**	0,74	0,14	**80%**	1,87	2,83	**-52%**	0,10	0,43	**-330%**
Ofloxacin	0,13	0,12	**8%**							0,06	0,00	**100%**	0,20	0,47	**-137%**	31,99	0,27	**99%**	3,34	0,08	**98%**	0,29	0,03	**89%**			
Ronidazole										0,05	0,04	**24%**	0,11	0,27	**-147%**					0,19	*	2,26	0,31	**86%**			
Sulfamethoxazole	0,07	0,01	**86%**										0,20	0,02	**88%**	5,30		**100%**	1,41	0,00	**100%**	0,61	0,09	**86%**			
Tetracyclin										0,13	0,59	**-354%**	0,91	0,08	**91%**				0,01	0,02	**-36%**	0,03	0,05	**-92%**			
Trimethoprim	0,05	0,01	**87%**													2,57	0,64	**75%**	0,85	0,00	**100%**	0,06	0,05	**18%**			
SUBTOTAL	0,89	0,57	**36%**	0,38	0,00	**100%**				1,06	0,84	**21%**	4,56	3,86	**15%**	56,80	7,93	**86%**	23,91	2,23	**91%**	60,72	7,00	**88%**	0,59	0,66	**-12%**
Psychiatric drugs	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**
10,11- EpoxyCBZ							75,50	16,20	**79%**	3,33	3,91	**-18%**				338,89	17,25	**95%**	8,98	0,00	**100%**						
2-Hydroxycarbamazepine				0,50	0,27	**46%**	163,80	0,60	**100%**							25,20	3,75	**85%**									
Acridone				1,01	0,00	**100%**		0,05	*								0,29	*									
Alprazolam													0,06	0,02	**66%**												
Carbamazepine	0,03	0,05	**-81%**	0,70	0,96	**-37%**		0,05	*	0,54	1,37	**-155%**	0,77	0,76	**1%**	0,44	0,46	**-5%**	0,06	0,16	**-191%**	4,62	2,36	**49%**		0,34	*
Citalopram	0,10	0,05	**47%**	0,10	0,00	**100%**	0,04	0,00	**100%**	0,77	0,37	**52%**	0,56	0,04	**93%**	0,44	0,63	**-42%**	0,26	0,05	**79%**					0,00	*
Diazepam										0,04	0,02	**51%**				0,01	0,01	**54%**				0,51	0,03	**94%**	1,00	0,04	**96%**
Fluoxetine										0,22	0,19	**13%**	0,22	0,00	**100%**												
Lorazepam													0,54	0,28	**49%**	0,05	0,64	**-1147%**	0,20	0,24	**-22%**						
Norfluoxetine																0,02		**100%**									
Olanzapine													0,49	0,48	**0%**				0,14	0,03	**76%**	0,01	0,02	**-349%**	0,01	0,01	**0%**
Paroxetine	0,59	0,18	**70%**																								
Sertraline										0,18	0,19	**-6%**							0,07	0,00	**100%**						
Trazodone	0,08	0,04	**48%**													0,04	0,00	**100%**									
Venlafaxine	4,11	2,66	**35%**							1,36	1,04	**23%**	0,97	0,44	**55%**	0,40	0,21	**48%**	0,68	0,04	**95%**						
SUBTOTAL	4,90	2,98	**39%**	2,31	1,23	**47%**	239,34	16,90	**93%**	6,43	7,10	**-10%**	3,61	2,02	**44%**	365,50	23,22	**94%**	10,39	0,53	**95%**	5,13	2,41	**53%**	1,00	0,39	**61%**
X-ray contrast agent	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**
Iopromide	0,06	0,20	**-215%**							2,41	0,81	**67%**				104,64	25,60	**76%**	420,00	276,88	**34%**				1,34	0,18	**86%**
SUBTOTAL	0,06	0,20	**-215%**							2,41	0,81	**67%**				104,64	25,60	**76%**	420,00	276,88	**34%**				1,34	0,18	**86%**

Table 4.4 (Cont) - Concentrations and removals of the PhACs analyzed in each wastewater sample.

	Concentrations (μg L^{-1})																							
	CAS treatment			Fungal reactor																				
	Urban wastewater			University village I			University village II			R.O. Concentrate I			R.O. Concentrate II			Hospital wastewater I			Hospital wastewater II			Veterinay hospital I			Veterinay hospital II		
Others	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**
Warfarin																											
Glibenclamide													0,10	0,00	**98%**				0,00	0,00	**100%**						
Albendazole																			0,03	0,00	**100%**				0,01	0,00	**56%**
Levamisol	0,02	0,05	**-88%**							0,25	0,13	**50%**	0,56	0,48	**15%**							1,07	3,25	**-205%**			
Thiabendazole		0,00	*							0,12	0,02	**80%**	0,00	0,00	**100%**												
Amlodipine													0,10	0,09	**10%**											0,48	*
Irbesartan	0,28	0,25	**12%**							0,05	0,01	**71%**	1,31	0,00	**100%**	0,24	0,01	**97%**	0,57	0,01	**98%**	0,45	0,00	**100%**	0,25	0,03	**89%**
Losartan	0,21	0,16	**23%**							2,67	0,03	**99%**	0,16	0,03	**81%**	0,67	4,41	**-559%**	1,06	0,00	**100%**						
Valsartan	1,51	0,10	**93%**							3,09	0,20	**94%**	0,22	0,06	**75%**	8,22	0,40	**95%**	3,35	0,39	**89%**						
Clopidogrel													0,01	0,00	**100%**										0,11		**100%**
Diltiazem										0,01	0,01	**25%**	0,06	0,00	**100%**	0,05	0,01	**77%**	0,43	0,00	**100%**						
Norverapamil																											
Verapamil																0,02	0,00	**100%**	0,00	0,00	**100%**						
Furosemide	1,90	0,29	**85%**							1,00	0,00	**100%**	1,63	0,13	**92%**	15,81	0,00	**100%**	3,84	0,67	**83%**	0,12	0,01	**88%**	0,31	1,29	**-316%**
Hydrochlorothiazide	1,37	1,04	**24%**							0,85	0,46	**46%**	1,15	1,00	**13%**				1,17	0,59	**50%**						
Torasemide										0,08	0,03	**68%**	0,38	0,11	**73%**	0,04	0,01	**70%**	0,08	0,01	**94%**						
Cimetidine																0,04	0,00	**100%**									
Desloratadine																						0,00	0,00	**69%**			
Famotidine																0,08	0,00	**100%**									
Loratadine																											
Ranitidine	1,17	0,18	**85%**							0,17	0,12	**29%**	0,83	0,00	**100%**	11,50	0,02	**100%**	0,64	0,00	**100%**	0,04	0,01	**67%**	0,45		**100%**
Atorvastatin	0,10	0,01	**91%**													0,14	0,00	**100%**	0,52	0,00	**100%**				0,03	0,03	**-14%**
Bezafibrate	0,12	0,00	**98%**																								
Fluvastatin																						0,02	0,00	**100%**	0,05	0,07	**-61%**
Gemfibrozil	1,01	0,18	**82%**							1,03	0,02	**98%**	0,27	0,00	**100%**	13,48	0,00	**100%**	2,85	0,00	**100%**	0,57	0,00	**100%**	0,04	0,04	**6%**
Pravastatin																			0,38	0,00	**99%**				0,01		**100%**
Tamsulosin																											
Xylazine	0,06	0,03	**47%**																								
Atenolol	2,22	0,27	**88%**							3,23	0,62	**81%**	2,12	0,52	**75%**	4,52	2,66	**41%**	2,99	0,76	**75%**						
Carazolol																			0,05	0,00	**100%**	0,00	0,00	**100%**			
Metoprolol	0,39	0,17	**57%**							0,22	0,09	**57%**	0,24	0,18	**25%**	0,07	0,05	**36%**	0,02	0,00	**100%**						
Nadolol	0,03	0,02	**33%**							0,09	0,07	**16%**	0,05	0,04	**19%**	0,06	0,05	**20%**	0,02	0,00	**93%**						
Propranolol										0,14	0,07	**50%**	0,12	0,01	**89%**	0,12	0,03	**73%**				0,19	0,00	**100%**			
Sotalol										0,48	0,16	**67%**	0,29	0,20	**29%**												
Dexamethasone																			0,36	0,40	**-11%**	0,01	0,00	**38%**			
Salbutamol																0,63		**100%**	0,13	0,00	**100%**						
Azaperol													0,42	0,00	**100%**							0,02	0,00	**100%**			
Azaperone																											
SUBTOTAL	10,39	2,74	**74%**							13,47	2,03	**85%**	10,02	2,85	**72%**	55,69	7,64	**86%**	18,47	2,81	**85%**	2,48	3,28	**-32%**	1,26	1,95	**-54%**
TOTAL	56,71	8,24	**85%**	56,10	2,82	**95%**	243,21	16,90	**93%**	51,53	27,73	**46%**	21,80	9,42	**57%**	730,61	65,33	**91%**	623,41	283,81	**54%**	69,91	17,40	**75%**	10,24	5,17	**50%**

For all wastewaters, their corresponding HQs and HQs removal values were calculated (Table 4.5) based on the abundance of the PhACs and their PNEC values.

According to PNEC values (Materials and methods, Table 3.2) it can be asserted that the most hazardous group is the antibiotics group due to the extremely low PNEC values of some of them such as clarithromycin (0.002 μg L^{-1}), sulfamethoxazole (0.027 μg L^{-1}), ciprofloxacin (0.005 μg L^{-1}), trimethoprim (0.0058 μg L^{-1}), ofloxacin (0.016 μg L^{-1}), azithromycin (0.019 μg L^{-1}) and erythromycin (0.02 μg L^{-1}). In point of fact, antibiotics are the major contributors to the total HQ in more than half of the raw water samples, even though when they are at very low concentration (Figures 4.1 and 4.2). For instance, in the hospital wastewater I and II, antibiotics concentration represents 8% and 4% respectively of the total PhACs concentration; in contrast, they represent 90% and 93% respectively of the total HQs.

Analgesics/anti-inflammatories also present high HQ values in urban and university village I wastewaters, due to their high concentrations and relatively low PNEC values. In contrast, the X-ray contrast agent iopromide, despite being at a very high concentration in the hospital wastewater I and II (14% and 67% of all the PhACs measured respectively) only contributes to less than 1% to the total HQ measured in those water samples. This is caused by the high PNEC value of iopromide, 256 μg L^{-1}.

Therefore, it can be highlighted that the most hazardous effluent were the hospital wastewaters (HQ values 5601.3 and 4477.3) and the veterinary hospital I (HQ value 1254.8) (Figure 4.2), summarizing, those with larger antibiotic concentrations. Also the R.O. concentrate present high HQ values (527.0 and 547.8), due to the presence of some compounds such as azithromycin, diclofenac, ibuprofen or irbesartan with low PNEC values. Urban wastewater and university village wastewater samples, which could be considered as urban wastewater too, differ in their HQ values, depending on the specific PhACs present in each sample. Within these samples, the urban wastewater is the most hazardous, with a total HQ value of 285.5; meanwhile the university village wastewater I and II samples are the less hazardous; 106.1 and 18.6 respectively.

Table 4.5 - Hazard quotients and their removal rates in the respective wastewater samples

	HQ																											
	CAS treatment			Fungal reactor																								
	Urban wastewater			University village I			University village II			R.O. Concentrate I			R.O. Concentrate II			Hospital wastewater I			Hospital wastewater II			Veterinay hospital I			Veterinay hospital II		
Analgesics/anti-inflamatories	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**
Acetaminophen	1,87	0,01	**100%**	0,56		**100%**	0,23		**100%**	0,19	0,01	**95%**	0,02		**100%**	16,54	0,08	**99%**	15,79	0,04	**100%**	0,01		**100%**	0,00		**100%**
Codeine	0,01	0,00	**26%**	0,00		**100%**				0,01	0,00	**63%**	0,01	0,01	**54%**	0,01	0,00	**94%**	0,04		**100%**						
Diclofenac	14,40	15,45	**-7%**							58,80	4,40	**93%**	80,72	0,55	**99%**	50,25	1,75	**97%**	15,80		**100%**				5,90	17,06	**-189%**
Ibuprofen	53,76	0,14	**100%**	63,05	0,20	**100%**	11,15		**100%**	105,83	34,81	**67%**				51,50		**100%**	177,50		**100%**	0,40		**100%**	1,71	0,75	**56%**
Indomethacine	0,00	0,00	**-36%**										0,02	0,00	**76%**										0,00	0,00	**-781%**
Ketoprofen	0,25	0,07	**71%**	0,24	0,16	**35%**	0,04		**100%**	0,24	0,61	**-152%**	0,57	0,25	**56%**	4,12	0,15	**96%**	1,09	0,05	**95%**	0,62	2,24	**-264%**	0,23	0,25	**-8%**
Meloxicam	0,11	0,04	**65%**																								
Naproxen	2,07	0,07	**97%**	9,62		**100%**							0,02		**100%**	3,70		**100%**	0,44		**100%**				0,02		**100%**
Oxycodone										0,00	0,00	**57%**															
Phenazone	0,00	0,00	**62%**							0,00		**100%**	0,00		**100%**	0,00	0,00	**71%**	0,00	0,00	**96%**						
Piroxicam	0,01	0,00	**31%**										0,00		**100%**					0,00		0,00	0,00	**-28%**	0,00	0,00	**-56%**
Propyphenazone																											
Salicylic acid	0,18	0,00	**99%**	0,02	0,03	**-46%**				0,10	0,24	**-141%**				0,00		**100%**	0,02	0,02	**-38%**				0,14	0,02	**86%**
Tenoxicam	0,01	0,00	**27%**																			0,00		**100%**			
SUBTOTAL	72,66	15,79	**78%**	73,49	0,39	**99%**	11,42	0,00	**100%**	165,17	40,06	**76%**	81,36	0,81	**99%**	126,11	1,98	**98%**	210,67	0,12	**100%**	1,04	2,25	**-117%**	8,01	18,08	**-126%**
Antibiotics	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**
Azithromycin	6,79	7,53	**-11%**										90,78	64,79	**29%**				72,11	53,63	**26%**	3,47	3,26	**6%**			
Cefalexin																						0,03		**100%**			
Ciprofloxacin	78,40	35,20	**55%**							126,20		**100%**	126,48	89,80	**29%**	2409,80	754,80	**69%**	2600,00	34,60	**99%**	1131,12		**100%**	47,04		**100%**
Clarithromycin	50,00	49,50	**1%**										22,77	2,50	**89%**				1100,00	218,50	**80%**	5,96		**100%**			
Dimetridazole																0,00	0,00	**0%**	0,00		**100%**						
Erythromycin	0,75	0,90	**-20%**	16,50		**100%**							7,73		**100%**				0,40		**100%**						
Metronidazole				0,00		**100%**							0,00		**100%**	0,05	0,04	**31%**	0,02	0,00	**81%**	0,12	0,09	**23%**	0,01	0,01	**11%**
Metronidazole-OH										0,00	0,00	**-9%**	0,00	0,00	**-193%**	0,00	0,00	**39%**	0,00	0,00	**80%**	0,00	0,00	**-52%**	0,00	0,00	**-330%**
Ofloxacin	8,00	7,38	**8%**							3,75		**100%**	12,30	29,19	**-137%**	1999,56	17,00	**99%**	208,75	5,13	**98%**	18,22	2,04	**89%**			
Ronidazole										0,00	0,00	**24%**	0,00	0,00	**-147%**					0,00	*	0,00	0,00	**86%**			
Sulfamethoxazole	2,59	0,37	**86%**										7,43	0,89	**88%**	196,22		**100%**	52,22		**100%**	22,54	3,22	**86%**			
Tetracyclin										0,13	0,59	**-354%**	0,91	0,08	**91%**				0,01	0,02	**-36%**	0,03	0,05	**-92%**			
Trimethoprim	9,31	1,21	**87%**													442,93	109,48	**75%**	147,07		**100%**	11,13	9,14	**18%**			
SUBTOTAL	155,84	102,08	**34%**	16,50	0,00	**100%**				130,08	0,59	**100%**	268,40	187,25	**30%**	5048,57	881,32	**83%**	4180,58	311,88	**93%**	1192,61	17,81	**99%**	47,05	0,01	**100%**
Psychiatric drugs	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**
10,11- EpoxyCBZ							0,16	0,03	**79%**	0,01	0,01	**-18%**				0,72	0,04	**95%**	0,02		**100%**						
2-Hydroxycarbamazepine				0,00	0,00	**46%**	0,74	0,00	**100%**							0,11	0,02	**85%**									
Acridone				0,01		**100%**		0,00	*								0,00	*									
Alprazolam													0,02	0,01	**66%**												
Carbamazepine	0,01	0,02	**-81%**	0,35	0,48	**-37%**		0,03	*	0,27	0,69	**-155%**	0,38	0,38	**1%**	0,22	0,23	**-5%**	0,03	0,08	**-191%**	2,31	1,18	**49%**		0,17	*
Citalopram	14,96	7,87	**47%**	15,75		**100%**	6,30	0,00	**100%**	120,94	58,58	**52%**	88,74	6,61	**93%**	69,29	98,43	**-42%**	40,94	8,50	**79%**					0,64	*
Diazepam										3,80	1,85	**51%**				1,30	0,60	**54%**				50,50	3,00	**94%**	99,50	4,07	**96%**
Fluoxetine										76,21	66,55	**13%**	75,59		**100%**												
Lorazepam													0,01	0,00	**49%**	0,00	0,01	**-1147%**	0,00	0,00	**-22%**						
Norfluoxetine																0,01		**100%**									
Olanzapine													0,01	0,01	**0%**				0,00	0,00	**76%**	0,00	0,00	**-349%**	0,00	0,00	**0%**
Paroxetine	1,02	0,31	**70%**																								
Sertraline										14,79	15,62	**-6%**							6,12		**100%**						
Trazodone	0,00	0,00	**48%**													0,00		**100%**									
Venlafaxine	0,40	0,26	**35%**							0,13	0,10	**23%**	0,09	0,04	**55%**	0,04	0,02	**48%**	0,07	0,00	**95%**						
SUBTOTAL	16,40	8,47	**48%**	16,11	0,48	**97%**	7,20	0,06	**99%**	216,15	143,40	**34%**	164,84	7,06	**96%**	71,70	99,34	**-39%**	47,18	8,59	**82%**	52,81	4,18	**92%**	99,50	4,87	**95%**
X-ray contrast agent	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**
Iopromide	0,00	0,00	**-215%**							0,01	0,00	**67%**				0,41	0,10	**76%**	1,64	1,08	**34%**				0,01	0,00	**86%**
SUBTOTAL	0,00	0,00	**-215%**							0,01	0,00	**67%**				0,41	0,10	**76%**	1,64	1,08	**34%**				0,01	0,00	**86%**

Table 4.5 (Cont) - Hazard quotients and their removal rates in the respective wastewater samples

	HQ																											
	CAS treatment			Fungal reactor																								
	Urban wastewater			University village I			University village II			R.O. Concentrate I			R.O. Concentrate II			Hospital wastewater I			Hospital wastewater II			Veterinay hospital I			Veterinay hospital II		
Others	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**	**t=0**	**t=f**	**Removal**
Warfarin																											
Glibenclamide													0,11	0,00	**98%**				0,00		**100%**						
Albendazole																			0,00		**100%**				0,00	0,00	**56%**
Levamisol	0,00	0,00	**-88%**							0,01	0,01	**50%**	0,03	0,03	**15%**							0,06	0,19	**-205%**			
Thiabendazole		0,01	*							0,40	0,08	**80%**	0,00		**100%**												
Amlodipine													0,00	0,00	**10%**											0,00	*
Irbesartan	0,97	0,85	**12%**							0,17	0,05	**71%**	4,51		**100%**	0,81	0,02	**97%**	1,96	0,03	**98%**	1,55		**100%**	0,85	0,10	**89%**
Losartan	0,06	0,04	**23%**							0,72	0,01	**99%**	0,04	0,01	**81%**	0,18	1,20	**-559%**	0,29		**100%**						
Valsartan	0,19	0,01	**93%**							0,39	0,03	**94%**	0,03	0,01	**75%**	1,05	0,05	**95%**	0,43	0,05	**89%**						
Clopidogrel													0,00		**100%**										0,03		**100%**
Diltiazem										0,00	0,00	**25%**	0,01		**100%**	0,01	0,00	**77%**	0,05		**100%**						
Norverapamil																											
Verapamil																0,04		**100%**	0,01		**100%**						
Furosemide	1,90	0,29	**85%**							1,00	0,00	**100%**	1,63	0,13	**92%**	15,81		**100%**	3,84	0,67	**83%**	0,12	0,01	**88%**	0,31	1,29	**-316%**
Hydrochlorothiazide	0,00	0,00	**24%**							0,00	0,00	**46%**	0,00	0,00	**13%**				0,00	0,00	**50%**						
Torasemide										0,00	0,00	**68%**	0,00	0,00	**73%**	0,00	0,00	**70%**	0,00	0,00	**94%**						
Cimetidine																0,00		**100%**									
Desloratadine																						0,01	0,00	**69%**			
Famotidine																0,00		**100%**									
Loratadine																											
Ranitidine	29,13	4,40	**85%**							4,28	3,05	**29%**	20,87		**100%**	287,50	0,38	**100%**	15,93	0,05	**100%**	0,92	0,31	**67%**	11,31		**100%**
Atorvastatin	0,61	0,06	**91%**													0,84		**100%**	3,23		**100%**				0,18	0,20	**-14%**
Bezafibrate	0,26	0,01	**98%**																								
Fluvastatin																						0,02		**100%**	0,07	0,11	**-61%**
Gemfibrozil	3,36	0,61	**82%**							3,42	0,07	**98%**	0,88		**100%**	44,93		**100%**	9,50		**100%**	1,91		**100%**	0,13	0,12	**6%**
Pravastatin																			0,21	0,00	**99%**				0,01		**100%**
Tamsulosin																											
Xylazine	0,08	0,04	**47%**																								
Atenolol	0,11	0,01	**88%**							0,16	0,03	**81%**	0,11	0,03	**75%**	0,23	0,13	**41%**	0,15	0,04	**75%**						
Carazolol																			0,00		**100%**	0,00		**100%**			
Metoprolol	3,93	1,69	**57%**							2,16	0,93	**57%**	2,42	1,81	**25%**	0,73	0,47	**36%**	0,19		**100%**						
Nadolol	0,01	0,00	**33%**							0,02	0,01	**16%**	0,01	0,01	**19%**	0,01	0,01	**20%**	0,00	0,00	**93%**						
Propranolol										2,82	1,41	**50%**	2,48	0,28	**89%**	2,32	0,62	**73%**				3,73		**100%**			
Sotalol										0,04	0,01	**67%**	0,02	0,02	**29%**												
Dexamethasone																			1,42	1,57	**-11%**	0,02	0,02	**38%**			
Salbutamol																			0,00		**100%**						
Azaperol													0,02		**100%**							0,00		**100%**			
Azaperone																											
SUBTOTAL	40,61	8,04	**80%**							15,59	5,68	**64%**	33,18	2,32	**93%**	354,47	2,88	**99%**	37,20	2,41	**94%**	8,35	0,53	**94%**	12,89	1,83	**86%**
TOTAL	285,51	134,37	**53%**	106,10	0,87	**99%**	18,61	0,06	**100%**	527,00	189,74	**64%**	547,78	197,43	**64%**	5601,26	985,62	**82%**	4477,27	324,07	**93%**	1254,81	24,76	**98%**	167,45	24,79	**85%**

Efficiency of fungal treatment

When evaluating total removal of PhACs after fungal treatment of the different wastewater samples (Table 4.3), it can be stated that the R.O. concentrate samples are the most difficult to degrade (57% and 46% of removal as it is shown in Table 4.3 and Figure 4.3). The ROC is the fraction containing the contaminants rejected from the reverse osmosis filtration of secondary effluent wastewater, namely those contaminants that have not been degraded during the conventional wastewater treatment. Therefore, recalcitrant compounds such as diclofenac, losartan, azithromycin, tetracycline and levamisole are present at higher concentration than other more biodegradable compounds.

In contrast to ROC samples, university village wastewater samples are the ones that reach the better removal values with the fungal treatment (95% and 93%). This is attributed to the high concentration of some specific compounds in such effluent, like ibuprofen, naproxen or carbamazepine metabolites (10,11-epoxycarbamazepine and 2-hydroxycarbamazepine) [191], which are easily degraded with the fungal treatment (as well as in conventional WWTP) (Table 4.4).

Removal values obtained in the two bioreactors used with hospital wastewater were very encouraging overall [205]. In spite of the high initial PhACs concentrations, 730.6 $\mu g\ L^{-1}$ in the first experiment and 623.4 $\mu g\ L^{-1}$ (Table 4.4) in the second one performed with hospital wastewater, the removal values achieved after the treatment were 91% and 54% respectively. The removal difference between the two experiments can be attributed to the large amount of the X-ray contrast agent, iopromide, detected in the samples from the second experiment; 420 $\mu g\ L^{-1}$ (representing 67% of total PhACs detected in the samples). Fungal treatment removed only 34% of this compound in that experiment. Composition of the wastewater effluent varies a lot from one day to another according to the type of treatments carried out in the hospital and in consequence, so will do the removal efficiency of the fungal treatment.

Removal values obtained in the two experiments performed with veterinary hospital wastewater were 75% for the first experiment, performed in batch bioreactors; and 50% for the second one, performed with continuous bioreactors [195]. As it happens with the hospital wastewater samples, the veterinary hospital composition varies a lot from one

day to another depending on the medical treatments performed in the facilities. Therefore depending on the compounds present in the samples, the removal value may vary.

Hospital and veterinary hospital wastewaters exhibit good removal values but similar or even worse than the ones obtained in the CAS treatment of the urban wastewater. Overall, the CAS treatment showed a slightly better removal value (85%) than the average removal (76%) obtained from the fungal treatment of all wastewaters (excluding the R.O. concentrate samples due to their special characteristics and composition).

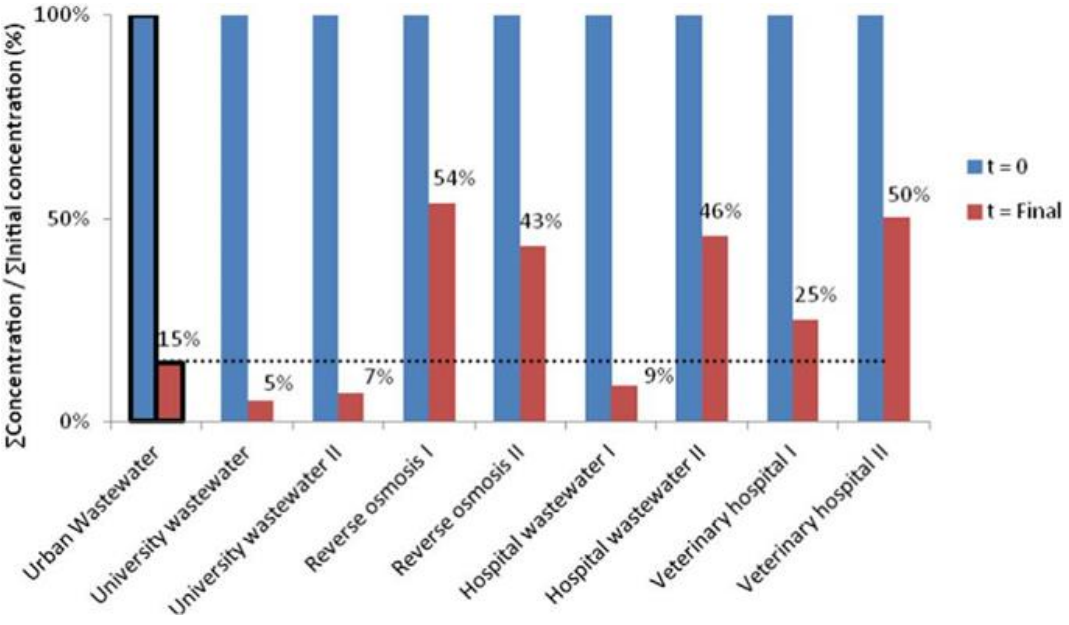


Figure 4.3 - Normalized PhAC concentration from all analyzed samples. Urban wastewater samples from the WWTP are highlighted.

Removal efficiency of fungal treatment was also evaluated in terms of reduction of total HQ after the treatments (Table 4.6). The biggest effectiveness of the fungal treatment was observed in the university village I and II, with HQ removal values of 99% and 100% respectively (Table 4.6 and Figure 4.4), in line with the also highest removal rates observed in terms of PhACs removal (Table 4.3). However these great results are not so remarkable compared with those obtained with other wastewaters evaluatedsince the urban wastewater used in these experiments had very low initial HQ values (106.1 and 18.6) compared with the other wastewaters: hospital (5601.3 and 4477.3) veterinary hospital (1254.8 and 167.5) and ROC (527.0 and 547.8) .

This HQ reduction is mainly attributable to the total elimination of ibuprofen, citalopram and erythromycin (Table 4.6); this copmunds represent the 90% and 94% of the total HQ of these samples before their treatment. Hospital and veterinary hospital wastewaters exhibited quite good HQ removal values after fungal treatment, ranging from 82% in the hospital wastewater I to 98% in the veterinary hospital wastewater I.

Table 4.6 - Sum of hazard quotient (HQ) values of PhACs in each water sample analyzed and their corresponding removals

		t = 0	t = final	
		HQ	HQ	Removal
CAS treatment	Urban wastewater	285.5	134.4	53%
Fungal treatment	University village I	106.1	0.87	99%
	University village II	18.6	0.06	100%
	R.O. concentrate I	527	189.7	64%
	R.O. concentrate II	547.8	197.4	64%
	Hospital wastewater I	5601.3	985.6	82%
	Hospital wastewater II	4477.3	324.1	93%
	Veterinary hospital I	1254.8	24.8	98%
	Veterinary hospital II	167.5	24.8	85%

In spite of the good PhAC removal (85%), in terms of HQ removal the CAS treatment showed the lowest value: 53% (Table 4.6). This might be attributed to the fact that CAS treatment is not able to degrade efficiently some specific compounds such as azythromycin, clarithromycin and erythromycin, which are very important from an ecotoxicological point of view.

Finally, R.O. concentrate samples, with their relatively high concentration of recalcitrant compounds, showed a low HQ removal efficiency (64% for both samples) (Table 4.6), in line with their PhAC removal values obtained (Table 4.4). However this HQ removal value is still better than the one obtained with the CAS treatment, 53%.

The HQ removal value of the fungal treatment with this type of wastewater bear great interest from an environmental point of view since the management of the concentrates is one of the main drawbacks of the advanced wastewater treatment technologies based on filtration processes (as explained in section 1.5)

Taking into account all the samples analyzed, with the exception of the R.O. concentrate samples, the fungal treatment showed a clearly better HQ removal value (93%) than the CAS treatment (53%) (Figure 4.4).

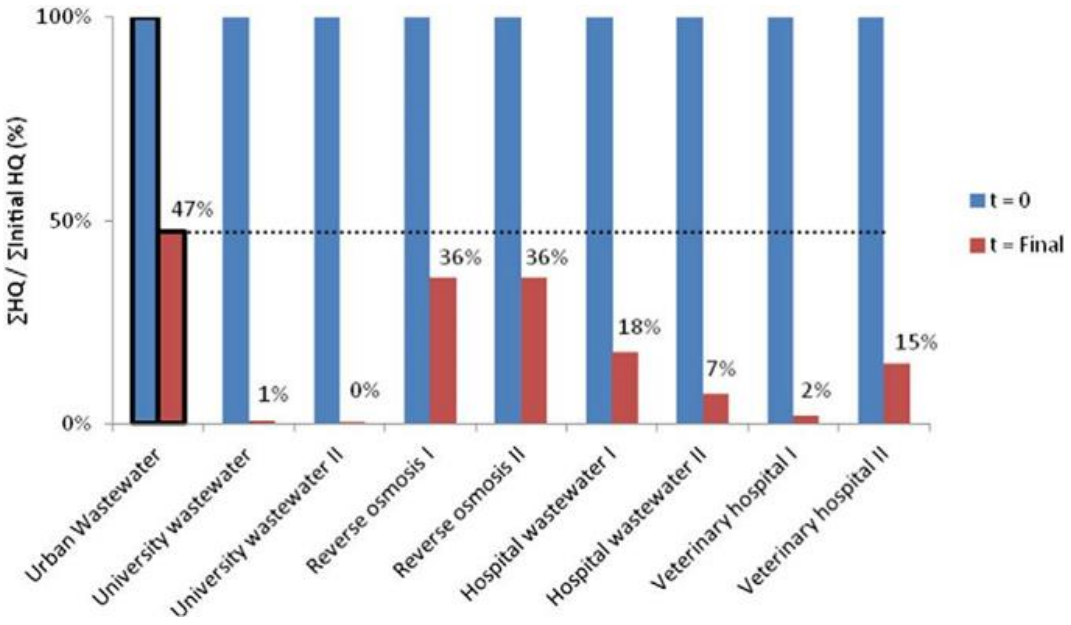


Figure 4.4 - Normalized HQ values from all analyzed samples. Urban wastewater samples from the WWTP are highlighted.

According to our results, the main difference between both treatments seems to lie in the fungal capability to degrade certain pollutants. Those compounds contributing the most to such efficiency differences are antibiotics and psychiatric drugs, and so are usually the most problematic ones due to their low PNECs values and to their high concentration respectively. Taking into account only antibiotics and the psychiatric drugs, the mean concentration removal achieved with the CAS treatment is 37% (36% for antibiotics and 39% for psychiatric drugs as seen in Table 4.4); however, with the fungal treatment the removal value achieved is a 58% for these two groups (56% for antibiotics and 59% for psychiatric drugs). In terms of HQs the differences between both treatments are even more remarkable; the HQ removal achieved for antibiotics and psychiatric drugs with the CAS treatment is 41% (34% for antibiotics and 48% for psychiatric drugs; Table 4.5) whereas with the fungal treatment the removal achieved is 77% (86% for antibiotics and 69% for psychiatric drugs).

4. Conclusions

Among all wastewaters considered in this study, hospital wastewaters were the most hazardous ones due to the significant presence of compounds such as antibiotics. Antibiotics have the lowest PNECs values compared to other families of contaminants

and are usually present in hospital wastewater at high concentrations because of their massive consumption. Because of this, hospital wastewaters should be strictly controlled and a decentralized treatment of these effluents to remove hazardous contaminants is strongly recommended.

ROC must be also taken into consideration since it contains recalcitrant compounds. In addition, because of the low removal efficiency of fungal treatment (in terms of PhACs and HQ elimination), the final effluent remains as one of the most toxic and so it should be controlled too.

According to the results obtained, fungal treatment of wastewaters is confirmed as a very promising technology, especially from the point of view of environmental risk. This acknowledges the great degradative capacity of fungi over hazardous and recalcitrant compounds (antibiotics and psychiatric drugs) particularly.

Regarding the environmental risk assessment methodology, another consideration should be mentioned. Some compounds, such as antibiotics, might have certain side effects which would go unnoticed if only the three common species (fish, daphnia or algae) are used to determine the PNEC. Antibiotics can cause changes in the microbiota ecosystem and even modify certain natural processes such as nitrification [436], methanogenesis, acetogenesis and sulfate reduction [437]. In addition they may be involved in the spread of ARGs. Therefore it would be convenient to add bacteria as a fourth bioindicator organism in the ERA studies.

Chapter 5

Chapter 5 – Antibiotics and ARGs

Based on the publication:
Fungal treatment for the removal of antibiotics and antibioticresistance genes in veterinary hospital wastewater
D. Lucas, M. Badia-Fabregat, T. Vicent, G. Caminal, S. Rodríguez-Mozaz,
J.L. Balcázar, D. Barceló
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Abstract

The emergence and spread of antibiotic resistance represents one of the most important public health concerns and has been linked to the widespread use of antibiotics in veterinary and human medicine. The overall elimination of antibiotics in conventional wastewater treatment plants is quite low; therefore, residual amounts of these compounds are continuously discharged to receiving surface waters, which may promote the emergence of antibiotic resistance. In this study, the ability of a fungal treatment as an alternative wastewater treatment for the elimination of forty-seven antibiotics belonging to seven different groups (β-lactams, fluoroquinolones, macrolides, metronidazoles, sulfonamides, tetracyclines, and trimethoprim) was evaluated. 77% of antibiotics were removed after the fungal treatment, which is higher than removal obtained in conventional treatment plants. Moreover, the effect of fungal treatment on the removal of some antibiotic resistance genes (ARGs) was evaluated. The fungal treatment was also efficient in removing ARGs, such as *ermB* (resistance to macrolides), *tetW* (resistance to tetracyclines), bla_{TEM} (resistance to β-lactams), *sul*I (resistance to sulfonamides) and *qnrS* (reduced susceptibility to fluoroquinolones). However, it was not possible to establish a clear link between concentrations of antibiotics and corresponding ARGs in wastewater, which leads to the conclusion that there are other factors that should be taken into consideration besides the antibiotic concentrations that reach aquatic ecosystems in order to explain the emergence and spread of antibiotic resistance.

Keywords

Antibiotics, antibiotic resistance genes, degradation, fungal treatment, wastewater, veterinary hospital

1. Introduction

Antimicrobial agents have been used in large quantities for several decades; these compounds have been widely used not only to treat infectious diseases in human and veterinary medicine, but also as growth promoters in animal production [25, 67, 385]. Antibiotics may therefore be found in different environmental compartments due to their extensive use and the continuous drainage of surface runoff and release from wastewater treatment plants (WWTPs) [138].

It is well known that antibiotics pose a significant risk to environmental and human health, even at low concentrations [25]. In addition, the overuse and misuse of antibiotics has led to the emergence of antibiotic resistant bacteria, compromising the effectiveness of antimicrobial therapy because the infectious organisms are becoming resistant to commonly prescribed antibiotics [45, 46].

According to recent studies, WWTPs are considered important hotspots for the spread of antibiotic resistance [41, 386, 387], because conventional treatments, where environmental bacteria are continuously mixed with resistant bacteria and antibiotics from anthropogenic sources, offer an environment potentially suitable for the emergence and spread of antibiotic resistance [68, 189, 388, 389]. Although the levels of antibiotics found in WWTPs are often below minimum inhibitory concentration, they may exert a selective pressure on microbial populations.

Antibiotic resistance genes (ARGs) and antibiotics compounds are pollutants that have different modes of action and are subject to different fate processes in the environment [387]. They are also likely to respond differently to treatment processes designed to remove them from liquid and solid wastes [391]. Although the efficiency of ARGs removal by sewage treatment procedures is such an important issue, very few studies have addressed this topic until the last decade [51, 67, 68, 392] and only few of them have focused on both antibiotics and ARGs [51, 58, 60, 138]. However, none of the studies available in literature so far have studied their fate in non-conventional biological treatments, such as fungal treatment.

The aim of our study was therefore to evaluate a fungal treatment of veterinary hospital wastewater with regards to the presence of antibiotics and ARGs. Veterinary hospital effluent was selected because its high antibiotic concentration expected.

A broad range of antibiotics covering different families were selected and monitored along the study. Culture-independent approaches were also used to determine the prevalence of selected ARGs encoding resistance to the main antibiotic families, such as *bla*$_{TEM}$ and *bla*$_{SHV}$ (resistance to β-lactams), *qnrS* (reduced susceptibility to fluoroquinolones), *ermB* (resistance to macrolides), *sul*I (resistance to sulfonamides) and *tetW* (resistance to tetracyclines) genes.

2. Materials and methods

Bioreactors

Two 1.5 L air-pulsed fluidized bed glass bioreactors were set up in parallel to treat VHW: one inoculated with *T. Versicolor* in form of pellets and the other one, non-inoculated, used as a control with no biomass except the wastewater-associated bacterial communities. A completely sterile reactor could not be set up to measure the abiotic degradation due to the inability to sterilize the water (heat, enzyme treatment, filtration, etc.) without affecting the stability of antibiotics. Pellets of *T. versicolor* were added at 2.0 g DCW L^{-1}. Temperature was set up at 25 ºC and pH was controlled to be constant at 4.5 ± 0.5 by HCl 1 M or NaOH 1 M addition. Bioreactors were operated in fed-batch mode for nutrients: glucose and ammonia tartrate were added at 277 mg g DCW^{-1} d^{-1} and 0.619 mg g DCW^{-1} d^{-1} respectively in pulses of 0.6 min h^{-1} from a concentrated stock. Addition rate was adjusted to avoid glucose accumulation in the media. Glucose concentration and laccase activity were monitored to assure the good performance of the bioreactor. Liquid samples of approximately 50 mL were taken at the beginning and at the end of the experiment (after 15 days) by triplicate for the analytical procedures. The samples were kept at -20 ºC and in the dark to avoid the photodegradation of some of the antibiotics.

DNA extraction

Samples were filtered under sterile conditions through low protein-binding 0.22-mm-pore-size membranes (Millipore). The collected bacterial cells were then resuspended in lysis buffer (1.2% Triton X-100, 1M Tris-Cl, 0.5 M Na2EDTA), followed by enzymatic digestion with lysozyme and proteinase K. Genomic DNA was extracted using the

DNeasy Blood & Tissue Kit (Qiagen; Valencia, CA, USA), according to the manufacturer's instructions. All DNA samples were stored at 20º C until analysis.

Quantification of ARGs

Real-time PCR (qPCR) assays were used to quantify the copy number of selected ARGs, such as *bla*$_{TEM}$, *bla*$_{SHV}$, *ermB*, *qnrS*, *sul* I and *tetW*, according to the method described by Marti et al. (2013) [358]. Copy number of the 16S rRNA gene was also quantified for normalization of the data. All qPCR assays were performed using the Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA), with the exception for the *bla*$_{TEM}$ gene, which was amplified using the SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) due to non specific amplification. All qPCR assays were conducted on a MX3005P system (Agilent Technologies). Each gene was amplified using specific primer sets (Table 5.1) and the PCR conditions included an initial denaturation at 95ºC for 3 min, followed by 40 cycles at 95ºC for 15 s and at the annealing temperature given in Table 3.3 for 20 s.

Table 5.1 - Primers and qPCR conditions used in this study.

Target gene	Primers	Sequence	Conditions
16S rRNA	F1048	GTGSTGCAYGGYTGTCGTCA	95 ºC 3 min (1 cycle); 95 ºC 15 s and 60 ºC 1 min (35 cycles)
	R1194	ACGTCRTCCMCACCTTCCTC	
qnrS	qnrSf-RT	ATGCAAGTTTCCAACAATGC	95 ºC 3 min (1 cycle); 95 ºC 15 s and 62 ºC 20 s (40 cycles)
	qnrSr-RT	CTATCCAGCGATTTTCAAACA	
bla$_{TEM}$	bla-TEM, FX	GCKGCCAACTTACTTCTGACAACG	95 ºC 3 min (1 cycle); 95 ºC 15 s and 60 ºC 20 s (40 cycles)
	bla-TEM, RX	CTTTATCCGCCTCCATCCAGTCTA	
bla$_{SHV}$	RTblaSHVF	CGCTTTCCCATGATGAGCACCTTT	95 ºC 3 min (1 cycle); 95 ºC 15 s and 64 ºC 30 s (40 cycles)
	RTblaSHVR	TCCTGCTGGCGATAGTGGATCTTT	
sul I	sul(I)-FW	CGCACCGGAAACATCGCTGCAC	95 ºC 3 min (1 cycle); 95 ºC 15 s and 65 ºC 20 s (40 cycles)
	sul(I)-RV	TGAAGTTCCGCCGCAAGGCTCG	
tetW	tet(W)-FW	GAGAGCCTGCTATATGCCAGC	95 ºC 3 min (1 cycle); 95 ºC 15 s and 60 ºC 20 s (40 cycles)
	tet(W)-RV	GGGCGTATCCACAATGTTAAC	
ermB	erm(B)-91f	GATACCGTTTACGAAATTGG	95 ºC 3 min (1 cycle); 95 ºC 15 s and 58 ºC 20 s (40 cycles)
	erm(B)-454r	GAATCGAGACTTGAGTGTGC	

In the case of the 16S rRNA gene, amplification conditions were 35 cycles at 95º C for 15 s, followed by an annealing temperature at 60º C for 1 min. A dissociation curve was

then constructed by increasing the temperature from 65 to 95ºC in order to confirm the specificity of the amplified products. Standard curves were generated by cloning the amplicon from positive controls into the pCR2.1-TOPO vector from Invitrogen (Carlsbad, CA, USA), and the corresponding copy number was calculated as previously described [60]. The copy number of each ARG was also normalized to the 16S rRNA gene copy number in order to obtain relative quantification.

3. Results and discussion

Quantification of antibiotics

In the chemical analysis 32 out of 47 antibiotics analyzed were detected in water samples collected from veterinary hospital used to feed both bioreactors. For an accurate quantification, extraction recoveries were determined in triplicate for each sample and applied to the values obtained in the extracts, for data correction. Those compounds, whose recovery values did not range between 50 and 150%, were discarded. Quantification limits (LOQs) of each compound were estimated between 1 and 14 ng L^{-1} (Table 5.2).

Table 5.2 - Concentrations, detection limits and removal values of the detected antibiotics.

Antibiotic group	Antibiotic	Concentrations (ng L^{-1}) ± SD			LOD	Removals	
		t=0d	With fungi t=15d	Control t=15d	(ng L^{-1})	With fungi t=15d	Control t=15d
Quinolones	Ciprofloxacin	13881 ± 2410	1325 ± 305	8680 ± 231	0,46	91%	38%
	Enrofloxacin	982 ± 224	241 ± 13	430 ± 3	1,6	76%	56%
	Ofloxacin	220 ± 29	118 ± 15	320 ± 5	1,35	46%	-46%
	Marbofloxacin	191 ± 24	25 ± 2	86 ± 59	13,51	87%	55%
	Enoxacin	190 ± 42	8 ± 1	8 ± 4	6,04	96%	96%
	Norfloxacin	91 ± 10	141 ± 17	161 ± 9	11,85	-55%	-77%
	Pipemidic Acid	88 ± 24	56 ± 8	63 ± 10	4,29	37%	29%
	Danofloxacin	23 ± 2	29 ± 7	32 ± 8	13,33	-25%	-38%
	Nalidixic Acid	16 ± 3	20 ± 1	28 ± 1	5,4	-26%	-76%
	Cinofloxacin	5 ± 1	18 ± 1	20 ± 2	0,9	-256%	-299%
SUBTOTAL		**15,701 ± 77**	**1980 ± 10**	**9838 ± 8**		**87%**	**37%**
β-Lactams	Cefazolin	4810 ± 152	22 ± 7	35 ± 12	0,83	100%	99%
	Ampicillin	2138 ± 100	ND	183 ± 66	0,41	100%	9%
	AmpicillinB	1965 ± 61	121 ± 37	107 ± 41	1,69	94%	95%
	Cefalexin	1178 ± 70	29 ± 4	40 ± 5	1,32	98%	97%
	Cefuroxime	162 ± 46	58 ± 7	15 ± 16	2,82	64%	91%
	PenicillinV	ND	186 ± 48	456 ± 252	13,57	*	*
SUBTOTAL		**10,253 ± 7**	**416 ± 2**	**835 ± 8**		**96%**	**92%**
Tetracyclines	Doxycycline	4697 ± 752	3345 ± 350	3439 ± 1	0,77	29%	27%
	Tetracyclin	63 ± 10	47 ± 1	95 ± 3	8,12	26%	-50%
	Oxytetracyclin	38 ± 6	17 ± 3	40 ± 1	11,95	57%	-4%
	Chlorotetracycline	8 ± 1	ND	ND	6,5	100%	100%
SUBTOTAL		**4807 ± 24**	**3409 ± 11**	**3574 ± 0**		**29%**	**26%**
Metronidazoles	Metronidazole	4588 ± 880	3815 ± 129	3603 ± 146	9	17%	22%
	Metronidazole OH	186 ± 49	640 ± 78	2154 ± 6	0,77	-244%	-1058%
SUBTOTAL		**4774 ± 28**	**4455 ± 5**	**5757 ± 5**		**7%**	**-21%**
Macrolides	Erythromycin	201 ± 54	267 ± 18	597 ± 205	11,82	-33%	-197%
	Tilmicosin	57 ± 18	72 ± 14	106 ± 11	3,2	-26%	-86%
	Azythromycin	23 ± 6	31 ± 7	38 ± 1	1,56	-34%	-61%
	Tylosin	14 ± 5	10 ± 2	9 ± 3	6,35	30%	35%
	Clarithromycin	13 ± 4	28 ± 7	86 ± 3	1,36	-115%	-553%
	Spyramycin	9 ± 3	ND	ND	5,42	100%	100%
SUBTOTAL		**309 ± 2**	**409 ± 1**	**836 ± 7**		**-32%**	**-170%**
Trimethoprim	Trimethoprim	52 ± 11	48 ± 3	67 ± 4	4,51	8%	-29%
SUBTOTAL		**52 ± 11**	**48 ± 3**	**67 ± 4**		**8%**	**-29%**
Sulphonamides	Sulfamethoxazole	16 ± 4	9 ± 1	ND	4,29	47%	100%
	Sulfadimethoxine	15 ± 5	7 ± 1	ND	3,26	55%	100%
	Sulfapyridine	ND	890 ± 72	908 ± 54	4,51	*	*
SUBTOTAL		**31 ± 0**	**905 ± 2**	**908 ± 2**		**-2846%**	**-2856%**
TOTAL		**35927± 85**	**7119 ± 16**	**15992 ± 14**		**77%**	**49%**

Among them, quinolones showed the highest concentration: 15701 ng L^{-1} as sum of the 10 compounds detected from this group, being fluoroquinolones the most concentrated ones. High concentrations of fluoroquinolones were also found in sewage water of human hospitals [8, 381, 393]. Such high values can be related to their high consumption, as these compounds are frequently used in veterinary hospitals [394]. After quinolones, the most abundant group of antibiotics wereβ-lactams with an initial concentration of 10253 ng L^{-1}, followed by tetracyclines, metronidazoles, macrolides, trimethoprim and sulfonamides, whose values were 4807, 4774, 309, 52 and 31 ng L^{-1}, respectively.

Despite several antibiotic compounds belong to the same family, each of them behave differently under the same treatment processes [8, 395, 396] and it is thus difficult to highlight a common degradation trend. Positive removal in both bioreactors (inoculated and non-inoculated) could be observed for 17 out of 32 compounds detected in the wastewater samples. In the case of ciprofloxacin, enrofloxacin, marbofloxacin and ampicillin, the removal rates achieved with the fungal treatment (91%, 76%, 87% and 100% respectively) were significantly higher ($p < 0.05$) than those obtained at the control treatment (38%, 56%, 55% and 9% respectively) whereas in the case of enoxacin, pipemidic acid, doxycycline, cefazolin, cefalexin, chlorotetracycline, tylosin, spyramicin, ampicillin B and metronidazole, no significant differences ($p > 0.05$) in degradation efficiency were observed.

Negative removal values were observed for clarithromycin, erythromycin, azythromycin, danofloxacin, tilmicosin, nalixidic acid, cinofloxacin, norfloxacin, sulfapyridyne, penicillin V and metronidazole-OH in both treatments (fungal and control); i.e., the concentrations measured after the treatments were higher than those found in raw water. Negative removals can be attributed to some particular processes taking place during wastewater treatment; some drugs e.g. clarithromycin, tetracycline and ofloxacin are excreted conjugated with other chemical compounds [8, 397-400] but can be further deconjugated by some enzymes present in wastewater bioreactor reverting them to their original form [401]. This effect was also detected in other fungal bioreactors previously studied [204]. Particularly remarkable is the case of sulfapyridine, usually supplied in a conjugated form, named sulfasalazine, which is composed of sulfapyridine conjugated to 5-aminosalicylate [402], and that it is usually for veterinary use [403]. That would explain why sulfapyridine was not detected in raw

wastewater but in the treated water (likely after deconjugation processes). Another possible explanation of these negative removals is related to the excretion pathway: some compounds such as erythromycin, azythromycin, ofloxacin and trimethoprim are mainly excreted with bile and faeces, so they are partly attached to particulate matter [8, 404, 405]. The load entering the bioreactors is therefore underestimated, since it is calculated considering only the dissolved fraction, obviating the sorption of the antibiotics to the suspended solids [398].

In the case of ofloxacin, oxytetracycline, tetracycline and trimethoprim, negative removals were only observed in the control treatment but not in fungal treatment, which might be attributed to the prevalence of biodegradation processes in general, higher in fungal than in control bioreactor in contrast to deconjugation and desorption processes happening in both bioreactors.

Considering all the antibiotics measured, the removal rates obtained with fungal treatment were in general better than those achieved in the control bioreactor (Table 5.2). Overall 77% of antibiotics were removed by fungi after 15 days, whereas only 49% were eliminated in the non-inoculated bioreactor after same period of time. Remarkable differences were identified in the case of quinolones: 87% removal at the inoculated and 37% at the non-inoculated bioreactor. Macrolides also showed a big disparity between both treatments, -32% in the fungal bioreactor against -170% at the non-inoculated bioreactor. For the rest of antibiotic families, removal rates were either slightly better with fungal treatment or no considerable difference was found between both bioreactors.

The efficiency of the fungal treatment was compared with data from conventional WWTPs for 23 antibiotics, out of the 32 found in veterinary effluent, based on the available literature (Table 5.3). Among the 23 compounds referred, 13 exhibited lower removal rates with conventional WWTPs than with the fungal treatment tested. This is important in the case of some recalcitrant compounds such as enrofloxacin, marbofloxacin, oxytetracycline, ampicillin and cefalexin. The removal rates obtained for these compounds in conventional WWTPs do not exceed 40% (39%, -146%, 9%, 7% and 38%, respectively). Meanwhile with the fungal treatment the values obtained are notably better: 76%, 87%, 57%, 100% and 98%, respectively (Table 5.3).

Table 5.3 - Antibiotic removal percentages obtained with fungal treatment in the present study and with conventional WWTPs, based on available literature references.

Related gene	Antibiotic group	Antibiotic	Fungal Treatment	Literature	References
ermB	Macrolides	Erythromycin	-33%	12%	[8, 60, 365]
		Azythromycin	-34%	-45%	[8, 60, 395]
		Clarithromycin	-115%	2%	[8, 60, 395, 406]
qnrS	Quinolones	Ciprofloxacin	91%	79%	[8, 60, 365, 395]
		Enrofloxacin	76%	39%	[8, 395]
		Ofloxacin	46%	46%	[8, 60, 365, 395]
		Marbofloxacin	87%	-146%	[395]
		Norfloxacin	-55%	67%	[8, 60, 365, 395]
		Pipemidic Acid	37%	22%	[60]
sulI	Sulfonamides	Sulfamethoxazole	47%	76%	[8, 51, 58, 60, 365, 395, 406]
		Sulfadimethoxine	55%	63%	[58, 60, 406]
		Sulfapyridine	*	18%	[8, 58, 395]
tetw	Tetracyclines	Doxycycline	29%	58%	[8, 51, 406]
		Tetracyclin	26%	21%	[8, 51, 58, 365]
		Oxytetracyclin	57%	9%	[8, 51, 58]
		Chlorotetracycline	100%	84%	[8, 51, 58]
$bla_{TEM/SHV}$	β-Lactams	Cefazolin	100%	65%	[60]
		Ampicillin	100%	7%	[395, 406]
		Cefalexin	98%	38%	[8, 60]
		Penicillin V	*	60%	[8]
	Others	Metronidazole	17%	35%	[60, 395]
		Metronidazole OH*	-244%	42%	[60, 395]
		Trimethoprim	8%	-4%	[60, 395, 406]

* Appearance of the compound

Quantification of ARGs

Six ARGs, bla_{TEM}, bla_{SHV}, *ermB*, *qnrS*, *sul*I and *tetW*, and the 16S rRNA gene were quantified using qPCR assays in water samples. High R^2 (average 0.991) and efficiency values (from 85% to 108%) were obtained from the standard curves showing the linearity and the sensitivity of each qPCR assay (Table 5.4). The 16S rRNA gene was also analyzed to quantify the total bacterial load and to normalize the abundance of ARGs in the collected samples.

Table 5.4 - Quality parameters of each qPCR assay.

	16S rRNA	***ermB***	***qnrS***	***sul* I**	***tetW***	***bla*$_{TEM}$**	***bla*$_{SHV}$**
R^2	0,999	0,996	0,999	0,979	0,991	0,993	0,978
Efficiency	85%	96%	85%	93%	108%	91%	107%

Four trends were observed regarding the presence of ARGs before and after fungal treatment (Figure 5.1). The first one corresponds to complete disappearance of the *ermB* and *tetW* genes, both after fungal treatment and in the non-inoculated control bioreactor. This result is not very surprising as previous studies have also demonstrated a completeremoval of these resistance genes in conventional WWTPs [51, 60, 392].

A second trend is represented by the *bla*$_{TEM}$ gene, in which the fungal treatment resulted in a marked decrease in the copy number of this gene. Likewise, similar observations have been found for this gene after conventional wastewater treatment [60, 61]. In contrast, no significant difference ($p = 0.256$) was found between samples before and after experimental period in the non-inoculated control bioreactor.

The third pattern corresponds to the *bla*$_{SHV}$ and *sul*I genes, whose copy numbers increased in both bioreactors; however, the increase was significantly lower ($p < 0.05$) in the fungal bioreactor than in the control bioreactor. These trends observed do not match with those reported in conventional WWTPs. Regarding *bla*$_{SHV}$ gene, an approximately thousand-fold increase was measured meanwhile no clear trends were found in the literature about these gene: in some WWTPs a reduction has been measured while in others WWTPsno reduction was detectec [59]. In the case of the *sul* I gene an approximately ten-fold increase was observed; mean while a hundred-fold reduction was observed in conventional WWTPs for the *sul* I gene [60, 407].

Finally, the *qnrS* gene presents an increase in the copy number in both treatments; however, no statistically significant difference was found between both bioreactors ($p = 0.56$). Opposite trends were obtained in conventional WWTPs in which the *qnrS* gene decreases its copy number almost completely [60].

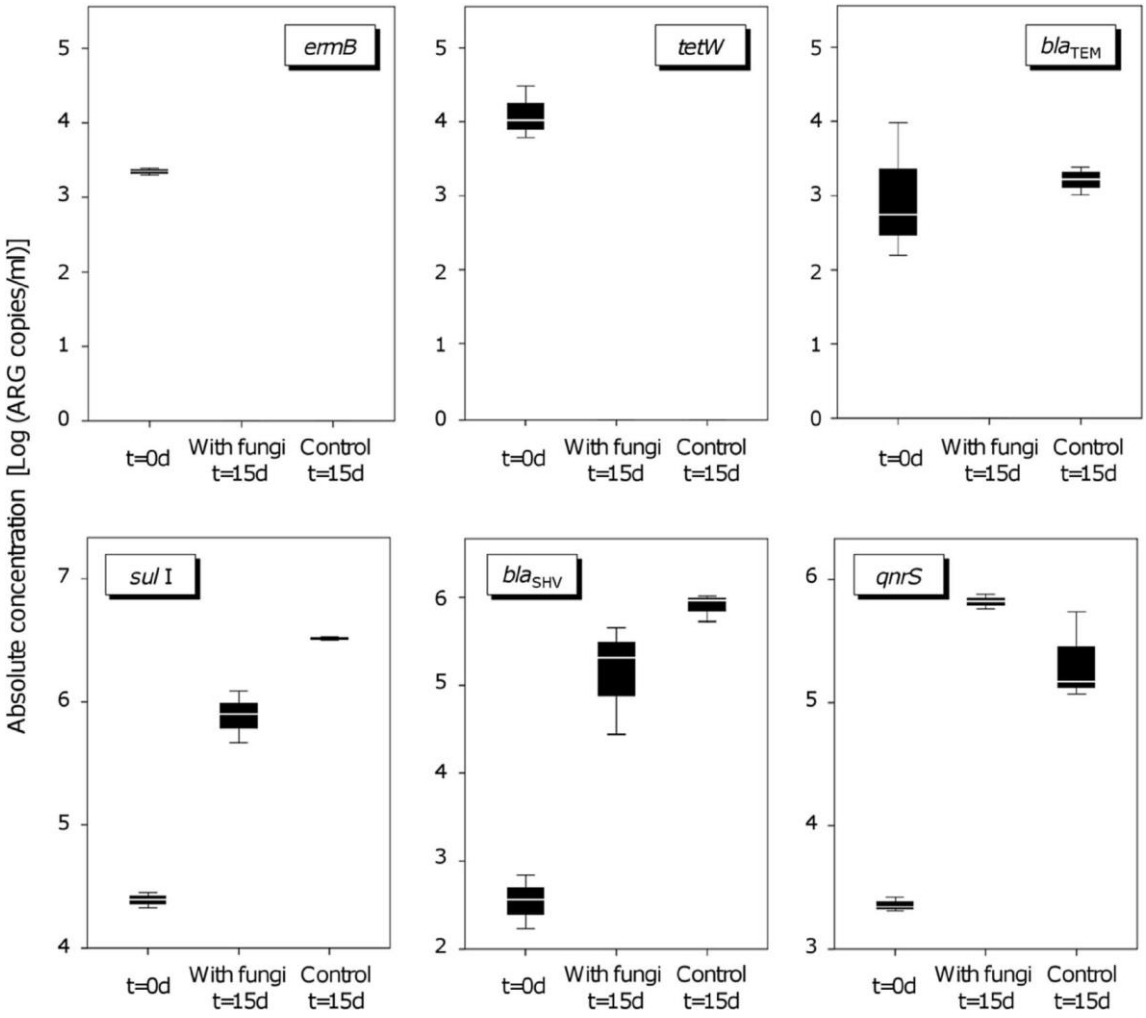

Figure 5.1 - Absolute concentration of ARGs in the different samples analyzed. Within the box plot chart the crosspieces of each box plot represent (from top to the bottom) maximum, upper-quartile, median (white bar), lower-quartile and minimum values.

The increase of the *sul*I, bla_{SHV} and *qnrS* genes observed in our studies could be related to the increase of total bacteria (measured by 16S rRNA gene copy number quantification). In fact, a ten-fold increase in the copy numbers of 16S rRNA genes was observed in both bioreactors, whereas data from conventional WWTPs showed ten- [129], hundred- [60], and even thousand-fold [51] reductions for these genes. The increase of bacterial population in our experiments can be attributed to the special conditions in which the bioreactors were set up to facilitate the growth of the fungus: nutrients addition, stable pH and temperature. It should also be noted that the increase of the 16S rRNA gene was slightly higher in the inoculated bioreactor, possibly due to the presence of the fungi, which produce extracellular degradative enzymes, e.g. laccase and peroxidases [191, 323]. These enzymes break complex compounds increasing the nutrients bioavailability for all microbial community members.

Therefore, according to the normalized data (Figure 5.2), the fungal treatment showed better relative removal rates for ARGs than those obtained from a conventional WWTP (Table 5.5).

Table 5.5 - Comparison of relative removal rates of the genes analyzed between this study and previous reports. *Conventional WWTP data was the mean value obtained from Lachmayr et al.(2009){Lachmayr, 2009 #61}; Gao et al. (2012){Gao, 2012 #51} and Rodriguez-Mozaz et al. (2015){Rodriguez-Mozaz, 2015 #60}.

Treatments	*ermB*	*qnrS*	*sul* I	*tetW*	bla_{TEM}	bla_{SHV}
Fungal treatment	100%	-163%	56%	100%	100%	-843%
Conventional WWTP *	82%	-302%	-58%	87%	-156%	33%

The fungal treatment resulted in a very good removal of the *sul*I and bla_{TEM} genes, reaching removal rates of 56 and 100%, respectively, as compared with data obtained from a conventional WWTP, in which the removal rates were negative, -156 and -58%, respectively [51, 60, 61]. The *qnrS* gene showed a large difference between the treatments, but with negative removal values in both of them; -163% for the fungal treatment and -302% for the conventional WWTPs [60]. The difference between treatments is not so remarkable with the *ermB* and *tetW* genes; however, better removal rates were obtained with the fungal treatment (100% for both genes) than at conventional WWTPs (82 and 87%, respectively) [51, 60]. Finally, the removal efficiency of the bla_{SHV} gene also showed a large difference between the fungal treatment (-843%) and the values obtained from conventional WWTPs (33%) [59]. In this case it must be highlighted the great difference in the absolute copy number of these gene at the end and at the beginning of the experiment. At the initial stages the absolute copy numbers were $1.5 \cdot 10^4$ in the fungal treatment and $1.2 \cdot 10^7$ in the conventional WWTP; at the end the absolute copy numbers were $1.4 \cdot 10^5$ in the fungal treatment and $8 \cdot 10^6$ in the conventional WWTP of each treatment showing that an important point for emoval evaluation is also always the initial concentration.

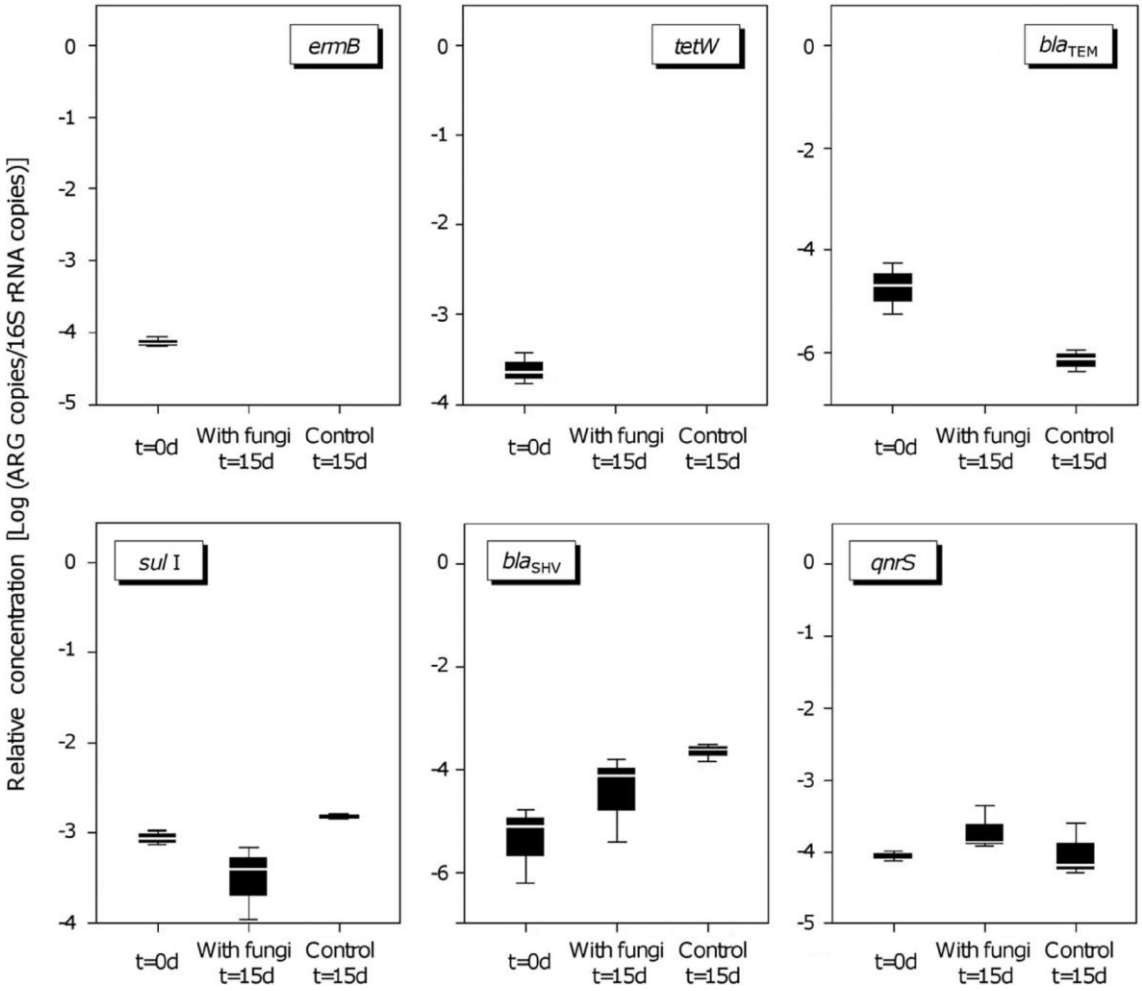

Figure 5.2 - Relative concentration of ARGs in the different samples analyzed. Within the box plot chart the crosspieces of each box plot represent (from top to the bottom) maximum, upper-quartile, median (white bar), lower-quartile and minimum values.

Relationship between antibiotics and ARGs

In view of the differences in behavior found for the ARGs and in order to reach a better understanding of the processes involved in the spread of antibiotic resistance, a possible correlation between antibiotics and ARGs has been studied. Unfortunately, the number of samples did not allow carrying out a statistical correlation analysis.Nevertheless with experimental data and those obtainedfrom the literature, some ideas could be highlighted regarding each ARG

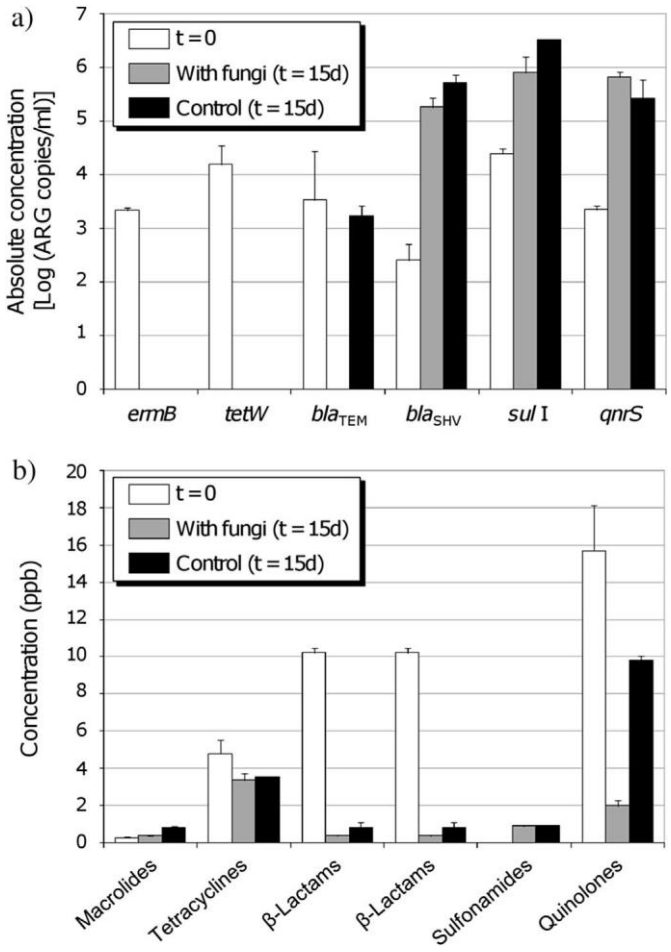

Figure 5.3 - Absolute concentration of a) ARGs and b) antibiotics grouped into families.

The first association was found between the *ermB* gene and macrolides (Figure 5.3). Whilst the gene disappeared completely in both bioreactors, macrolides experienced a slight increase in their concentration. This observation, contrary to the classical knowledge [36, 408], is in agreement with the findings of a recent study [60], where the concentration of the *ermB* gene decreased by 3 orders of magnitude in the presence of even higher concentrations of macrolides than those measured in our bioreactor.

The *tetW* gene disappeared totally in both bioreactors, even though tetracycline antibiotics were hardly removed along the treatment (29% and 26% removal in the fungal and the control bioreactors, respectively). The concentration of this gene has also been reported to decrease by three or four orders of magnitude in presence of small amounts of tetracycline antibiotics, although higher than those detected in the bioreactors in this work [51, 58, 409].

The concentration of β-lactam antibiotics in raw wastewater was quite high (c.a. 10 mg L^{-1}) although removal in both bioreactors was very efficient, reaching values close to zero. Nevertheless, levels of *bla*$_{TEM}$ and *bla*$_{SHV}$ along the treatment were quite different. In the fungal bioreactor, the *bla*$_{TEM}$ gene disappeared (100% removal), wich is in agreement with previous studies [60], whereas ARG concentration in control bioreactor did not undergo noteworthy change after treatment. In contrast, the *bla*$_{SHV}$ gene increased in both bioreactors almost to the same extent, in agreement with the assumption that ARGs increase is favored by the presence of selective agents, such as antibiotics [36, 408]. The hypothesis here is that despite the decrease in the concentration of β-lactams in both bioreactors, the concentration was high enough to exert a selective pressure; however further studies are required to understand the relationship between the evolution of the *bla*$_{TEM}$ and *bla*$_{SHV}$ genes and the concentration of β-lactam antibiotics, including the exposure to sub-therapeutic concentrations.

The concentration of sulfonamides and the *sul*I gene increased in both bioreactors, whereas in another study in a urban WWTP [60] both antibiotics and the gene decreased their concentrations. These positive correlations between the gene and antibiotics are in line with the classical knowledge about the emergence of antibiotic resistance [36, 408].

The relationship between the *qnrS* gene and quinolones showed a similar trend to that found between the *bla*$_{SHV}$ gene and β-lactams. An increase of the *qnrS* genes was observed, whereas the antibiotic decreased. Quinolones are the most abundant group in wastewater and therefore, despite their depletion, they may exert enough selective pressure to increase the gene concentration. Some studies have also suggested that *qnr* genes may have other functions (e.g. regulation of cellular DNA-binding proteins) in addition to the antibiotic resistance that contribute to its spread [410].

4. Conclusions

In this study, antibiotics and ARGs from the effluent of a veterinary hospital were measured in order to analyze the efficiency of an alternative wastewater treatment. Based on removal rates of both antibiotics and ARGs, fungal treatment emerges as an interesting technology; however, an optimization or combination with other methods is needed in order to reduce the amount of associated microbiota. This treatment offers good results in terms of elimination of certain compounds such as ciprofloxacin,

enrofloxacin, marbofloxacin and ampicillin, which are recalcitrant in conventional WWTPs.

It has also been observed that the presence of antibiotic compounds is not the only factor influencing the fate of antibiotic resistance. Therefore, other factors should also be taken into account such as the operational parameters of bioreactors, the wastewater-associated bacterial communities and their interaction with fungi, which may contribute to the spread of resistance genes associated with certain families of microorganisms.

Chapter 6

Chapter 6 – Sorption processes

Based on the publication:
The role of sorption processes in the removal of pharmaceuticals by fungal treatment of wastewater
D. Lucas, F. Castellet-Rovira, M. Villagrasa, M. Badia-Fabregat, D. Barceló, T. Vicent, G. Caminal, M. Sarra and S. Rodríguez-Mozaz.
Science of the Total Environment (2018) 610-611: pp. 1147-1153
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Abstract

The contribution of the sorption processes in the elimination of pharmaceuticals (PhACs) during the fungal treatment of wastewater has been evaluated in this work. The sorption of four PhACs (carbamazepine, diclofenac, iopromide and venlafaxine) by 6 different fungi was first evaluated in batch experiments. Concentrations of PhACs in both liquid and solid (biomass) matrices from the fungal treatment were measured. Contribution of the sorption to the total removal of pollutants ranged between 3% and 13% in relation to the initial amount. The sorption of 47 PhACs in fungi was also evaluated in a fungal treatment performed in 26 days in a continuous bioreactor treating wastewater from a veterinary hospital. PhACs levels measured in the fungal biomass were similar to those detected in conventional wastewater treatment (WWTP) sludge. This may suggest the necessity of manage fungal biomass as waste in the same manner that the WWTP sludge is managed.

Keywords

Wastewater treatment, *Trametes versicolor*, emerging contaminants, water quality, chromatography, sludge

1. Introduction

Many studies have been performed focusing on the fungal treatment of PhACs [195, 204, 257, 327, 374, 390, 413, 414] showing quite good PhACs removal values specially when using the white rot fungi *Trametes versicolor*. However, there are still many questions to be answered regarding the fungal treatment, e.g., the role of the sorption processes in contrast to biodegradation processes in the elimination of the pollutants. Sorption in this article gathers both absorption (entry of pollutants inside the biomass) and adsorption (adhesion of pollutants to the biomass surface). Sorption processes in fungal treatment have been studied for contaminants such as textile dyes [289, 338, 415], personal care products [416] and some specific PhACs [263, 326, 327]. However, in these studies, sorption evaluation was done indirectly, i.e., setting up a control flask with thermal inactivated fungus, and measuring the pollutants concentration in the water at the beginning and at the end of the experiment; the difference in concentration is then attributed to sorption processes assuming that biodegradation processes are not taking place. In contrast, any study in the literature so far has investigated the sorption of organic micropollutants in fungal biomass by means of a direct measurement of these compounds in the solid phase.

The main objective of this chapter is to evaluate the role of the sorption processes in the removal of PhACs during fungal treatment. Direct measurement of contaminants in fungal biomass were performed considering two different types of experiments; namely batch experiments with different fungi performed with spiked synthetic water and experiments in a continuous bioreactor with the fungus *T. versicolor* for the treatment of VHW.

2. Materials and methods

Batch experiments with fungi and spiked synthetic medium

Concerning the first experiment 6 fungi where selected to evaluate their PhACs biodegradation capacities in order to find a proper candidate to be used in further experiments at a bigger scale. The 6 fungi where selected following different criteria; *T. versicolor* as the most studied fungus in terms of PhACs elimination in wastewater treatments [187, 211, 257]; *G. lucidum* and *I. lacteus* were selected due to previous studies in which the capacity to remove certain PhACs was proved [263, 438, 439]. Finally *S. rugosoannulata*, *G. luteofolius* and *A. erebia* were selected due to their ecophysiological resemblance with *T. versicolor* and previous degradation studies of them eliminating industrial dyes, explosives and pesticides respectively [440-442].

The experiments were performed in 250 mL Erlenmeyer flasks, where an amount of mycelial pellets (a mean of 0.5 ± 0.1 g in dry weight) was added in 100 mL of defined medium, consisting of 8 g L^{-1} of glucose, 3.3 g L^{-1} of ammonium tartrate, 1.168 g L^{-1} of 2,2-dimethylsuccinate buffer and 1 and 10 mL of a micro and macronutrient solution from Kirk medium [407]. The inoculums were always the same mass of pellets but measured as wet weight and later translate to dry weight by the ratio wet weight/dry weight measured in each set of experiments and each fungus. The medium was prepared in ultrapure water, so initial COD was related to the glucose concentration, this is about 8500 mg O_2 L^{-1}, but this is mainly reduced up to the end of the experiment because the glucose was uptaked by fungi and so no glucose was detected at day 6. Four selected PhACs were added to the defined medium reaching a final individual concentration between 47 and 184 μg L^{-1}. The water was spiked at slightly higher concentrations than those commonly found in wastewater treatment plants. However, concentrations were low enough to avoid any possible toxic effect of the PhACs on the fungi [408]. The pollutants present in the stock solution included diclofenac (anti-inflammatory), carbamazepine (anti-convulsant), venlafaxine (antidepressant) and iopromide (media contrast agent). These PhACs were selected based on its ubiquity in hospital wastewater effluents and the low biodegradability exhibited in previous fungal treatments performed with *T. versicolor* [257, 409]. After fungus inoculation and PhACs addition,

flasks were incubated under orbital shaking (135 rpm) at 25 °C for 6 days in dark conditions to prevent a possible photodegradation on compounds.

Apart from the experimental treatment (*Exp*) above described, heat-killed (*KC*) control, and abiotic control were performed. Heat-killed controls consisted on autoclaved cultures, which were set-up under identical conditions to those of the *Exp* cultures. These experiments allowed us to evaluate sorption differences between *Exp* and *KC* samples. Abiotic control cultures were performed in the same manner as the *Exp* culture but without fungus. These experiments were intended to account the degradation of the contaminants due to physico-chemical processes. Each treatment was done in triplicate for each fungus.

Water samples and fungal biomass samples were taken, after 15 min and 6 days of PhACs addition in the cultures. Fungal biodegradation is a quite slow process, so 15 min are considered enough time to ensure homogeneity in the concentration of the flask and too short to detect a significant adsorption process. On the contrary, according our experience, 5 days is the time that takes to uptake the added glucose and to decrease the degradation rate. So we decided to take the sample after 6 days of treatment to ensure the accomplishment of both objectives: the glusoce uptake and to measure degradation yield. Biomass samples were taken at the end of the experiment since in the original experiment In both cases, the whole content of the flasks was sacrificed at each sampling time. All samples were filtered through 1 μm fiberglass in order to separate the fungal biomass from water fraction. Liquid samples were kept frozen at -20 ºC whereas solid samples were first freeze dried and then kept frozen until their analysis.

Continuous bioreactor with *T. versicolor* and VHW

Two 1.5 L air-pulsed glass bioreactors were set up in parallel, one inoculated with pellets of *T. versicolor* and the other non-inoculated as a control, for 26 days. In the inoculated bioreactor, fungal pellets were maintained fluidized through the air pulses. The bioreactors were filled with 1.5 L of VHW, temperature was set up at 25 °C, and pH kept constant at 4.5 ± 0.5 by adding HCl 1 M or NaOH 1 M. Fungal pellets were kept inside the bioreactor by means of a mesh. *T. versicolor* was initially added at 3.7 g DCW L^{-1}. However, every 2–5 days, approximately 1/3 of the biomass was replaced by fresh one as determined by Blánquez et al. (2006) [345]. HRT was stablished at 3.3

days. Glucose and ammonia tartrate were added at rates of 343– 1040 and 0.77–433 mg g DCW^{-1} day^{-1}, respectively, according to the nutritional requirements [195], and in pulses of 0.6 min h^{-1} from a concentrated stock. VHW in the feed storage tank was replaced every 2–3 days by fresh one stored at 4 °C[195].

Aproximately 150 ml liquid samples were taken from the effluent on days 0, 5,10, 12, 14, 15, 17, 18, 21, and 26 and kept frozen at -20 ºC until their analysis. Finally, both fungal biomass from the inoculated bioreactor (inoculated biomass) and biomass from the non-inoculated control bioreactor (control biomass), were collected through a filtration system at the end of the experiment (26 days) and then were frozen and kept at −20 °C until analysis. Samples were taken at the end of the experiment because no stationary state was achieved because different nutrient additions were being tested during the process to determine optimal conditions for fungal survival. It was at the end of the experiment when the conditions were fixed and no removal of biomass was done; therefore it was the period that can be considered as stationary. [195].

Biomass samples

For the analysis of micropollutants in the fungal biomass samples, an extraction methodology was previously adapted and implemented. Three extraction methodologies, initially developed for the analysis of several pollutants (biocides and PhACs) in solid samples, were selected from the literature to be tested [346-348]. A fourth methodology was also tested following the scheme of Capone et al. (1996) [346]; but using a different extraction solvent (MeOH-Na_2EDTA). Methods description is summarized in table 6.1.

Table 6.1 - Extraction methods tested for the determination of PhACs in fungal biomass

Target compounds	Matrix	Biomass used	Extraction method	Solvents	Clean up	Reference
PhACs	Sludge	0.2 g	Accelerated solvent extraction	Water : Methanol (2:1 v/v)	Solid phase extraction	Jelić et al., 2009
Pentachlorophenol	Soil	10 g	Agitation and ultrasonic bath	Hexane : Acetone (1:1 v/v)	-	Rubilar et al., 2011
Antibiotics	Marine sediments and animal tissue	1 g	Ultrasonic bath	Acetone	Filtration	Capone et al., 1996
PhACs	Fungal biomass	0.25 g	Ultrasonic bath	Methanol : Na_2EDTA (50:1.5 v/v)	Filtration	-

The latest extraction methodology exhibited the best recovery values for the selected PhACs (Figure 6.1) and was thus used in the present work for the analysis of micropollutants in biomass samples.

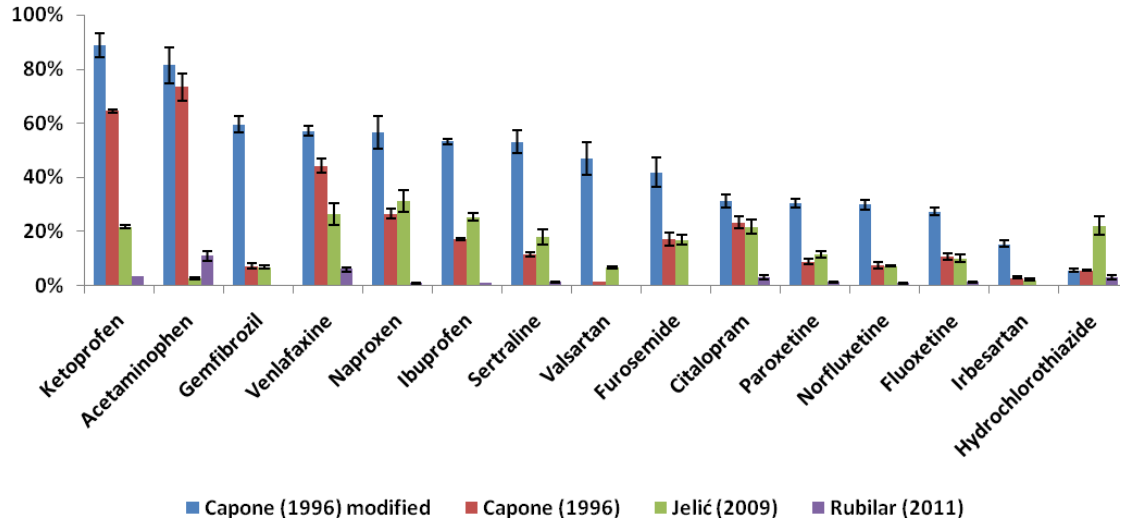

Figure 6.1 - Recovery values obtained for a selected set of PhACs by applying the 4 extraction methodologies tested

In Capone et al. (1996) [346] adapted methodology, the biomass was freeze dried and then homogenized using a Robot Coupe Blixer food processor (Robot Coupe, Jackson, MS, USA) and a mortar. For each sample, 1 g of biomass was used; then 4 mL of MeOH-Na_2EDTA (50:1.5 v/v) was added and vortexed for 30 s. Later on, samples were sonicated for 3 min and centrifuged at 1500 rpm for 5 min at 5 °C. The supernatant was stored while the procedure was repeated twice more with the pellet, using 3 mL of

MeOH-Na_2EDTA each time. The total resulting supernatant was centrifuged at 3200 rpm for 20 min, decanted, filtered with nylon membrane filters, evaporated under nitrogen stream using a Reacti-Therm 18,824 system (Thermo Scientific) and reconstituted with 1 mL of methanol-water (10:90 v/v). Lastly, 10 μL of internal standard mix at 10 ng μL^{-1} were added in the extracts for internal standard calibration. Na_2EDTA were added since it has been observed that it considerably improves extraction efficiency of antibiotics as well as other PhACs [349, 350]. This is attributed to the fact that these compounds can potentially bind residual metals present in the sample matrix and glassware, resulting in low extraction recoveries. By adding Na_2EDTA, soluble metals are bound to the chelating agent, increasing the extraction efficiency of PhACs [341].

Sorption and biodegradation calculation

In order to determine the role of biodegradation and sorption processes in the elimination of PhACs in the batch experiments with fungi, the following calculations were performed: Total elimination for each PhAC was first calculated as:

$$E = (C_{wi} - C_{wf}) \cdot V$$

where E is total elimination (ng), C_{wi} and C_{wf} the concentration (ng mL^{-1}) of each PhAC measured in the water samples at the beginning and at the end of the experiment respectively, and V is the volume of the water in each flask (mL).

PhACs sorbed for each compound can be expressed as:

$$A = C_{bf} \cdot B_f$$

where A is the amount of PhAC sorbed (ng), C_{bf} is the concentration (ng g^{-1}) of each PhAC measured in the biomass at the end of the experiment, and B_f the amount of biomass (g) at the end of the experiment.

Finally, the biodegradation for each PhAC were calculated as follows

$$Bd = E - A$$

where Bd is the quantity of PhAC (ng) eliminated by biodegradation, E is the total amount of PhAC eliminated (ng), and A is the quantity of PhAC (ng) eliminated by sorption processes.

Solid–water partition coefficients (K_d), [359] which defines the distribution of a compound between water and solid phase, were calculated from PhACs concentrations in water and biomass samples obtained at the end of the experiment in the continuous bioreactor. K_d takes into account both absorption and adsorption and is used to evaluate the overall sorption in solids exposed to different concentration of pollutants in the liquid phase. It was used with the biomass of the continuous bioreactor at the end of the experiment in order to have a picture of the sorption of contaminants at the end of the experiment;

$$K_d = \frac{C_{sorbed}}{C_{soluble}}$$

where C_{sorbed} is the sorbed PhACs concentration onto biomass (μg Kg^{-1}) and $C_{soluble}$ the dissolved concentration of the compound (μg L^{-1}).

3. Results and discussion

Batch experiments with fungi and spiked synthetic medium

In general terms, results obtained from the abiotic cultures reveal no PhACs degradation attributed to physico-chemical processes such as volatilization, thermal degradation and sorption in the bioreactor [343]. Therefore, PhACs elimination observed in Exp cultures can only be associated to biodegradation and/or sorption processes. The role of sorption was thus calculated based on the results obtained from the measurements of PhACs both, in liquid and fungal phases after 6 days of treatment (Table 6.2).

Table 6.2 - Concentrations measured in both water and biomass samples from experimental and killed control cultures of the batch experiments performed with spiked ultrapure water.

	***Trametes versicolor* (m= 0.52 g DCW)**									
	Concentration					Standard deviation				
	t= 0	t= 6 days				t= 0	t= 6 days			
		Experimental		Killed control			Experimental		Killed control	
PhAC	Water (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})
Carbamazepine	47670	20260	3074	43856	3148	3000	3690	599	1907	309
Diclofenac	55000	2443	17371	32500	18270	12700	5650	619	4072	446
Iopromide	174400	93130	881	160971	1025	2100	1847	370	8720	1745
Venlafaxine	53570	24214	904	50356	965	2740	7443	314	3214	136

	***Ganoderma lucidum* (m= 0.44 g DCW)**									
	Concentration					Standard deviation				
	t= 0	t= 6 days				t= 0	t= 6 days			
		Experimental		Killed control			Experimental		Killed control	
PhAC	Water (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})
Carbamazepine	54800	35072	2945	47676	2912	6500	6028	978	3836	622
Diclofenac	61000	1100	24418	40000	30270	1600	256	28	1280	140
Iopromide	51100	35106	705	41902	580	2250	1073	215	1380	276
Venlafaxine	50500	35350	642	45097	476	800	4545	192	4545	192

	***Irpex lacteus* (m= 0.3 g DCW)**									
	Concentration					Standard deviation				
	t= 0	t= 6 days				t= 0	t= 6 days			
		Experimental		Killed control			Experimental		Killed control	
PhAC	Water (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})
Carbamazepine	83400	31692	3112	79230	7164	8400	1668	271	2502	406
Diclofenac	61000	1960	19135	45000	31784	2700	592	65	0	0
Iopromide	105000	76650	1603	99750	3214	8160	8400	1681	4200	840
Venlafaxine	104000	88400	1507	97600	3771	11000	0	0	0	0

	***Stropharia rugosoannulata* (m= 0.5 g DCW)**									
	Concentration					Standard deviation				
	t= 0	t= 6 days				t= 0	t= 6 days			
		Experimental		Killed control			Experimental		Killed control	
PhAC	Water (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})
Carbamazepine	123000	17220	2951	111930	2284	5000	3780	613	7380	1197
Diclofenac	113000	24182	11586	51980	508	3600	5824	598	6780	742
Iopromide	184700	11082	988	140372	428	9100	2001	385	20317	4065
Venlafaxine	82700	72776	810	74430	155	2700	7526	325	7443	314

	***Gymnopilus luteofolius* (m= 0.4 g DCW)**									
	Concentration					Standard deviation				
	t= 0	t= 6 days				t= 0	t= 6 days			
		Experimental		Killed control			Experimental		Killed control	
PhAC	Water (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})
Carbamazepine	71000	31950	1848	63900	6518	11000	8520	1382	7100	1152
Diclofenac	106000	25800	18162	47400	49946	10000	600	66	600	66
Iopromide	82000	3280	257	79540	1827	20000	5740	1149	2460	492
Venlafaxine	102000	90100	184	90100	1840	16000	13780	582	11660	492

	***Agrocybe erebia* (m= 0.38 g DCW)**									
	Concentration					Standard deviation				
	t= 0	t= 6 days				t= 0	t= 6 days			
		Experimental		Killed control			Experimental		Killed control	
PhAC	Water (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})	Water (ng L^{-1})	Biomass (ng L^{-1})
Carbamazepine	114000	62700	3292	106020	5232	2000	11400	1849	6840	1109
Diclofenac	104000	24000	34782	27000	75676	9100	728	80	624	68
Iopromide	159000	71550	568	139920	531	5000	17490	3500	9540	1909
Venlafaxine	106000	95400	702	103880	940	9000	10600	447	2120	89

The sorption contribution in the PhACs elimination ranged from 3% to 13% (Figure 6.2).

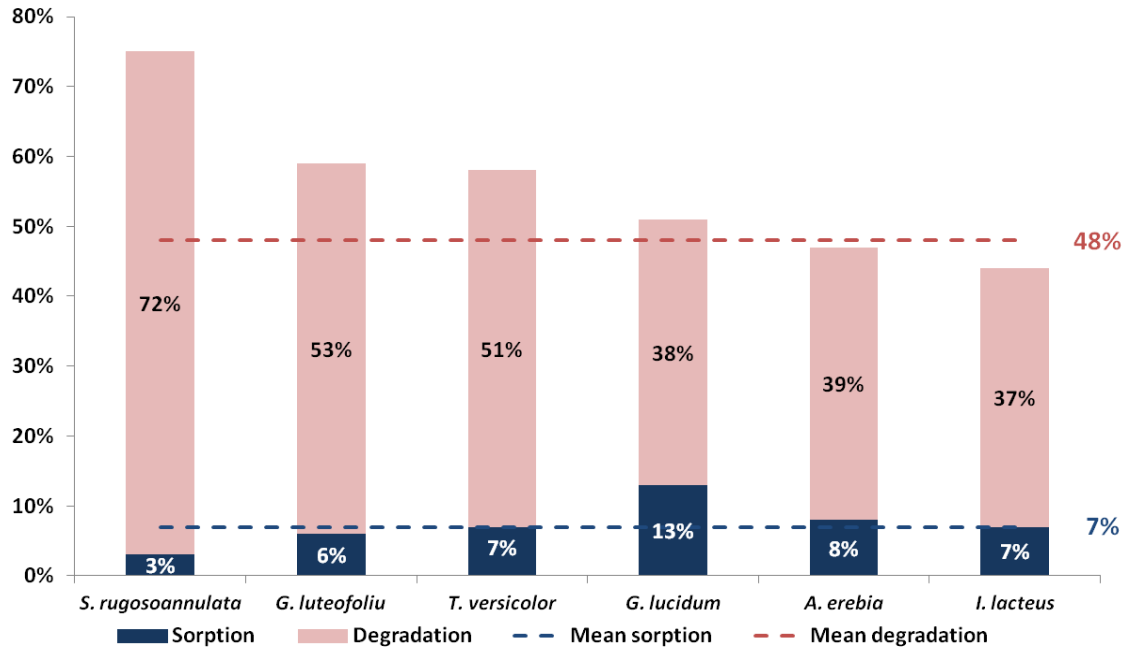

Figure 6.2 - Contribution of sorption and biodegradation (%) to the total removal PhACs for each fungus from *Exp*culture studied in the batch experiments with fungi and spiked synthetic medium. Data obtained from direct measurement method.

The contribution of the sorption processes to overall removal was different depending on the fungus considered; many factors are actually involved in the sorption process and specific interactions between PhACs and the surface components of each fungus can occur. The fungi can be ordered from most to least efficient as follows: *S.rugosoannulata*, *G.luteofolius*, *T. versicolor*, *G.lucidum*, *A.erebia* and *I.lacteus*. *S.rugosoannulata* was the most effective fungus in terms of elimination of the 4 selected PhACs (75%), but also in terms of biodegradation; 72% biodegradation + 3% sorption (Figure 6.1). On the contrary, *I.lacteus* exhibited the lowest elimination value, 44% (37% biodegradation + 7% sorption). Comparing biodegradation and sorption values it can be highlighted that, in the experiments with *S.rugosoannulata* the sorption processes has less relevance to the total elimination observed; only 4% of total elimination can be attributed to sorption processes while 96% of the elimination is due to biodegradation. In contrast; the relevance of the sorption processes achieves the highest values in the case of *G.lucidum*. For this fungus, 26% of total elimination can be

attributed to sorption processes and 74% can be attributed to biodegradation (Figure 6.3).

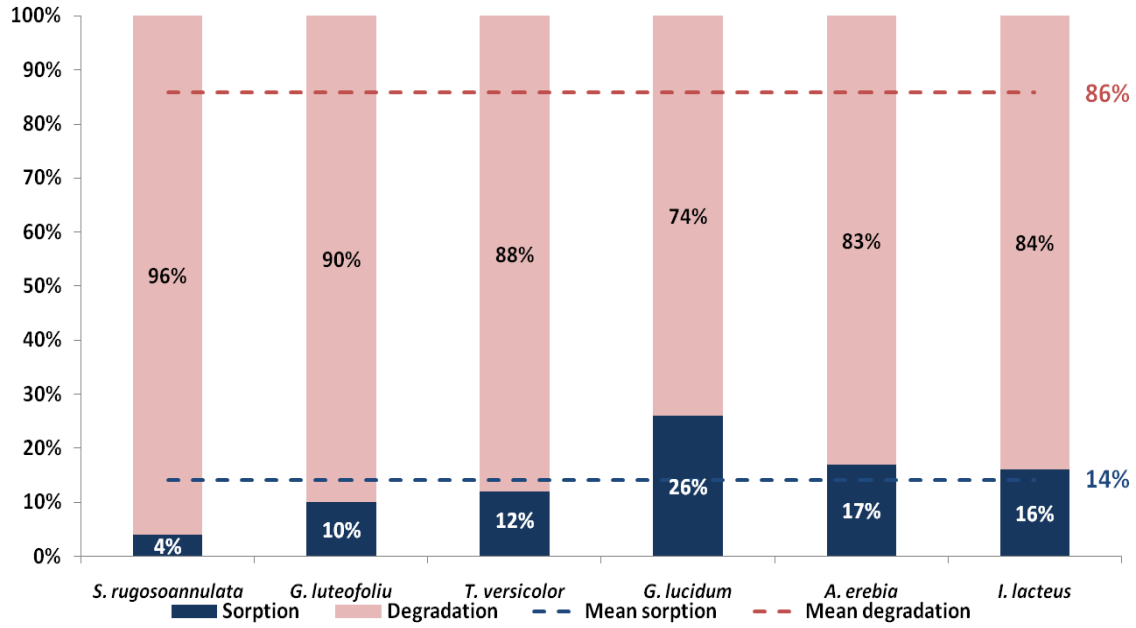

Figure 6.3 - Relative contribution of sorption and biodegradation (%) to the total removal PhACs for each fungus from *Exp*culture studied in the batch experiments with fungi and spiked synthetic medium. Data obtained from direct measurement method.

Behaviour of each compound in the six different fungal experiments was also evaluated (Figures 6.4 and 6.5). Diclofenac was the compound with the highest sorption in the fungal biomass. Up to 9153.2 ng g^{-1} (33% of initial amount) of this compound were retained in *A.erebia* biomass, being the average retained concentration of diclofenac, considering all fungi, 5213.2 ng g^{-1} (25% of initial amount). These high diclofenac sorption values are in agreement with those exhibited in previous fungal [326] and conventional activated sludge (CAS) treatments [179]. The great sorption of diclofenac in the present experiment might be due to its high partition coefficient (log Pat pH 4.5 = 3.6) indicating its hydrophobic character [417]. In contrast, iopromide and venlafaxine present the lowest concentrations in the fungal biomass (212.5 and 202.5 ng g^{-1}), c.a. 1% of the initial amount for each compound (Figure 6.4), which is in agreement with the low sorption shown by these compounds in sludge, reported in studies performed with CAS [170, 418]. The high hydrophilicity of iopromide (log P at pH 4.5 = −0.44) and venlafaxine (log P at pH 4.5 = −0.69) may explain the low concentrations found for these compounds in the fungal biomass.

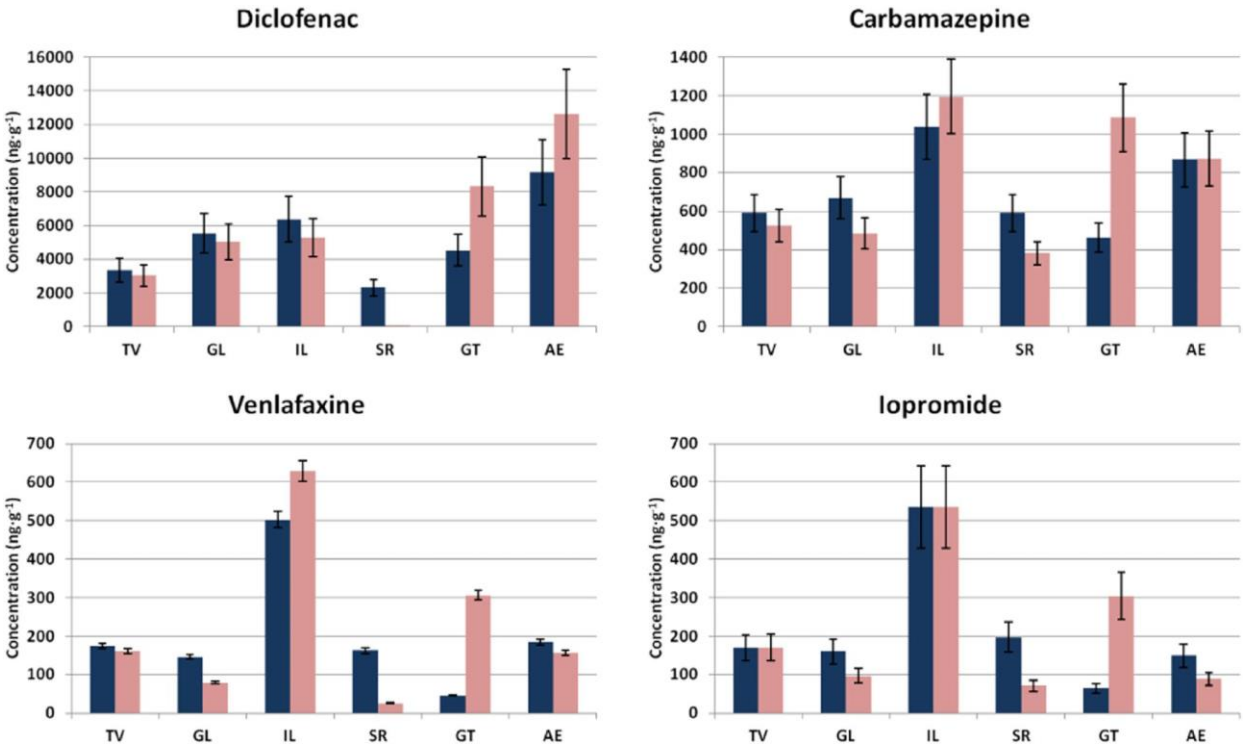

Figure 6.4 - Concentrations (ng g^{-1}) of each pollutant in the corresponding fungi at the end of the batch experiments performed with ultrapure spiked water; experimental (blue) and killed control (pink) cultures. TV: *Trametes versicolor*; GL: *Ganoderma lucidum*; IL: *Irpex lacteus*; SR: *Stropharia rugosoannulata*; GT: *Gymnopilus luteofolius*; AE: *Agrocybe erebia*. Error bars correspond to deviation of analytic results of the concentrations measured.

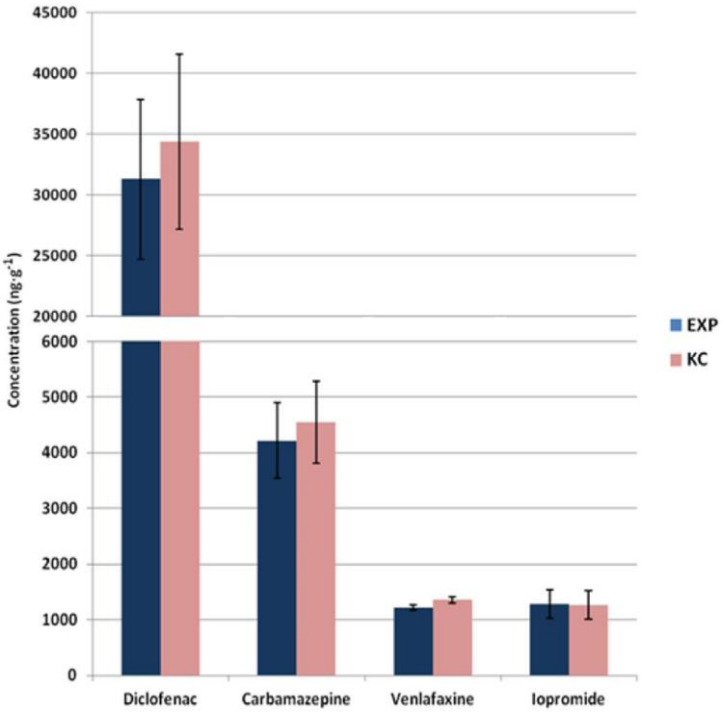

Figure 6.5 - Total concentration of each PhAC detected in the fungal biomass at the end of the batch experiments: *Exp* culture and *KC* culture, both performed with spiked synthetic medium. Error bars correspond to deviation of analytic results of the concentrations measured.

Sorption values obtained in the *Exp* treatments were compared with those sorption values of *KC* treatments, and the feasibility of performing *KC* experiments to indirectly calculate the sorption contribution in the total removal of PhACs was also evaluated. *KC* treatments are very common as control treatment in biodegradation experiments; they are frequently used to evaluate the contribution of the sorption processes in the elimination of the pollutants. Sorption of the active biomass is thus considered to be the same as the sorption of the inactive (killed control) biomass. Therefore, the difference in elimination in both treatments is attributed to biodegradation processes. However this approach might sometimes lead to some inaccuracies since the structure of biomass, and therefore their sorption capacities, may change according to the inactivation mechanism. It is indeed well known that, e.g., some fungal cells exposed to heat treatments alter the physico-chemical properties of their surfaces leading to a greater, equivalent or less bioadsorptive capacities than that of living cells depending on the pollutant [415, 419]. In addition, active transport in living cells which may play an important role in the sorption processes, is inactive in the killed control cultures. The biodegradation values obtained measuring both types of matrices (liquid and solid) from the *Exp* culture (direct method), were compared with the biodegradation values obtained from the *KC* culture (indirect method) [343]. For most of the compounds, degradation percentages calculated using both strategies were similar (Table 6.3). However, for some compounds the differences were quite remarkable, like in the case of the degradation of diclofenac by *S. rugosoannulate* (68% degradation calculated through the direct method and 25% with the indirect method), or the degradation of venlafaxine by *G.luteofolius* (15% of degradation calculated with the direct method and no degradation measured with the indirect method). These differences detected can be attributed, on one hand, to the different sorption values between the active and the inactive biomass. On the other hand, biodegradation processes of absorbed compounds can occur in the active biomass due to intracellular enzymes, whereas no degradation occurs in inactivated biomass, thus affecting the distribution of the contaminants in the two compartments.

Table 6.3 - Average removal, biodegradation and sorption values plus minus standard deviation calculated directly (with PhACs concentration in both liquid and biomass) and indirectly (subtracting the degradation value measured in the liquid from the killed control culture from the degradation measured in the liquid from the experimental culture [343] from the batch experiments with fungi and spiked synthetic medium)

Total removal

	T. versicolor	*G. lucidum*	*I. lacteus*	*S. rugosoannulata*	*G. luteofolius*	*A. erebia*
Carbamazepine	58 ± 8%	36 ± 7%	62 ± 6%	86 ± 7%	55 ± 12%	45 ± 10%
Diclofenac	96 ± 24%	98 ± 15%	97 ± 14%	79 ± 7%	76 ± 13%	77 ± 20%
Iopromide	47 ± 7%	31 ± 7%	27 ± 12%	94 ± 8%	96 ± 25%	55 ± 20%
Venlafaxine	55 ± 8%	30 ± 5%	15 ± 4%	12 ± 8%	15 ± 3%	10 ± 3%

Biodegradation

	T. versicolor		*G. lucidum*		*I. lacteus*		*S. rugosoannulata*		*G. luteofolius*		*A.erebia*	
	Direct method	Indirect method	Direct method	Indirect method	Direct method	Indirect method	Direct method	Indirect method	Direct method	Indirect method	Direct method	Indirect method
Carbamazepine	51 ± 7%	50 ± 7%	31 ± 6%	23 ± 2%	58 ± 5%	57 ± 7%	84 ± 7%	77 ± 12%	52 ± 11%	45 ± 10%	42 ± 9%	38 ± 3%
Diclofenac	64 ± 16%	54 ± 15%	58 ± 8%	64 ± 6%	65 ± 6%	71 ± 9%	68 ± 6%	25 ± 5%	59 ± 9%	20 ± 2%	43 ± 8%	3 ± 0%
Iopromide	46 ± 7%	39 ± 3%	30 ± 7%	13 ± 1%	25 ± 11%	22 ± 3%	93 ± 8%	70 ± 13%	96 ± 25%	93 ± 36%	55 ± 20%	43 ± 4%
Venlafaxine	53 ± 8%	49 ± 9%	29 ± 5%	19 ± 1%	14 ± 3%	9 ± 1%	11 ± 7%	2 ± 0%	15 ± 3%	0 ± 0%	9 ± 3%	8% ± 1%

Sorption

	T. versicolor		*G. lucidum*		*I. lacteus*		*S. rugosoannulata*		*G. luteofolius*		*A.erebia*	
	Direct method	Indirect method	Direct method	Indirect method	Direct method	Indirect method	Direct method	Indirect method	Direct method	Indirect method	Direct method	Indirect method
Carbamazepine	6 ± 1%	8 ± 1%	5 ± 1%	13 ± 1%	4 ± 0%	5 ± 1%	2 ± 0%	9 ± 1%	3 ± 1%	10 ± 2%	3 ± 1%	7 ± 1%
Diclofenac	32 ± 8%	42 ± 12%	40 ± 6%	34 ± 3%	31 ± 4%	26 ± 3%	10 ± 1%	54 ± 11%	17 ± 3%	56 ± 6%	33 ± 9%	74 ± 10%
Iopromide	1 ± 0%	8 ± 1%	1 ± 0%	18 ± 1%	2 ± 1%	5 ± 1%	1 ± 0%	24 ± 4%	0 ± 0%	3 ± 1%	0 ± 0%	12 ± 1%
Venlafaxine	2 ± 0%	6 ± 1%	1 ± 0%	11 ± 1%	1 ± 0%	6 ± 1%	1 ± 0%	10 ± 1%	0 ± 0%	15 ± 3%	1 ± 0%	2% ± 0%

Continuous bioreactor with *T. versicolor* and VHW

28 out of 47 compounds analyzed, were detected in the biomass sampled at the end of the experiment performed in the continuous bioreactor (Table 6.4).

Table 6.4 - PhACs concentrations measured in the fungal and control biomass at the end of the experiment with the continuous bioreactor treating wastewater from veterinary hospital and PhACs concentrations in sludge from CAS reported in the literature

	Inoculated biomass (ng g^{-1}±SD)	**Control biomass (ng g^{-1}±SD)**	**WWTP sludge Mean value (ng g^{-1})**	**References**
Acridone	0.7±0.1	1.2± 0.2		
Atenolol	0.9±0.1	4.2± 0.6	20.4	[147, 179, 390, 420]
Bezafibrate	0	2.1± 0.4	9.7	[147, 390, 420]
Carbamazepine	19.1±3.1	0.9± 0.2	68.6	[147, 176, 179, 187, 390, 420-425]
Ciprofloxacin	0	6.5± 0.8	58.6	[421-423]
Codeine	0	1.9± 0.3	8.9	[187, 422, 424]
Furosemide	32.5± 7.4	3.2± 0.7	43.5	[147, 187, 331, 390]
Irbesartan	0.2± 0.0	0.1± 0.0		
Ketoprofen	3.5± 1.8	6.5± 2.3	40.9	[176, 179, 390]
Loratadine	21.6± 2.2	1.5± 2.0	87.0	[179]
Meloxicam	0	0.1± 0.0		
Metronidazole	4.1± 0.4	11.8± 1.1		
Metronidazole-OH	4.1± 0.5	10.5± 1.3		
Metropolol	5.6± 0.3	5.6± 0.3	6.3	[422]
Nadolol	2.9± 0.4	3.9± 0.6	3.7	[147]
Ofloxacin	7.0± 0.4	14.4± 0.7	56.0	[179, 421, 422]
Olanzapine	0.1± 0.0	0.2± 0.0	19.8	[187]
Piroxicam	2.7± 0.5	1.5± 0.3		
Pravastatin	0	2.0± 0.1		
Propanolol	35.2± 2.2	36.8± 2.3	32.3	[176, 179, 422, 425]
Ranitidine	3.8± 0.7	6.1±1.1	11.6	[147, 331, 390, 422]
Ronidazole	1.2± 0.1	2.0± 0.2	71.2	[187]
Salbutamol	0	1.3± 0.2		
Salycilic acid	23.0± 4.1	42.3± 7.5	61.2	[176, 422]
Tamsulosin	3.6± 0.3	5.8± 0.5		
Tenoxicam	0.4± 0.1	11.3± 2.3		
Trazodone	0.1± 0.0	0.3± 0.1		
Warfarin	2.2± 0.4	3.0± 0.5	46.0	[424]

As it can be seen in Figure 6.6 the sorption of the compounds was different between the biomass from the inoculated bioreactor (obtained in the experiment performed with inoculated fungi) and the biomass obtained in the experiment performed without any fungal inoculation (control bioreactor), which can be considered as mainly bacterial biomass with some fungal strains detected at very low concentrations [195].

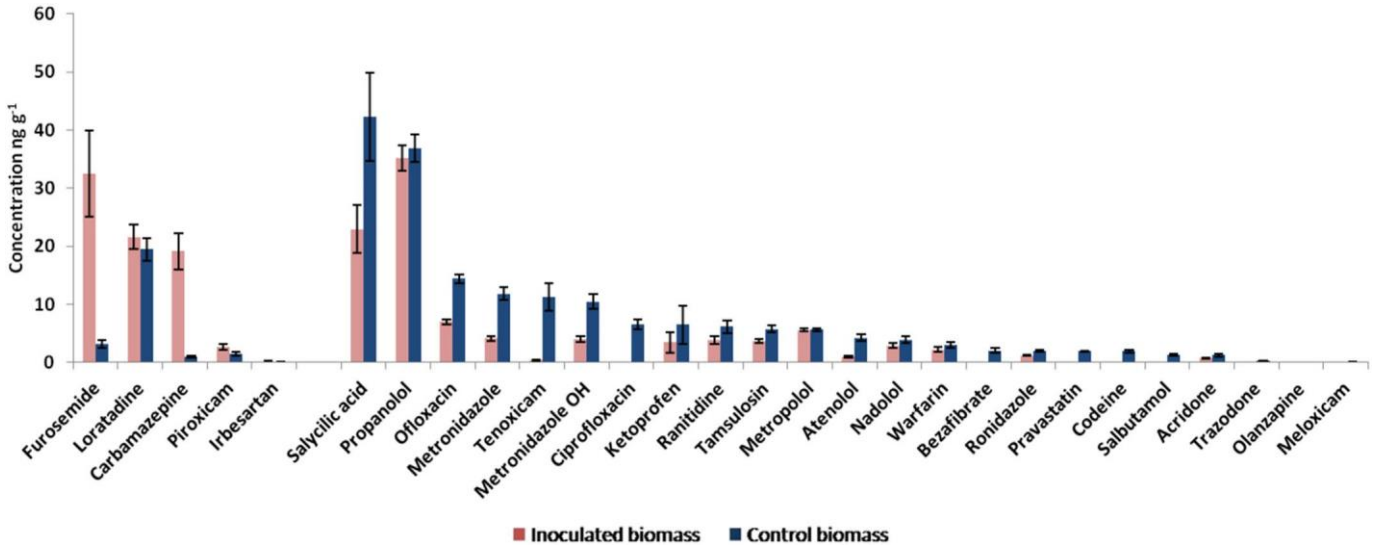

Figure 6.6 - Concentration of the PhACs detected in the biomass samples from the continuous bioreactor with *T. versicolor* and VHW at the end of the experiment. Error bars correspond to deviation of analytic results of the concentrations measured.

Most of the compounds were better sorbed into the control biomass (23 out of 28 compounds), whereas for only 5 of them the sorption was higher in the inoculated biomass. This can be explained due to the fungal capacity of degrading sorbed compounds by its internal enzymatic degradative system [261-263]. However, in global terms, no significative differences can be pointed out in the total PhACs concentration measured in both biomasses; 174.6 ± 5.0 ng g^{-1} in the inoculated biomass and 204.9 ± 5.2 ng g^{-1} in the control biomass.

Then again, PhACs levels in sludge from conventional WWTPs reported in other studies (Table 6.4) were higher than levels encountered in the control biomass in our experiment, with the exception of propranolol, with similar concentrations in all the samples analyzed; 35.2 ng g^{-1}, 36.8 ng g^{-1} and 32.3 ng g^{-1} for inoculated biomass, control biomass and sludge from conventional WWTPs, respectively. However, comparison between levels of PhACs in the biomass from the different treatments without taking into account the levels in the water phase (which can vary enormously from one WWTP to the other) is not accurate since both are closely related. As biomass concentration in the treatments is likely to vary between the treatments, the sorption was compared in terms of K_d coefficients [359]. Using this coefficient we assumed that the bioreactor has achieved a stationary state, which is the case in CAS, since the samples selected were taken, at the end of the experiment where, no removal of biomass was performed.

For example, K_d values (Table 6.5) calculated for salycilic acid were similar in the inoculated biomass and in the biomass of the control bioreactor (33 and 14 respectively) and in the sludge of a CAS treatment (23) [426]. Also for carbamazepine, K_d values were quite similar: 48 in the inoculated biomass, 31 in the control biomass from the control bioreactor, 43 and 61,2 from CAS treatment [179, 426]. Considering these results, it could be suggested that there are no major differences in the sorption of PhACs in the inoculated biomass in comparison to sludge from CAS treatments ($p < 0.05$). Therefore, inoculated biomass after fungal treatment might be considered a potential waste (as it is the case of wastewater sludge in a conventional WWTP), and thus be treated accordingly before being released into the environment [187]. However, this is a preliminary experiment and further studies would be necessary to confirm these findings.

Table 6.5 - PhACs concentrations measured in the fungal and control biomass at the end of the experiment with the continuous bioreactor

	Inoculated biomass K_d	Control biomass K_d
Acetaminophen	0	0
Acridone	*	*
Alprazolam	-	-
Atenolol	*	*
Atorvastatin	0	0
Bezafibrate	*	*
Carazolol	-	-
Carbamazepine	48	31
Cimetidine	-	-
Ciprofloxacin	0	41
Codeine	*	*
Dexamethasone	-	-
Diclofenac	0	0
Dimetridazole	-	-
Famotidine	-	-
Fluoxetine	-	-
Fluvastatin	0	0
Furosemide	15	40
Ibuprofen	0	0
Indomethacine	0	0
Iopromide	0	0
Irbesartan	*	*
Ketoprofen	7	10
Loratadine	*	*
Lorazepam	-	-
Meloxicam	*	*
Metronidazole	4	4
Metronidazole OH	7	5
Metropolol	*	*
Nadolol	*	*
Ofloxacin	*	*
Olanzapine	7	6
Piroxicam	12	9
Pravastatin	0	66
Propanolol	*	*
Ranitidine	0	15
Ronidazole	*	*
Salbutamol	*	*
Salycilic acid	33	14
Sotalol	-	-
Tamsulosin	*	*
Tenoxicam	*	*
Trazodone	*	*
Valsartan	-	-
Venlafaxine	-	-
Warfarin	*	*
Xylazine	-	-

* PhAC in water = 0
- PhAC in water and in the biomass = 0

Alternatively, the appearance and removal of transformation products (TP) of PhACs has been addressed by some authors [336, 427, 428]. According to studies performed in our research group [429] ligninolytic fungi degradation rely on mechanisms occurring

inside and outside the fungal cells, though Blanquez et al. (2004) [338] evidenced that most of the biological transformation occurs at intracellular level.Therefore, the great majority of transformation products might be found in the fungal biomass. However, the occurrence of these TP in fungal biomass has not been reported so far and calls for further studies in order to reach a better understanding on the subject.

4. Conclusions

According to the results obtained in our studies, some ideas can be highlighted: i) It is worthwhile to measure target compounds in both, liquid and solid (biomass) phases in order to determine the role of the sorption and biodegradation mechanisms when the elimination of pollutants is evaluated, ii) Sorption processes accounted for a 7% of the PhACs elimination (mean value) in our batch experiments. However, the contribution of the sorption processes to overall removal is different depending on the fungus considered, ranging from 4% in the case of *S.rugosoannulata* to 26% for *G.lucidum*. Iii) Sorption of PhACs on fungal biomass was similar to that observed into the sludge from conventional CAS treatments. Therefore, based on this preliminary study, fungal biomass should be managed in the same way as the sludge from CAS treatments.

Chapter 7

Chapter 7 – Conclusions

The work carried out during this thesis has led to the following conclusions:

1. The elimination rates achieved with the fungal treatment are quite similar values in global terms to those from conventional WWTP.

2. The main benefit of the fungal treatment is the high elimination rate achieved with antibiotics and psychiatric drugs (86% and 69% respectively), which are those posing a higher environmental risk among PhACs.

3. In addition to a better removal of antibiotics, the fungal treatment has also shown better ARGs removal than conventional WWTPs regarding their relative concentrations. In contrast some ARGs such as *sulI*,*bla*$_{SHV}$ and *qnrS* showed an increase in their absolute concentrations, associated with the increase of the bacterial population during the fungal treatment

4. The selective pressure of the antibiotics favoring the spread of ARGs during the fungal treatment is not the main factor involved in the fate of antibiotic resistance during the wastewater treatment. Other factors should be taken into account such as the operational parameters of bioreactors, the wastewater-associated bacterial communities and their interaction with fungi.

5. As expected, among all treated effluents, hospital wastewater was the most toxic one due to the higher levels of antibiotics.Fortunately fungal treatment of this effluent lead to a high antibiotic removal and thus great HQ removal.

6. As a side effect, the fungal treatment causes an increase in bacteria population in the wastewater. This is due to the fungal ability of degrading macromolecules, releasing bioavailable nutrients for the bacteria.

7. A successful method has been developed for the extraction and further analysis of up to 47 PhACs in fungal biomass.

8. For the evaluation of PhACs removal it is worthwhile to measure target compound in both liquid and solid (biomass) phases in order to determine the role of sorption and biodegradation mechanisms in the elimination of target pollutants.

9. Sorption processes in fungal biomass must be taken into account when analyzing the elimination of PhACs. In our batch experiments sorption processes represent a mean value of 7% of the elimination measured in fungal treatments. However for some fungus, e.g. in *G. lucidum*, sorption accounted for 26% of total elimination measured. In addition, for compounds such as diclofenac, the mean sorption value was 25% of the initial concentration.

11. PhACs concentrations in the fungal biomass aresimilar to those measured in sludgeof conventional WWTPs, so the fungal biomass must be handle in the same way as the sludge from CAS treatments.

Chapter 8

Chapter 8 – Future perspectives

After all the studies performed with the fungus, it could be confirmed that the fungal treatment is a very promising technology to be used in the treatment of wastewaters contaminated with PhACs. As explained previously, the fungal treatment is particularly effective with certain compounds, especially antibiotics, which are quite recalcitrant in conventional treatments. In addition to the elimination of PhACs it has also been shown that the fungal treatment has a positive effect by reducing the spread of resistance genes during the wastewater treatment.

Therefore, the fungal treatment could be applied not as a replacement treatment, but as a complementary treatment to conventional ones thanks to the ability to remove certain compounds that are not efficiently eliminated in conventional treatments; mainly antibiotics and psychiatric drugs. Taking into account all information obtained during this thesis, an ideal place to locate a fungal treatment would be at the outlet manifold of hospital facilities for different reasons. First of all, these kind of wastewaters have a high concentration of PhACs, which in some cases could potentially affect the microorganisms involved in degradation processes in conventional WWTPs; whereas fungi have a high tolerance to toxic compounds like PhACs, due to the presence of extracellular enzymes [211]. In addition, hospital waters usually contain a high concentration of antibiotics, a group of PhACs effectively removed by fungi based on our studies. In this type of effluent also a high concentration of ARGs was found; and ARGs are other type of emerging pollutant for which fungal treatment has been particularly effective regarding the relative values of ARGs concentration measured.

To date, all experiments performed with fungal treatment were performed in a small scale, Erlenmeyer flasks or little bioreactors. The next step should focus on the use of this technology on a larger scale. However before applying this technology to eliminate PhACs from wastewaters, there are still certain issues that must be addressed.

a. PhACs conjugation and deconjugation

   For some compounds, the concentration measured at the end of the fungal treatment was higher than at the beginning of the experiment. That kind of behavior was observed in almost all fungal experiments performed. This is

usually attributed to both desorption and deconjugation processes [195, 204, 432]. As explained previously (introduction, section 1.1) when treating humans or animals with PhACs, those PhACs dose administered were not totally absorbed by the organism; part of them were assimilated and metabolized whereas other part were excreted modified or as the parent compound. Therefore both, parent compounds and PhACs metabolites excreted via urine or faeces find their way to raw sewage and further into wastewater treatment systems. One of the most common metabolization route is the conjugation of PhACs with specific chemical molecules, like glucuronic acid, sulfates, etc. in order to increase the solubility of the compound and facilitate their urinary excretion [399]. During the fungal treatment, some of these conjugated compounds can be deconjugated back into its original form, by either enzymatic or physicochemical action [204]. As analytical methods do not target those conjugated compounds, they will only be detected once they are cleaved back to parent compound, namely after fungal treatment. Therefore an increase in the concentration of parent compound can be measured. That is the reason why, for certain PhACs e.g. carbamazepine, salicylic acid and ketoprofen; the removal values observed are negative. Deconjugation processes and consequent negative removals have also been described for some PhACs after conventional WWTPs; eg. carbamazepine, triclosan, trimethoprim and roxithromycin [8]. It is known that some compounds like carbamazepine and ketoprofen are excreted to some extent, conjugated [399].

Two approaches are suggested to overcome this problem. The first one is to develop an analytical method covering conjugated compounds and other metabolites, so that concentration of PhACs and related compounds could be known at each step. However, there are no standards for many of the PhACs conjugates or they are very expensive. The second possible approach would be to find a sample pre-treatment method (hydrolysis, solvolysis, enzymatic lysis, etc.) able to deconjugate PhACs conjugates, so that available analytical methods can measure total amount of parent compounds along the treatment. This is a challenging issue, since in many occasions these types of sample pretreatment strategies also alter the concentration of other target pollutants, as it has been observed in the preliminary experiments carried out during the thesis.

b. Polysaccharides fungal secretion

During the treatment of the wastewater samples it was observed that the fungus as part of their metabolism, generate a mucous substance (polysaccharides) [443]. These substances eventually may causes bioreactor operation difficulties such as growth on the reactor walls and agitators, foaming and increased need of mixing and oxygen supply [444].

It would be desirable to obtain a solution to avoid the accumulation of these polysaccharides during wastewater treatment. One possibility would be to remove these compounds periodically; another option is to try to isolate just the fungal enzymes involved in the degradative processes, and use them for the wastewater treatment, as recently has been tested by our research group [324]. However, up to now the removal rates achieved are not as satisfactory as those obtained with the fungal treatment.

c. Nutrient supply during the treatment

One factor not yet fully optimized is the nutrients supply needed by the fungus to degrade the PhACs efficiently. As it was demonstrated, the most of WRF requires a minimal amount of nutrients (carbon and nitrogen) to generate some degradative enzymes such as laccase [445]; and usually, wastewaters are not able to provide a sufficient amount of nutrients [257]. However, recent studies have demonstrated that some WRF, like *Pleurotus ostreatus*are able to assimilate degradation organic components (measured aschemical oxygen demand, COD) from wastewater, performing a proper PhACs degradation [446]. However, the degradation with this fungal, has been tested just with three compunds, and further studies should be necessary in order to consider *P. ostreatus* as a good candite for wastewater treatment.

Regarding this issue, some authors [444] have proposed some possible solutions: i) optimizing the nutrient addition, adjunsting it to the consumption rate; ii) replacing the nutrients (usually glucose and ammonium tartrate/chloride) by cheaper products; iii) using the fungal treatment but just with small volumes of micropollutant-contaminated wastewater. In this case, the nutrient addition would be lower. Finally, iv) immobilizing the fungal biomass onto lignocellulosic materials (usually cheap byproducts of other industries [447,

448]); these substrates act also as carbon and nitrogen sources for WRF, thus avoiding the need of nutrient addition [444]. However the use of these kind of materials may lead to the release of recalcitrant compounds, e.g. tannins or phenolic compounds [449].

d. Over-growing of bacteria along with the fungal treatment.

The increase of bacterial population during fungal treatment can be attributed to the increase of nutrients availability; fungi produce extracellular degradative enzymes, e.g. laccase and peroxidases [191, 323], able to break complex compounds such as ligninand thus increasing the nutrients bioavailability for all microbial community members.The bacterial over-growth has been identified as one of the reasons of the decline in micropollutant removal [444]. Indeed after the first moments of the water treatment, bacteria has been shown to exert competitive pressure for the nutrients, thus leading to the loss of fungal biomass. Because of this, different proposals have been studied in order to solve this limitation.

- Favouring the fungal growth

  It involves setting up the proper conditions that distinctively favor WRF over bacteria. These strategies includes i) operation at an acidic pH, the optimal pH for the viability and activity of WRF; ii) immobilization of fungal biomass, in form of pellets allowing a high concentration of fungus inside the reactor, thus hindering bacterial colonization [450]. Immobilization can be carried outon lignocellulosic carriers, in that case the fungal concentration tends to be lower, but most bacterial species find it difficult to grow on such lignocellulosic substrates [444]; iii) periodical biomass renewal, in summary, the substitution of old biomass by fresh one achieved a more stable fungal population in the reactors, maintaining enzymatic activity for a longer period of time [345]; and iv) optimizing the carbon-to-nitrogen ratio (C/N ratio) of the nutrients adition according to the specific needs of the fungus ant its enzymatic production [444].

- Washing out bacteria

The purpose of these strategies is to keep the fungal biomass in the reactor while washing out the bacteria and other microorganisms, therefore increasing the retention time of WRF (SRT) while keeping an HRT able to wash out the other microorganisms. This goal can be achieved by immobilizing the fungi on inert carriers, lignocellulosic substrates, membranes [451-453].

Nevertheless, while fungal survival might be improved with low HRTs it oftens mean lower degradation contaminants [454]. Moreover, washing out of bacteria comes inevitably with the washout of extracellular enzymes and mediators produced by the fungus [453, 455]. In summary, both HRT and SRT must be optimized in order to achieve a compromise between bacteria-and-enzyme washout, micropollutant removal and fungal survival [444].

- Wastewater pretreatments

In order to reduce the initial amount of bacteria present in the wastewater, some pretreatments of the raw wastewater, before the fungal treatment has been tested, e.g.: ozonation [456] or coagulation and flocculation processes. The first option presents some drawbacks like its high economic cost and also the fact that adding disinfection with ozone can lead to some inaccuracies in the calculation of the removal values reached with the fungus [444]. The second option has been recently tested with quite good results [457], but further experiments would be necessary to confirm those good results.

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