Universitat Autònoma de Barcelona

Fungal biodegradation of pharmaceutical active compounds in wastewater

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PhD Thesis

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MONTSERRAT SARRÀ ADROGUER, Professora Titular del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona (UAB), i MARIA RAMOS MARTÍNEZ ALONSO, Professora Titular del Departament de Genètica i Microbiologia de la Universitat Autònoma de Barcelona (UAB),

CERTIFIQUEM:

Que el llicenciat en Biotecnologia Francesc Castellet Rovira ha realitzat sota la nostra direcció, tant en els laboratoris del Departament d'Enginyeria Química, Biològica i Ambiental com en els del Departament de Microbiologia i Genètica, el treball que amb el títol "**Fungal biodegradation of pharmaceutical active compounds in wastewater**" es presenta en aquesta memòria, la qual constitueix la seva Tesi per a optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

I perquè en prengueu coneixement i consti als efectes oportuns, presentem a l'Escola d'Enginyeria de la Universitat Autònoma de Barcelona l'esmentada Tesi, signant el present certificat a:

Bellaterra (Barcelona), Juny 2017

Agraïments

No he sentit mai ningú dir que escriure una tesi fos fàcil, i aquesta no ha estat pas una excepció. Durant quatre anys he notat, amb més o menys intensitat, el pes de l'estrès, la impotència de no poder avançar o la incapacitat per a resoldre problemes que semblaven inabastables; però també he tingut temps per a recollir i acumular coneixements, ajudes, vivències i aprenentatges ben diversos i de tanta gent diferent que de ben segur és fa difícil fer un recopilatori complet i definitiu de totes les persones a les quals dec alguna "pàgina" d'aquest document.

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Abstract

Pharmaceutical active compounds are an important, large and diverse group of chemical compounds designed to cause therapeutic effects on health, mainly human but also animal. Because of their composition, they are bioactive and difficult to be degraded even when present at a minimal dose. Their variety of structure, composition and effects is huge, while some of them are completely removed at wastewater treatment plants, others are only partially or non-removed. Furthermore, despite of being strictly regulated by clinical trials before their market distribution, specific restrictions for their release in the environment are almost non-existent, and there is not a standard quantification method yet.

Regarding this situation, there is a need to implement alternative technologies in wastewater treatment plants to remove pharmaceuticals from water before entering the environment. One possible treatment is the use of ligninolytic fungi to degrade these compounds. In relation with this approach, the white-rot fungus *T. versicolor* has been widely studied in the removal of many pharmaceutical active compounds in wastewater under non-sterile conditions. However, there are still several questions to tackle: recalcitrant compounds unable to be degraded, the generation of transformation products, and operational bottlenecks can be highlighted among many other.

Certain pharmaceuticals are considered recalcitrant due to its stable structure when treated for their removal. In fungal treatment, this stability depends mainly on factors such as the degradation mechanism, which is directly affected by the metabolism of the fungal species. A screening experiment among 6 different species of fungi attempted to discover new candidates in the removal and biodegradation of recalcitrant compounds. Scarcely studied species such as *S. rugosoannulata* and *G. luteofolius* obtained considerable removals linked to biodegradation processes.

The removal of pharmaceutical active compounds has also been studied in terms of biodegradation and sorption mechanisms in submerged cultures. Regarding sorption processes, the analyses of the pharmaceutical active compounds present in liquid phase and in fungal biomass showed similar results respect to the sorption estimated by heat

killed control biomass. It has been proven that 6 days is generally insufficient time for fungal biomass to degrade the adsorbed contaminants.

Concerning the generation of transformation products from parental compounds, o-desmethylvenlafaxine and n-desmethylvenlafaxine were identified as the two main biological transformation products generated during the degradation of the recalcitrant compound venlafaxine. On the other hand, transformation products generated by the fungus *P. ostreatus* in the degradation of diclofenac and ketoprofen coincide with the molecules generated by *T. versicolor* in the same treatment according to their nuclear magnetic resonance profiles. Besides, studies on two main fungal enzymatic mechanisms showed that the extracellular enzyme laccase is involved in the degradation of recalcitrant compounds such as atenolol, whereas the intracellular enzymatic complex cytochrome P450 is involved in diclofenac transformation.

Operational bottlenecks, such as microbial competition by native microbiota for nutrients in fungal bioreactors, cause several problems in applying continuous treatment. In order to prevent bacteria proliferation, the implementation of a biosand filter for the reduction of a high bacterial load in hospital wastewater was evaluated. This pretreatment was not able to achieve enough reduction in bacterial load of the fungal reactor's inlet. Instead, the native microbiota present in the biosand filter was able to remove spiked concentrations of the antibiotic ciprofloxacin and the analgesic ibuprofen. This opened the way for a molecular biology study with denaturing gradient gel electrophoresis (DGGE) to analyse which changes in the microbiota could entail the presence of potential candidates for pharmaceutical removal and which could be those microorganisms.

Other operational issues to be tackled in *T. versicolor* reactors were the need for carbon supply and the pH control. In this thesis, *P. ostreatus* and *S. rugosoannulata* have proven able to self-obtain its carbon source from wastewaters achieving high chemical oxygen demand (COD) reductions, avoiding the need for carbon supply. Besides, S. rugosoannulata was also able to lower the pH of hospital wastewaters to its optimal, and hence showing promising features to its full implementation in reactors.

Resum

Els fàrmacs constitueixen un grup gran, important i divers, de compostos químics dissenyats per a causar efectes terapèutics sobre la salut principalment humana, però també animal. Per la seva composició, són bioactius i difícils de degradar encara que presentin una dosi mínima. La seva varietat estructural, de composició i efectes és enorme i mentre que alguns d'ells són eliminats completament a les depuradores, d'altres romanen inalterats o parcialment transformats. A més a més, malgrat ser estrictament regulats per assajos clínics abans de la seva distribució al mercat, no existeixen restriccions específiques per al seu alliberament en el medi ambient, i encara manca un mètode de quantificació estàndard per a detectar-los.

Cal implementar tecnologies alternatives en les depuradores per a eliminar aquests principis actius de l'aigua abans de ser abocats al medi ambient. Un possible tractament és l'ús de fongs ligninolítics per a degradar aquests compostos. En relació amb aquest tractament, el fong *T. versicolor,* membre del grup de fongs de podridura blanca, ha estat àmpliament estudiat en l'eliminació de molts fàrmacs en aigües residuals i condicions no estèrils. Tanmateix, encara hi ha diverses qüestions a resoldre d'entre les quals en destaquen: els compostos recalcitrants, la generació de productes de transformació i colls d'ampolla operacionals.

Es consideren recalcitrants aquells fàrmacs que romanen inalterats a causa de la seva estructura estable durant els tractaments d'eliminació. En el cas dels tractaments amb fongs, aquesta estabilitat depèn principalment de factors com el mecanisme de degradació, directament afectat pel metabolisme de les espècies de fong emprades. Per tal de descobrir candidats potencials per a l'eliminació i biodegradació de compostos recalcitrants es va realitzar un experiment de cribatge entre 6 espècies diferents de fongs. Espècies poc estudiades, com ara *S. rugosoannulata* i *G. luteofolius*, van permetre obtenir importants eliminacions vinculades amb processos de biodegradació.

També es va estudiar l'eliminació de fàrmacs tenint en compte els diferents mecanismes de biodegradació i sorció en cultius submergits. Pel que fa a la sorció, les anàlisis dels fàrmacs presents en la fase líquida i en la biomassa del fong mostren resultats semblants respecte de la sorció estimada a partir de controls amb biomassa de fong inactivats per

calor. S'ha demostrat que 6 dies no és temps suficient per al fong per a poder degradar els contaminants adsorbits.

Pel que fa a la generació de productes de transformació a partir dels compostos principals, es van identificar o-desmethylvenlafaxine i n-desmethylvenlafaxine com els dos principals productes de transformació biològica generats durant la degradació del compost recalcitrant venlafaxina. D'altra banda, els productes de transformació generats pel fong *P. ostreatus* en la degradació de diclofenac i ketoprofè coincideixen amb les molècules generades per *T. versicolor* en el mateix tipus de tractament, segons els seus perfils de ressonància magnètica nuclear. A més a més, estudis sobre dos dels principals mecanismes enzimàtics dels fongs, van demostrar que l'enzim extracel·lular lacasa està implicat en la degradació de compostos recalcitrants com l'atenolol, mentre que el complex enzimàtic intracel·lular citocrom P450 intervé en l'eliminació de diclofenac.

Els colls d'ampolla operacionals com la competència de la microbiota nativa pels nutrients en els bioreactors fúngics provoquen diversos problemes en l'aplicació del tractament en continu. Per a prevenir la proliferació de bacteris, es va avaluar la implementació d'un biofiltre de sorra per a la reducció de la càrrega bacteriana en aigües residuals d'hospital. Malgrat aconseguir certa reducció bacteriana, aquest pretractament no va permetre reduir prou la càrrega bacteriana d'entrada del reactor amb el fong. En canvi, la microbiota nativa present en el biofiltre de sorra va ser capaç d'eliminar concentracions de l'antibiòtic ciprofloxacina i l'analgèsic ibuprofè. Això va motivar l'anàlisi molecular mitjançant electroforesi en gel de gradient desnaturalitzant (DGGE) per tal d'analitzar els canvis que es produïen en la microbiota com a conseqüència de l'exposició continuada a fàrmacs i quines poblacions microbianes eren candidats potencials responsables de l'eliminació dels fàrmacs en qüestió. Un altre dels problemes operatius a tractar en reactors amb *T. versicolor* va ser la necessitat de proveir de carboni el reactor per una banda, i del control del pH per l'altra. En aquesta tesi, *P. ostreatus* i *S. rugosoannulata* han demostrat poder-se autoproveir de carboni a partir d'aigües residuals amb alts valors de demanda química d'oxigen (DQO), evitant així la necessitat del seu subministrament. A més a més, *S. rugosoannulata* també va poder reduir el pH de les aigües residuals hospitalàries al seu nivell òptim i, per tant, va mostrar trets prometedors de cara a la seva futura plena implementació en reactors.

Resumen

Los fármacos son un grupo grande, importante y diverso, de compuestos químicos diseñados para causar efectos terapéuticos sobre la salud principalmente humana, pero también animal. Por su composición, son bioactivos y difíciles de degradar aunque presenten una dosis mínima. Su variedad estructural, de composición y efectos es enorme y mientras que algunos de ellos son eliminados completamente en las depuradoras, otros permanecen inalterados o parcialmente transformados. Además, a pesar de ser estrictamente regulados por ensayos clínicos antes de su distribución en el mercado, no existen restricciones específicas para su liberación en el medio ambiente, y aún falta un método de cuantificación estándar para detectarlos.

Hay que implementar tecnologías alternativas en las depuradoras para eliminar estos principios activos del agua antes de ser vertidos al medio ambiente. Un posible tratamiento es el uso de hongos ligninolíticos. En relación con este tratamiento, el hongo *T. versicolor*, perteneciente al grupo de hongos de podredura blanca, ha sido ampliamente estudiado en la eliminación de muchos principios activos farmacéuticos en aguas residuales y condiciones no estériles. Sin embargo, aún hay varias cuestiones a resolver entre las cuales destacan: los compuestos recalcitrantes, la generación de productos de transformación y cuellos de botella operacionales.

Se consideran recalcitrantes aquellos fármacos que permanecen inalterados debido a su estructura estable durante los tratamientos de eliminación. En el caso de los tratamientos con hongos, esta estabilidad depende principalmente de factores como el mecanismo de degradación, directamente relacionado con el metabolismo de las especies de hongo empleadas. En ese sentido se realizó un experimento de cribado entre 6 especies diferentes de hongos para descubrir candidatos potenciales para tratar compuestos recalcitrantes. Especies hasta ahora poco estudiadas, como *S. rugosoannulata* y *G. luteofolius*, proporcionaron importantes eliminaciones, vinculadas a procesos de biodegradación.

También se estudió la eliminación de fármacos teniendo en cuenta los diferentes mecanismos de biodegradación y sorción en cultivos sumergidos. En cuanto a la sorción, los análisis de los fármacos presentes en la fase líquida y en la biomasa del hongo

muestran resultados similares respecto a la sorción estimada a partir de controles con biomasa de hongo inactivada por el calor. Se ha demostrado que 6 días no es tiempo suficiente para que el hongo pueda degradar los contaminantes adsorbidos.

En cuanto a la generación de productos de transformación a partir de compuestos principales, se identificaron o-desmethylvenlafaxine y n-desmethylvenlafaxine como los dos principales productos de transformación biológica generados durante la degradación del compuesto recalcitrante venlafaxina. Por otra parte, los productos de transformación generados por el hongo *P. ostreatus* en la degradación de diclofenaco y ketoprofeno coinciden con las moléculas generadas por *T. versicolor* en el mismo tratamiento, según sus perfiles de resonancia magnética nuclear. Además, estudios sobre dos de los principales mecanismos enzimáticos de los hongos, demostraron que enzima extracelular lacasa está implicada en la degradación de compuestos recalcitrantes como el atenolol, mientras que el complejo enzimático intracelular citocromo P450 interviene en la eliminación de diclofenaco.

Los cuellos de botella operacionales como la contaminación microbiana y la competencia por los nutrientes en los biorreactores fúngicos provocan varios problemas en el mantenimiento de los mismos. Para prevenir la proliferación de bacterias se evaluó la implementación de un biofiltro de arena para la reducción de la carga bacteriana en aguas residuales de hospital. A pesar de conseguir cierta reducción bacteriana, este pretratamiento no permitió reducir suficientemente la carga bacteriana de entrada del reactor con el hongo. En cambio, la microbiota nativa presente en el biofiltro de arena fue capaz de eliminar concentraciones del antibiótico ciprofloxacina y el analgésico ibuprofeno. Esto motivó el análisis molecular mediante electroforesis en gel de gradiente desnaturalizante (DGGE) para analizar los cambios que se producían en la microbiota como consecuencia de la exposición continuada a fármacos y que poblaciones microbianas eran candidatos potenciales responsables de la elimincación de dichos fármacos. Otro de los problemas operativos a tratar en reactores con *T. versicolor* es la necesidad de proveer el reactor de carbono por un lado, y del control del pH por el otro. En esta tesis, *P. ostreatus* y *S. rugosoannulata* han demostrado poderse autoproveer de carbono a partir de aguas residuales con altos valores de demanda química de oxígeno (DQO), evitando así la necesidad de su suministro. Además, *S. rugosoannulata* también

pudo reducir el pH de las aguas residuales hospitalarias a su nivel óptimo y, por tanto, mostró rasgos prometedores para su futura implementación completa en reactores.

List of acronyms and abbreviations

In order to understand the abbreviations and acronyms used in this document, a collection of the most frequent terms is listed below:

ATL	Atenolol
BAC	Biological Activated Carbon
BLAST	Basic Local Alignment Search Tool
BRF	Brown rot fungi
CAS	Conventional Activated Sludge
CBZ	Carbamazepine
CFD	Cyclophosphamide
COD	Chemical Oxygen Demand
CPX	Ciprofloxacin
DCF	Diclofenac
DDT	Dichlorodiphenyltrichloroethane
DGGE	Denaturizing Gradient Gel Electrophoresis
FBB	Fluidized Bed Bioreactor
GAC	Granular Activated Carbon
HPLC	High Precision Liquid Chromatograph
HRT	Hydraulic retention time
HWW	Hospital Wastewater
IBP	Ibuprofen
IFD	Ifosfamide
IPD	Iopromide
KTP	Ketoprofen
Lac	Laccase
LDF	Litter Decomposing Fungi
LiP	Lignin Peroxidase
MBR	Membrane bioreactor

MDP	Minimal Degradation Percentage
MEC	Micropollutants of emerging concern
MEC	Measured Environmental Concentration
MnP	Manganese Peroxidase
MS/MS	Tandem Mass Spectrometry
MTP	Metoprolol
MTPA	Metoprolol acid
NSAIDs	nonsteroidal anti-inflammatory drugs
ODMVFX	O-desmethylvenlafaxine
PAC	Powder Activated Carbon
PAH	Polycyclic aromatic hydrocarbons
PCR	Polymerase Chain Reaction
PhAC	Pharmaceutical Active Compound
PNEC	Predicted No Effect Concentration
RP	Removal Percentage
SD	Standard deviation
SP	Sorption Percentage
SPE	Solid Phase Extraction
SRF	Soft rot fungi
SRT	Sludge retention time
STP	Sewage Treatment Plants
TNT	2,4,6-Trinitrotoluene
TOC	Total Organic Carbon
TP	Transformation product
TSS	Total Suspended Solids
UPLC	Ultra Performance Liquid Chromatograph
VFX	Venlafaxine
WRF	White Rot Fungi
WWTP	Wastewater Treatment Plant


GENERAL ASPECTS

1. Background and Introduction

1.1. Pollutants of emerging concern in water: an historical problem

At the end of the 19th century, as cities became more populated in industrialized areas across Europe and the United States, the first occurrence of chemical and industrial pollution in aquatic environments was detected. In 1897, a report to the Royal Commission on River Pollution detailed the gross industrial contamination of the Tawe River in Wales, noting that it was polluted by "alkali works, copper works, sulfuric acid liquid, sulphate of iron from tin-plate works, and by slag, cinders and small coal" (Markham, 1994).

However, several decades passed until the first reactions towards a definitive solution to fight chemical pollution in water took place. In early 1960s, environmental pollution awareness became a public issue in the USA thanks to denounce works such as Rachel Carson's Silent Spring book (Carson, 1962), and from then on until nowadays, water chemical pollution, along with air and soil contamination, has become an increasingly complex subject to study for scientific community.

Highly persistent and ecotoxic chemical substances began to be regarded as pollutants during the second half of twentieth century, when the industrial boom after the second World War massively spread the use of synthetic pesticides or organic solvents, among others. Their low biodegradability in the environment but also their potential toxic effects both for wildlife and human health, invoked the birth of modern pollution legislation and environmental regulatory systems all over the world, as well as an eventual implementation of municipal sewage treatment plants (Shifrin, 2005).

However, by the end of the century a new group of pollutants began to gain notorious attention. Loosely called emerging pollutants, these substances became frequently mentioned since mid-1990s by USA's Environmental Protection Agency (EPA) and other scientific agencies and communities, referring to chemicals and substances without any regulatory standard, discovered recently and mainly in natural ecosystems (US Environmental Protection Agency, 2008).

Nowadays emerging pollutant qualification has shifted from recently discovered pollutants into a broader meaning. What was considered as an emerging pollutant a decade ago may no longer be emerging today in case its lack of information and regulation has been solved, whereas contaminants already known from centuries ago may become emerging due to recent discoveries on its environmental or health impacts (e.g. acting as a xenobiotic[1]). Besides, pollutants concentration is no longer as important as its potential hazard to cause an impact on human health or environment, since a great variety of pollutants have been found to be active at trace concentrations. On regarding to this topic, some authors and agencies (including EPA) suggest using the expression "contaminants of emerging concern" instead, in order to refer to any chemical substance or even microorganism, man-made or naturally produced, that has been recently identified as a potential hazard source, and hence not submitted to regulation yet or whose regulation does not consider its recently discovered hazardous effects (Sauvé and Desrosiers, 2014).

Improvements in analytical chemistry (such as LC-MS/MS) allowed scientists to determine the presence of contaminants of emerging concern at a very low concentration range (ng/L) in the aquatic environment, most of them persistent and bioactive, also polar compounds that could never be detected before. These certain contaminants of emerging concern have been called micropollutants by some authors (Kümmerer, 2011), although other authors consider the expression "micropollutant" to be equivalent to "contaminant of emerging concern" indistinctly (Luo et al., 2014).

These large and extremely diverse mix of contaminants, are not successfully treated by Conventional Aerobic Sludge (CAS) treatments in wastewater treatment plants (WWTP), which are mainly focused on removing carbon, nitrogen, phosphorus and pathogens. However, these contaminants concerning effects on human health and environment are still investigated and mostly unknown. Its appearance as a complex mixture in the environment makes more difficult to understand crucial aspects such as their occurrence,

[1]Xenobiotic: A xenobiotic (Greek, *xenos* "foreign"; *bios* "life") is a compound that is foreign to a living organism or biological system. Principal xenobiotics include: drugs, carcinogens and various compounds that have been introduced into the environment by artificial means. (IUPAC, 1997)

fate, distribution, toxicity, biological or physicochemical transformation and even the synergies among each and every compound. (Kurwadkar et al., 2015).

According to EU's Directive 2008/105/EC (the Environmental Quality Standards Directive, EQSD), amended in 2013 by the Directive 2013/39/EU a new mechanism is needed to provide high-quality monitoring information on the concentrations of polluting substances in the aquatic environment across the EU (Ribeiro et al., 2015). Legislation about contaminants and/or micropollutants of emerging concern is very different across EU's countries, but on 31st July 2015 a first watch list of ten of these compounds (including 4 pharmaceuticals[2]) was issued, to monitor and identify their concentrations across European aquatic environment for up to 4 years.

1.2. Micro-pollutants of emerging concern: Pharmaceuticals

Among the different micro pollutants of emerging concern, Pharmaceutical Active Compounds (PhACs) need to be paid special attention, especially when more than 70 compounds can be detected at concentrations up to several μg/L in WWTP effluents (Rosal et al., 2010). PhACs are an important, large and diverse group of chemical compounds designed to cause therapeutic effects on (mainly human but also animal) health, being bioactive and reluctant to biodegradation at a minimal dose (Bolong et al., 2009; Gavrilescu et al., 2015). Their variety of structure, composition and effects is huge, while some of them are completely removed at WWTP conventional treatments, others are only partially or non-removed. Moreover, despite of being strictly regulated by clinical trials before their market approval, specific restrictions for their release in the environment are almost non-existent, and even their quantification lacks of standard methods.

Thousands of pharmaceuticals are available in the market and susceptible to reach the environment nowadays, but less than 2% has been detected and investigated to be removed (Taheran et al., 2016). Far from being solved, their release to the environment has been growing year by year as the pharmaceutical worldwide production (the annual values already exceed hundreds of tons) and consumption increased due to factors such

[2] Diclofenac (DCF), 17-β-estradiol (E2), 17-α-ethynilestradiol (EE2) and antibiotics from the family of erythromycin, clarithromycin and azithromycin.

as changes in demography, the discovery of new drugs and more affordable prices thanks to patent expirations (Kümmerer and Hempel, 2010; Sim et al., 2011).

Regarding this situation, some studies predicting PhACs environmental concentration have been done (Hansen, 2007; Ortiz de García et al., 2013) taking into account several factors such as consumption rates, use, WWTP treatments and legislation, although its accuracy can be relative and not always adjusted to reality.

After all, it is clear enough that the ultimate fate for PhACs produced and consumed in human and animal health treatments are environmental water systems. In spite of this evidence, the sources of these PhACs are complex and can be found in households, livestock farms, hospitals or veterinary wastewater effluents and even in pharmaceutical production plants (Figure 1.1): this heterogeneous situation, with so many different source points of micropollutants at different concentrations, pose a challenge that might only be solved with complex and tailored solutions (Dietrich et al., 2002).

Pharmaceuticals should not be completely persistent throughout their life-cycle, although according to their use, they do need to be functionally persistent. Their chemical structure and activity should remain unaltered during its shelf life, but especially when consumed and transported through the body until reaching its final biological target. In case of oral ingested drugs, compounds have to be stable enough to pass through acidic stomach and sometimes even remain unchanged by other complex metabolic pathways. Hence, from the exclusive point of view of eco-friendly pharmacy, the ideal PhAC should be a compound only susceptible to begin its break down after its excretion, however this implies the need of complex mechanisms to be implemented (Kümmerer and Hempel, 2010). On the contrary, in current PhACs a significant fraction of their parent compound is metabolized as transformation products (TPs), usually hydroxylated or conjugated, that can cause different biological activities or side effects, and end up excreted into raw sewage or wastewater treatment plants. Conventional treatments in WWTP expose PhACs to different degrees of natural attenuation (dilution, sorption, direct and indirect photolysis and aerobic biodegradation), although they fail to completely remove most of PhACs (Petrovic et al., 2009), and this has proven to be the main cause of micropollutants release in surface waters. For instance, in recent studies about PhACs pollution in surface waters, some of the most frequently reported compounds were nonsteroidal anti-

inflammatory drugs (NSAIDs), carbamazepine (CBZ), sulfamethoxazole and triclosan (Luo et al., 2014).

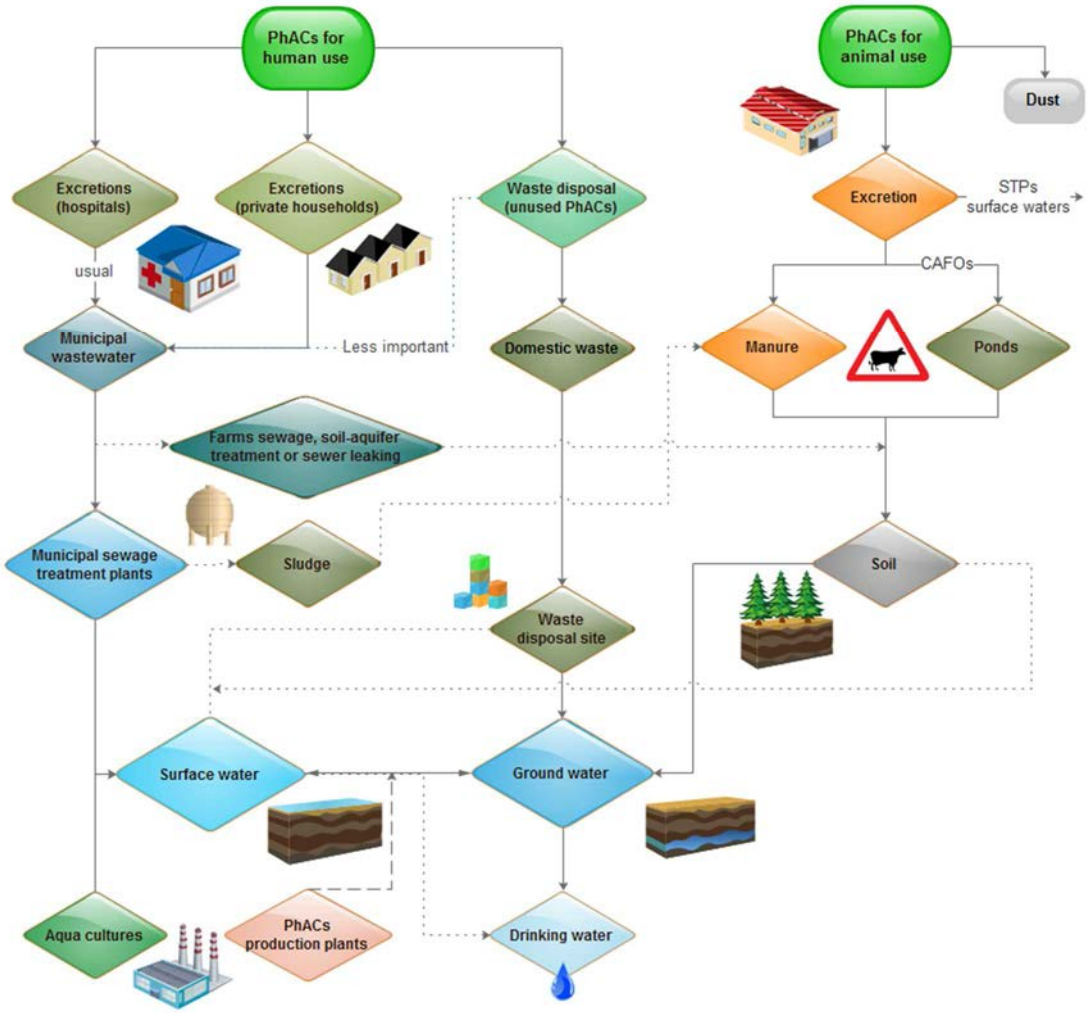

Figure 1.1 Possible sources and pathways for the occurrence of PhACs reaching the aquatic systems. Adapted from Lapworth et al. (2012). (CAFO: Concentrated animal feeding operation, STPs: Sewage Treatment Plants)

To assess the impact of PhAC concentration in human health and environment, two main factors must be taken into account. Preliminary exposure assessment of PhACs can be calculated by factors such as Predicted Environmental Concentration (PEC) (Equation 1.1), Measured environmental concentration (MEC) provided by the direct measurement of PhAC concentration in water samples, and Predicted No Effect Concentration (PNEC), being this last factor the parameter to define preliminary ecotoxicological risk characterization (Besse et al., 2012; Luo et al., 2014; Zhang et al., 2013). Considering these

three factors, there have been several attempts to model PhACs fate in the environment, however most of them still lack of fitness, since MEC can differ considerably from any predictions, especially because data for PhAC consumption may be highly disperse.

Concerning PEC according to Besse et al. (2012) it can be calculated with the following equation (expressed in mg/L):

$$PEC = \frac{Consumption \cdot F_{excreta} \cdot F_{stp}}{WW\ in\ hab \cdot 365 \cdot hab \cdot Dilution} \quad \text{(Equation 1.1)}$$

Where consumption is the quantity of the PhAC consumed by the studied population over 1 year (in mg); $F_{excreta}$ is the excretion fraction of the PhAC; F_{stp} is the fraction of emission of the drug from WWTPs directed to surface water; WWinhab is the volume of wastewater per person per day (in L); 365 is the number of days per year; hab is the number of population's inhabitants and finally Dilution is the dilution factor from WWTP effluents to surface water (in worst-case scenario = 1).

WWTP effluent has been considered as the primary source for most of micropollutants in aquatic systems. Hence, this effluent constitutes the main target to prevent PhACs entering the water bodies all across the world. Only by implementing new alternatives to CAS treatments, PhACs will be effectively degraded (Luo et al., 2014).

In accordance with those facts, there is an urgent need to find effective treatments to completely remove PhACs from wastewaters.

1.3. Treatments

Nowadays, research on wastewater treatment has already provided several processes able to remove PhACs from wastewater (Table 1.1). The most common treatments in PhACs removal belong to the physicochemical type of processes (either individually or combined) such as physical separation and chemical transformation (including advanced oxidation processes, photocatalysis or electro-degradation among others). Despite of being today the most used treatments to eliminate PhACs, their cost-effectiveness and their ecological impact to be implemented worldwide is considered unaffordable.

Physical methods rely on micropollutant concentration and do not imply degradation, requiring a secondary step of either chemical/biological elimination or a waste disposal management (Taheran et al., 2016). There are mainly three different treatments: coagulation, ion exchange, adsorption and membrane methods.

Table 1.1 Removal processes of PhACs in water and wastewater (Adapted from (Luo et al., 2014; Taheran et al., 2016)

Process	Subcategories	Examples
Physical separation	**Membrane separation**	Reverse osmosis (RO)
		Forward osmosis (FO)
		Nanofiltration (NF)
	Adsorption	Zeolite
		Activated carbon (PAC/GAC)
		Metal-organic frameworks
	Ion exchange	Anionic and cationic resin
	Coagulation-flocculation	$Al_2(SO_4)_3$ / $FeCl_3$
Chemical transformation	**Chemical oxidation**	Ozonation
		Fenton process
	Photo-oxidation	Photolysis
		Photocatalysis
		Photo-Fenton reaction
	Electrochemical process	Electro-degradation
		Electro-coagulation
	Biodegradation	Activated sludge
	Attached growth treatment	Biofiltration
		Fluidized Bed Bioreactor
	Enzymatic	Enzymatic bioreactor
	Ultrasonic treatment	Ultrasonic cavitation
Combinational treatment*	**Membrane separation**	Membrane bioreactor (MBR)
	+ biodegradation	Membrane immobilized enzyme
Integrative treatment*	**Membrane + photo-oxidation**	Membrane immobilized enzyme
		TiO_2 immobilized on membrane

*Other combinatorial and integrative processes can ve assembled by coupling each set of the subcategories in a series arrangement.

Coagulation-floculation process involve the addition of an inorganic coagulant that has to be removed, together with the removed PhACs, at the end of the process. These methods

showed poor results at removing PhACs from wastewater effluents, and only more apolar micropollutants such as diclofenac (DCF) showed removals up to 46% as maximum, being unsuitable for water-soluble PhAC, in spite of being used either as a pre-treatment or a post-treatment combined to CAS (Matamoros and Salvadó, 2013; Suarez et al., 2009). Besides, some authors have stated that this technique would deeply depend on factors such as wastewater composition (e.g. being inhibited by dissolved organic matter and promoted by high fat water content), pH and temperature among others (Luo et al., 2014).

In relation with adsorption treatments, zeolite, bentonite and activated carbon adsorption (both powder and granular) are the most popular technologies. In the case of activated carbon it is easy to be applied at full-scale municipal WWTP and it has shown higher efficiency in PhACs removal than coagulation-floculation with removals over 90% for DCF , CBZ and other PhACs when applied as CAS effluent post-treatment (Kovalova et al., 2013). Nevertheless these technologies present short life cycles and depend on adsorbate and adsorbent features, for instance contact time, adsorbent concentration or regular adsorbent regeneration (specially in granular activated carbon). Although achieving efficient removals in non-polar characteristics (K_{ow} > 2) they are not the best option for highly contaminated wastewaters (Verlicchi et al., 2010).

In comparison, powder activated carbon (PAC) presents higher removal yields than granular activated carbon (GAC) when used in WWTP, since the adsorption sites are more rapidly saturated in the second case (Bolong et al., 2009). These differences between PAC and GAC in the particle size, also entail particular operational requirements for each particular treatment such as the optimal dose in PAC, GAC regeneration, or contact time in both technologies.

Membrane processes are preferentially used in wastewater with low suspended solid (SS) content and include nanofiltration, reverse osmosis and forward osmosis. Sometimes microfiltration and ultrafiltration are included in this group, but because of their high molecular weight cut off, these two treatments are generally unable to retain PhACs by size exclusion. Thus, membrane based processes present higher removal rates of high molecular weight organic micropollutants, excellent quality of effluent, modularity and ability to integrate with other systems. However, they do display important drawbacks as membrane fouling and clogging and high energy consumption and operating costs or a

decrease in performance when increasing permeate recovery, as well as a poor understanding of PhACs removal mechanisms (Taheran et al., 2016).

Instead of physical separation treatments, chemical transformation allows micropollutant degradation, yielding, in the cases where it is completely achieved, CO_2 and inorganic ions. There are several treatments available to be implemented (Table 1.1), being redox based technologies the most common ones. Also known as advanced oxidation processes (AOP), and unlike traditional oxidation methods, oxidative reactions are triggered both directly and indirectly (usually by creating stronger oxidizing agents with lower selectivity such as $\cdot OH^-$) (Gerrity et al., 2011). These techniques include the combination of ozonation (O_3), Fenton, UV photolytic and photocatalytic processes, H_2O_2 and TiO_2, which not only degradate micropollutants but also disinfect waters. Alternative techniques to AOPs are electrodegradation, electro-coagulation and ultrasonic cavitation. Their main advantages are high removal rates in relatively short-time operation, lack of waste generation and unspecific reactivity; however, the efficiency of these technologies is affected by PhAC's chemical structure, and they may produce harmful degradation by-products affecting the final water quality. Besides, their operational costs can be quite high compared to other technologies (Luo et al., 2014; Ribeiro et al., 2015).

On the contrary, a few biological treatments are being studied to achieve new alternatives of PhAC removal from a more economical and sustainable approach. At the present day, the most common biodegradation treatments have been based on membrane bioreactors (MBRs), that combine at the same time biological activity of CAS treatments and membrane filtration membrane filtration, ultrafiltration and even nanofiltration recently (Taheran et al., 2016), but also attached growth bioreactors and immobilized enzymatic bioreactors.

Membrane bioreactors offer high effluent quality, easy scale up in WWTP, small carbon footprint and limited space requirement, and due to higher hydraulic and sludge retention time (HRT and SRT, respectively) and micropollutant's adsorption to the sludge, its removal efficiency can be as high as other physicochemical treatments. Operational costs are lower than other technologies offering the same performance, but membranes can increase the final capital and operational costs. Nonetheless, drawbacks of this technique are higher operating times, the need of an aged sludge (>100 days), and the high

dependence on biomass, that can be inactivated by temperature (according to Hai et al. (2011) optimal range is between 10-35ºC) or changes in pH, aeration and conductivity, among others (Kovalova et al., 2012). MBRs have been compared to CAS processes frequently, since both systems are cost effective technologies and their performance can be equivalent for certain micropollutant removal. However, greater and steadier elimination for a wide range of PhACs has only been obtained with MBRs (Luo et al., 2014).

Attached growth bioreactors are an alternative biological treatment were biomass grows attached on inert carriers, either fixed or mobilized in suspension (Guo et al., 2012). Better oxygen transfer is one of its main advantages, together with higher biomass concentration and its organic removals allow to work with wastewaters of high organic loading, providing lower operational costs and lower space requirements. As any other biological reactors, biomass age and HRT are also crucial factors. There are two main types of attached growth reactors: fixed bed or biofiltration processes (that include trickling filters, with sand and activated carbon as main immobilization supports) and moving bed bioreactors, containing a great variety of carriers. PhACs removal has not been deeply studied in attached growth systems, although preliminary results are promising. In moving bed biofilm systems, removals over 60% in 24 h for common PhACs such as DCF (Falås et al., 2012), whereas in fixed biological activated carbon, elimination rates of over 90% of CBZ, sulfamethoxazole, gemfibrozil and DCF were obtained (Reungoat et al., 2011).

Immobilized enzymatic bioreactors are being studied recently to integrate oxidation and membrane separation in one system, where the key factor is mass transfer phenomena. Enzymatic immobilization provides prolonged enzyme capacity, easier process scaling up, high flow rate capability and a reduction in operational and capital costs. However, there is a scarcity of studies related with PhACs degradation using these combined enzymatic techniques and it is not clear yet if capital and operational costs might still exceed MBR investment (Taheran et al., 2016).

The use of cell-free enzymes isolated from biological systems avoid low biomass content drawbacks of biological reactors such as long HRT and large volumes, and allow the concentration of enzymes for a selective and precise strategy to degrade micropollutants. Enzymatic remediation of PhACs has been proven to be effective with laccases and peroxidases from either fungi, bacteria or even plants (horseradish peroxidase) (Méndez

et al., 2017; Stadlmair et al., 2017). Other enzymes may also play important roles but need further research such as feruloyl esterases (Dilokpimol et al., 2016; Faulds, 2010), as "lesser understood" members of the phenolic-acting enzyme family, same as laccases and peroxidases, but being involved in the hydrolysis of ester linkages in phenolic compounds. However, one of the main drawbacks in these treatment is the need of mediators to guarantee enzymatic degradation catalysis, which sometimes can increase economical costs and even induce unspecific toxicity (Becker et al., 2016).

However, there are other promising biological technologies based on biodegradation that remain barely explored, and this thesis wants to contribute to the development of fungal biodegradation as a feasible alternative to remove micropollutants (especially PhACs) from the aquatic environment.

1.4. Fungal biodegradation

Fungal biodegradation has become a non-conventional promising approach among wastewater treatment technologies to remove micropollutants (including several contaminants of emerging concern such as endocrine disruptors or PhACs). Thanks to their nonspecific ligninolytic enzymatic system, fungi can remove a wide range of complex industrial contaminants, including xenobiotics (Harms et al., 2011). In addition, instead of bacteria that employ pollutants as carbon and nitrogen sources, fungi can co-metabolize certain pollutants. Thus, extra sources of C and N may be needed, but biomass may mineralize pollutants completely at the end (Badia-Fabregat et al., 2014). Fungi can also attack low-soluble compounds avoiding their toxicity, through secreted extracellular enzymes. Since more than thirty years ago, when fungi successfully proved to degrade organochlorines such as dichlorodiphenyltrichloroethane (DDT) and its toxic metabolites (Fernando et al., 1989), fungal biodegradation has been applied to remove recalcitrant pollutants from explosive 2,4,6-trinitrotoluene (TNT) (Anasonye et al., 2015) to polycyclic aromatic hydrocarbons (PAHs) (Steffen et al., 2003), including olive mill wastewater (Olivieri et al., 2006), dyes and pigments (Blánquez et al., 2004), UV filters (Badia-Fabregat et al., 2012b) and, at the end, pharmaceuticals (Marco-Urrea et al., 2009).

However, most of these studies were only performed at Erlenmeyer scale. But mainly during the last decade, bioreactor operation has been steadily implemented (either using

fungal biomass or enzymatical extracts of it) such as membrane bioreactors, with both fungi and bacteria (Taheran et al., 2016), enzymatic membrane bioreactors (Becker et al., 2016; de Cazes et al., 2016; Ji et al., 2016), or fluidized bed bioreactors (FBBs) (Blánquez et al., 2008). This last bioreactor, along with fungal pelleted reactors (Espinosa-Ortiz et al., 2016) have shown plenty of promising results, in particular for the biodegradation of many PhACs in FBBs with *T. versicolor*: from continuous sterile conditions with spiked water (Carlos E Rodríguez-Rodríguez et al., 2012), to non-sterile hospital wastewater (HWW) (Badia-Fabregat et al., 2016); or fedbatch with non-sterile urban water containing PhACs (Cruz-Morató et al., 2013a) and even for non-sterile hospital wastewater (HWW) (Gros et al., 2014). Nevertheless, in spite of these favourable results, most of fungal species used until today still permit some compounds to remain recalcitrant, which propels further research into finding new biodegradation candidates.

1.4.1. A brief insight into mycology

But first of all, what kind of microorganism are fungi? Fungi are heterotrophic eukaryotic organisms, with chitin in their cell walls and nutrition based on complex extracellular and intracellular enzymatic systems to digest polymers into less complex and easily available nutrients. The *hypha* is the characteristic growth form of fungal cells surrounded by cell walls, with filamentous structure, enabling the fungus to extend as it grows and exploit new environments and substrates. *Hypha* can also differentiate to form a wide variety of structures, such as sporangia, conidiophores, rhizoids and fruit bodies. A network of *hyphae* creates a macroscopic structure called *mycelium or mycelial mat*, gathering moisture and nutrients (Gooday, 1995; Goodell et al., 2008; Stamets, 2005). This mycelium can grow expanding networks of thousands of kilometres in cellular mats achieving the greatest mass of any individual organism on this planet (Stamets, 2005). However, in a lab scale and for certain fungi, by culturing it in submerged conditions and orbital shaking, this mycelium can grow in aggregated granules called pellets.

1.4.2. Ligninolytic fungi

Fungi play an important role in ecosystem's nutrient recirculation. Among many other substances, certain species of fungi are capable of degrading one of the most complex and recalcitrant substances found in nature: lignin. Lignin is the second most abundant

renewable organic compound just below cellulose (Liu et al., 2011) composing 15-25% land-produced biomass (Cui and Dolphin, 1989). Lignin is complex, heterogeneous and insoluble, being the first step in lignin's fungal biodegradation done by an extracellular oxidation using oxidoreductase enzymes (Martínez et al., 2005).

Ligninolytic or lignin-degrading fungi (LDF) are a heterologous group of saprophytic fungi. LDF are classified into three major categories based on the type of wood decay caused by these organisms: white-rot fungi (WRF), brown-rot fungi (BRF) and soft-rot fungi (SRF). Among these three groups, WRF are the most effective lignin degraders and have been the most extensively studied group. Taxonomically WRF comprise a heterogeneous collection of several hundreds of species of basidiomycetes (Ainsworth et al., 1973) and some ascomycetes (Eaton and Hale, 1993).

WRF, mainly basidiomycetes, are the most ubiquitous wood degrading organisms, and their enzymatic systems are able to decompose at the same time lignin, hemicelluloses, and cellulose. The final product obtained from rotten wood after a fungal attack is a cellulose-enriched white material, the so-called "white rot". Hence, independently from their enzymatic systems, white-rot fungi can degrade lignin selectively or simultaneously with cellulose (Riley et al., 2014b). Two white-rot patterns have been described in different types of wood, namely selective delignification, also called sequential decay in the case where lignin is degraded first and then cellulose, and simultaneous rot where both lignin and cellulose are decomposed at once. (Martínez et al., 2005)

1.4.3. Enzymatic systems involved in degradation of pollutants

Ligninolytic fungi rely on two main mechanisms for the biodegradation of compounds. The first primary attack takes place most of the time by extracellular oxidation, driven by lignin modifying enzymes such as laccase (lac) and other manganese (MnP) and lignin peroxidase (LiP) enzymes grouped in metal-containing oxidorreductases. The reactions catalysed by these enzymes include lignin depolymerization and dimethoxylation, decarboxilation, hydroxylation and breakdown of aromatic rings (Harms et al., 2011).

After extracellular oxidation, the second mechanism of enzymatic action involves intracellular degradation, including tyrosinases, transferases, dehalogenases, quinone

reductases, but mainly cytochrome P450 monooxigenases and nitroreductases. Enzymatic reactions occur via either oxidation and/or reduction, or by the conjugate formation by transferases (Jollow et al., 1977). However, when this enzymatic cocktail is applied to micropollutants and complex chemical substances, the final step is not always a complete mineralization of the compound but instead it may result in intermediate transformation products or only a bare sorption of the unaltered compound into the biomass (Badia-Fabregat et al., 2014; Golan-Rozen et al., 2015; Gros et al., 2014; Marco-Urrea et al., 2010a; Polesel et al., 2016)

During lignin degradation, WRF secrete mainly two different groups of enzymes acting synergistically: laccases and lignin-modifying peroxidases. The main difference is the electron acceptor (O_2 for laccases and H_2O_2 for peroxidases), but the secretion and activity of these enzymes is affected by many different factors such as concentration changes in the source of N and C, the presence of certain metals (such as Cu^{2+} or Mn^{2+}), pH or even temperature (Janusz et al., 2013).

Laccases (oxygen oxidoreductases, EC 1.10.3.2)

Present among fungi in both Ascomycota and Basidiomycota divisions, these enzymes target extracellular organic compounds and its reaction mechanism is O_2-dependent. They are able to directly oxidase phenols, aromatic compounds, and a wide range of pollutants in the presence of redox mediators, small molecular weight substrates, acting as electron donors. Its activity is mostly acidic. Protein structure and size differs between species and even among strains, however most of them are monomeric globular proteins of 60-70 kDa with an isolectric point around pH 4.0 (Giardina et al., 2010), its conserved core in its active site contains four copper atoms in different oxidation states, responsible for the catalysis of aromatic substrates oxidation as seen in Figure 1.2 (Harms et al., 2011). Laccases can also decarboxylate and demethylate, but oxidation of non-phenolic compounds only occur in the presence of mediators (Asgher et al., 2008a).

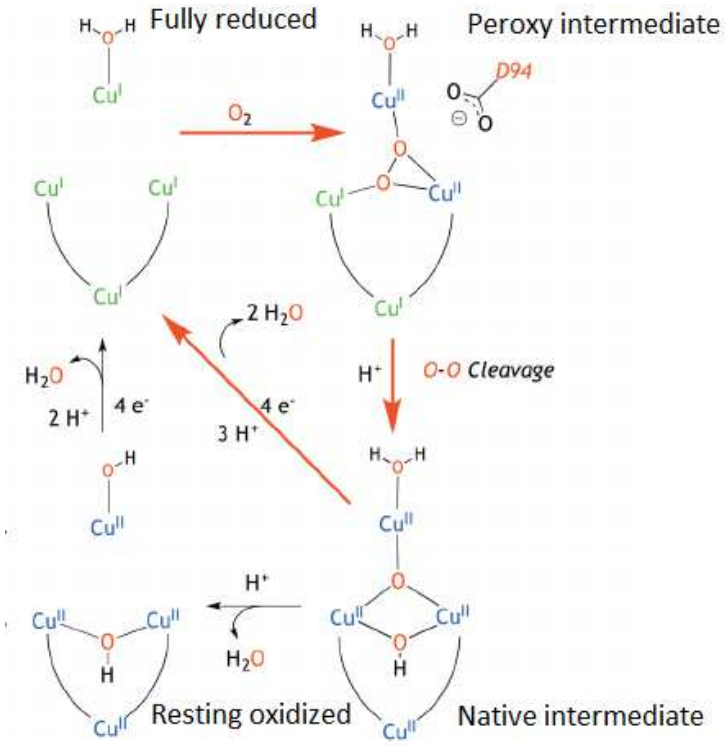

Figure 1.2. Redox reaction to reduce one molecule of oxygen to water by multicopper oxidases (Giardina et al., 2010). Using this mechanism, laccases generate highly reactive radicals (phenoxy radicals) that produce new oxidations in further reaction chains. Figure adapted from Heppner et al. (2014).

Lignin peroxidases (EC.1.11.1.14)

Lignin peroxidases are extracellular glycoproteins containing iron in its active site and requiring H_2O_2 for catalytic activity (Figure 1.3). Their redox potential is higher than laccases and can directly oxidase phenolic compounds, but unlike laccase they are rapidly inactivated. LiPs are capable of oxidizing recalcitrant phenolic and non-phenolic lignin-like substrates in the acidic pH range (Harms et al., 2011).

Manganese peroxidases (EC 1.11.1.13)

Manganese peroxidases (also known as MnP) are extracellular glycosylated enzymes containing a hemo group within its active site. They are involved in H_2O_2-dependent reaction, via one-electron oxidation of Mn^{2+} to Mn^{3+}, creating enough redox potential to oxidate aromatic amines and phenols (catalytic cycle can be seen in Figure 1.4). Its activity is acidic and its substrate range can be extended in the presence of co-oxidants (Harms et al., 2011). Catalytic cycle of MnP resembles LiP catalytic cycle, including the native ferric

enzyme as well as the reactive intermediates “compound I” and “compound II”. However, MnP uses Mn^{2+} as main substrate (electron donor) to oxidize it into highly reactive Mn^{3+}, stabilized by fungal chelators such as oxalic acid. The product Mn^{3+} forms a complex with organic acids and diffuses away from the enzyme to oxidize other materials (Hofrichter, 2002).

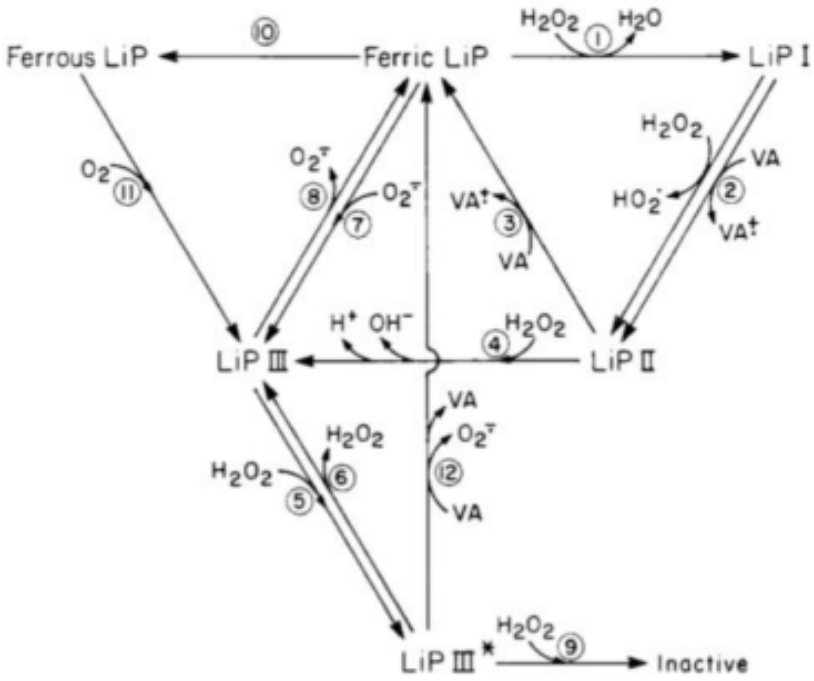

Figure 1.3. Catalytic cycle of lignin peroxidase. The catalytic cycle starts with H_2O_2 oxidizing LiP to form a two-electron intermediate (LiP-compound II), which oxidizes substrates by one electron completing the cycle. In spite of this activation, in cases where substrate concentration is low, LiP-compound II can react with H_2O_2 inactivating the enzyme (LiP-compound III) (Wariishi and Gold, 1990).

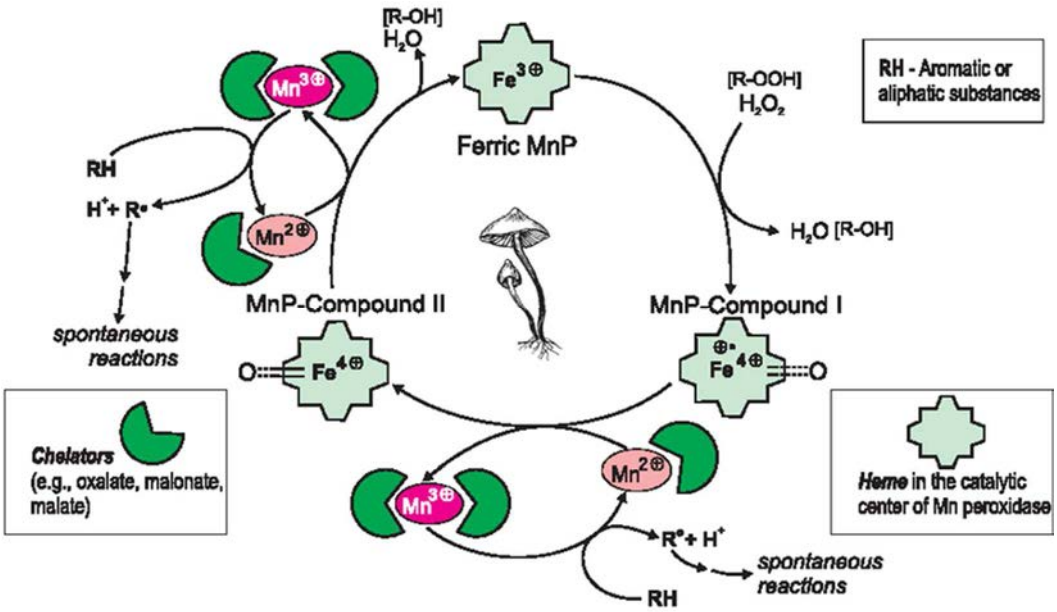

Figure 1.4. Catalytic cycle of MnP, from Tripathi and Dixit (2016), adapted from Hofrichter (2002).

Versatile peroxidases (EC 1.11.1.16)

Extracellular acidophilic enzymes present in *Pleurotus*, *Bjerkandera* and *Trametes* genera, sharing the catalytic properties of MnP and LiP, with high affinity for Mn^{2+} and catalysing its oxidation into Mn^{3+}, and subsequently oxidizing organic compounds. Although, unlike MnP, it can oxidize directly phenolic and non-phenolic substrates without Mn^{2+}. Its catalytic cycle is constituted by the sum of LiP and MnP cycles.

Cytochrome P450

Present in many fungi and other microorganisms, this conserved intracellular enzymatical complex catalyses hydroxylation, heteroatom monooxygenation (adding one atom of O_2 into a substrate molecule, with concomitant reduction of the left atom into H_2O), dealkylation, epoxidation of C=C bonds, reduction and dehalogenation, epoxidation and hydroxylation of many aromatic pollutants (Du et al., 2017; Harms et al., 2011).

Nitroreductases

Intracellular enzymes being NAD(P)H-dependent reductors of nitroaromatics to hydroxylamino and amino(nitro) compounds, and of nitro functional groups of N-containing heterocycles. They are widespread among fungi (Esteve-Núñez et al., 2001).

Quinone reductases and reductive dehalogenases

Present both in the cell bound of mainly Basidiomycota, the former performs quinone detoxification reducing it depending on NAD(P)H, whereas the latter involve two-component system of glutathione transferase and a soluble glutathione conjugate reductase allowing the chlorine removal and the release of reduced dechlorinated compounds (Harms et al., 2011).

Miscellaneous transferases

Intracellular enzymes widespread among fungi, they are involved in the production of glucoside, glucoronide, xyloside, sulphate or methyl conjugates from hydroxylated compounds (Chang et al., 2003; Mathieu et al., 2013).

Tyrosinases and Haem-thiolate peroxygenases

Both enzymes are active from acidic to alkaline pH range, Tyrosinases are mainly intracellular whereas Heme-thiolate peroxygenases are exclusively extracellular. While these second enzymes mainly perform a peroxygenation of aromatic, aliphatic and heterocyclic compounds (depending on H_2O_2), tyrosinases hydroxilate monophenol to o-diphenol and the oxidation of this last molecule into catechol, allowing the oxidation of phenols, even highly chlorinated (Harms et al., 2011).

1.4.4. Fungal species used in this thesis for PhACs removal.

All fungi used in this thesis are obligated aerobic and part of the Basidiomycota division and the agaricomycetes class, although they are divided in two main groups. *Trametes versicolor*, *Ganoderma lucidum, Irpex lacteus* and *Pycnoporus sanguineus* belong to polyporales order, which is a large order of fungi whose species have been commonly used for biodegrading micropollutants in water (Bernats and Juhna, 2015; Marco-Urrea et al., 2009; Vasiliadou et al., 2016), and in specific for *T. versicolor*, its PhAC removal capacities have been proved extensively (Badia-Fabregat et al., 2016; Cruz-Morató et al., 2013a; Carlos E Rodríguez-Rodríguez et al., 2012). In contrast, *Pleurotus ostreatus, Agrocybe erebia*, *Gymnopilus luteofolius*, and *Stropharia rugosoannulata* are included in the agaricales order, whose members have rarely been used for these types of alternative biological treatments (Anasonye et al., 2015; Kähkönen and Hakulinen, 2011), and specifically for the last three, its use in PhACs degradation at the present day has not been reported before. Fungi from the last order share many oxidative enzymes with polyporales, such as versatile peroxidase or manganese peroxidase, but may also have exclusive enzymes such as aromatic peroxygenases (heme-thiolate peroxidases) (Harms et al., 2011; Hofrichter et al., 2010).

Appearance of the studied fungal species in the wild can be seen in Figure 1.5, whereas particular aspects and curiosities can be consulted in Table 1.2.

Figure 1.5 Fruiting bodies from the fungal species used in this thesis. From up left, to down right: *A. erebia*, *G. lucidum*, *G. luteofolius*, *I. lacteus*, *P. ostreatus*, *P. sanguineus*, *S. rugosoannulata* and *T. versicolor* (Pictures credits: Tatiana Bulyonkova, Eric Steinert, Michael Wood, Otto Miettinen, James Lindsey, José Roberto Peruca, Ann F. Berger, Francesc Castellet-Rovira, respectively, all under CC-BY 2.0 license).

Table 1.2 Comparison of some of the main characteristics for the eight fungal species used in this thesis.

Fungus	Family	Distribution	Nutrition	Mushroom morphology	Spore colour	Curiosity	References
A. erebia	Strophariaceae	North America and Europe	Saprotrophic	Ringed and consistently dark brown cap	Dark	Slimy when fresh	(Singer, 1939)
G. lucidum	Ganodermataceae	Worldwide distribution across temperate and tropical regions	Saprotrophic	Soft texture, white when young, turning to brown afterwards	White/brown	Edible, used in traditional Chinese medicine.	(Bazzalo and Wright, 1982; Cao et al., 2012)
G. luteofolius	Strophariaceae	North America	Saprotrophic	Reddish brown cap with pale gills	Rusty orange	Psychoactive	(Stamets, 1996)
I. lacteus	Steccherinaceae	Temperate areas worldwide	Saprotrophic	White milk to pale grey crust	White	Very polymorphic depending on the growth conditions.	(Gilbertson and Ryvarden, 1986)
P. ostreatus	Pleurotaceae	Temperate and subtropical forests	Saprotrophic/ Nematophagous	Bright grey cap with pale gills	White	Edible, mycelia can kill and digest nematodes to obtain nitrogen	(Barron and Thorn, 1987; Stamets, 2000)
P. sanguineus	Polyporaceae	Tropical	Saprotrophic	Bright orange to red, corky and probably toxic	White	Inedible, tough, used in traditional medicine for heavy metal absorption	(Ryvarden and Johansen, 1980)
S. rugosoannulata	Strophariaceae	North America and Europe	Saprotrophic/ Nematophagous	Bright red cap, purple gills and white base	Purple	Edible, as *P. ostreatus*, its mycelia can kill and digest nematodes	(Luo et al., 2006; Santos da Silva et al., 2017)
T. versicolor	Polyporaceae	Worldwide	Saprotrophic	Multicoloured long cap without gills	White to yellow	Tough mushroom (even when fresh), non-edible	(Ryvarden and Johansen, 1980)

1.4.5. PhACs removal studies with agaricales fungi

In spite the publication of several studies regarding micropollutant removal with the four agaricales fungi and *P. sanguineus,* these fungi have been mostly related with micropollutant biodegradation for industrial and chemical waste, but studies aiming to apply them in PhACs removals are very scarce : *A. erebia* (and other species in the *A. praecox* cluster) showed potential biodegradability for liquid cultures of chlorinated DDT pesticide and polycyclic aromatic hydrocarbons (PAH) (Steffen et al., 2003; Suhara et al., 2011); *G. luteofolius,* on the contrary, has only been reported to degrade TNT in soil (Anasonye et al., 2015). Besides, *S. rugosoannulata* has proven able to degrade industrial dyes such as Basic Blue 22 and Acid Red 183 in liquid cultures (Jarosz-Wilkołazka et al., 2002). *P. ostreatus* was used for DDT bioremediation in soil (Purnomo et al., 2010). Laccase from this fungus degraded endocrine disruptors (Macellaro et al., 2014) whereas this same enzyme for P. sanguineus could remove up to 50% of DCF (Rodríguez-Delgado et al., 2016). However, only *P. ostreatus* and *P. sanguineus* have been clearly reported as removal agents for PhACs, in the case of CBZ, through degradation by *P. ostreatus* (Golan-Rozen et al., 2015), whereas laccase extract from *P. sanguineus* was able to remove up to 95% of triclosan and nonylphenol (Ramírez-Cavazos et al., 2014).

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2. Objectives and thesis overview

2.1. Objectives

The aim of this thesis was to address four main factors concerning the biodegradation of Pharmaceutical Active Compounds (PhACs) in wastewater:

I. Identification of new potential candidates for PhAC biodegradation.

- To study the potential PhAC removal capabilites for barely studied fungi such as *Agrocybe erebia*, *Stropharia rugosoannulata* and *Gymnopilus luteofolius* and to compare their performance to well-known and deeply studied fungi such as *T. versicolor*.

II. Assessment of biodegradation capabilities for several ligninolytic fungi in the treatment of recalcitrant compounds and the study of their metabolites and transformation products.

- To investigate the removal mechanisms (biodegradation versus sorption) of several recalcitrant PhACs by several fungal species.
- To assess the involvement of the main fungal enzymatic systems (laccase and cytochrome P450) in the degradation of PhACs.
- To evaluate fungal degradation for several recalcitrant PhACS (ATL, DCF, KTP, MTP, MTPA, ODMVFX and VFX) and to identify, when possible, their fungal metabolites and TPs.

III. Implementation of the treatment of real hospital wastewater (HWW) in bioreactor.

- To assess the removal of PhACs contained in HWW by new fungal candidates in fluidized bed bioreactors.

IV. Assessment of new strategies for the operation in fungal fluidized bed bioreactors.

- To evaluate the implementation of a Biosand Filter as a HWW pretreatment to prevent microbial competition in the fungal bioreactors.

- To estimate the requirements of carbon and nitrogen source supplementations, as well as pH control for fungal species (different from *T. versicolor*).

2.2. Thesis overview

Chapter 4:

In this chapter, a biosand filter was set in order to assess its capability to reduce bacterial load, aiming for its use as a fungal bioreactor pretreatment to avoid bacterial competition. Electrical conductivity and pH were assessed among other parameters, but cfu/ml determined the effectiveness of the application, which was lower than expected and hence rejected to accomplish its initial purpose. On the contrary, the biosand filter was fed with hospital wastewater with known pharmaceutical active compounds (PhACs) and its microbiota achieved considerable removals for ibuprofen and ciprofloxacin. A molecular study to identify potential PhACs degradation candidates among this microbiota was performed. This molecular study was performed in the Microbiological and Genetics Department of the UAB.

Biosand filter studies focused on molecular characterization are being written on a journal article called "*Biosand Filter: a new approach to Pharmaceuticual Active Compounds removal in wastewater*".

The work in this chapter, from the experimental design, to biosand filter's operation and molecular biology analyses have been entirely performed by the author.

Chapter 5

In this chapter, an screening experiment of six fungal species was performed to determine their removal capabilities for six different recalcitrant PhACs. Sorption and biodegradation removal mechanisms were studied by different methodology, and it was concluded that sorpted PhACs were not degraded within fungal biomass after 6 days of treatment. On the other hand, laccase purification methodologies were investigated for up to eight

different fungal species with the aim to assess the involvement of this enzyme in PhACs degradation.

This chapter was performed in collaboration with Daniel Lucas, responsible for PhACs analysis and fungal biomass sorption studies. On the other hand, the purification of laccase induced fungal extract was performed in collaboration with Dra. Rostro and Dr. Parra from the Environmental Bioprocesses Group of *Instituto Tecnológico de Monterrey* (Mexico) during a PhD internship. The rest of work, from experimental design to data analyses, was done by the author.

A manuscript called "Stropharia rugosoannulata *and* Gymnopilus luteofolius*: promising fungal species for pharmaceutical biodegradation in contaminated water*" has been accepted with minor revisions at the Journal of Environmental Management.

Another manuscript referring to sorption studies with fungal biomass called "*The role of sorption processes in the elimination of pharmaceuticals in fungal treatment of wastewater*" has been accepted with minor revisions at the journal Science of the Total Environment.

A short communication is being prepared on the basis of laccase purification studies.

Chapter 6

In this chapter the removal of four recalcitrant PhACs such as metoprolol (MTP), metoprolol acid (MTPA), venlafaxine (VFX) and o-desmethylvenlafaxine (ODMVFX), was evaluted for three different fungi: *G. lucidum*, *P. ostreatus* and *T. versicolor.* Besides, two main transformation products of VFX fungal degradation (o-desmethylvenlafaxine and n-desmethylvenlafaxine) were identified.

On regarding the promising results of G. lucidum in the degradaiton of MTP and MTPA, a batch bioreactor was set, in which its removals were higher than in Erlenmeyer scale.

Studies with VFX, ODMVFX and their transformation products, have been presented and discussed in a manuscript called "*Elimination of transformation products and NDMA precursors of Venlafaxine and O-desmethylvenlafaxine from contaminated waters by fungal biodegradation*", being under revision at the Biochemical Engineering Journal. In

relation with MTP and MTPA removal studies, the publication of the obtained results in a scientific article has also been projected.

The work in this chapter was done entirely by the author except for PhACs analysis that were carried out by Dr. (Marta) Llorca (in the case of VFX, ODMVFX and their respective TPs) and Adrián Gil (for MTP and MTPA).

Chapter 7

In this chapter, the collected information about PhACs removal gathered along the previous experiments was condensed in the implementation of three different fungal bioreactor applications.

In the first case, the removal of an antibiotic cocktail under non-sterile conditions was performed with a bioreactor of *T. versicolor*, firstly with osmosed water, and secondly with hospital waste water. When comparing the antibiotic removals obtained with an enzymatic membrane reactor (EMR), *T. versicolor* obtained higher removals in both cases, specially noticeable for hospital wastewaters.

In the second case, the elimination of three recalcitrant PhACs (atenolol, diclofenac and ketoprofen) was assessed in a batch reactor first, and a second continuous reactor with *P. ostreatus* with almost complete removals for all of the PhACs used. Ttransformation products for diclofenac and ketoprofen were also identified.

Finally, a set of bioreactors with *S. rugosoannulata* to eliminate carbamazepine, diclofenac and iopromide were performed with noticeable elimination rates and showing operational advantages for this fungus such as the ability to lower the wastewater pH or to obtain its carbon source from high-COD wastewater instead of external glucose supply.

This work was done in collaboration with Dr. Varela for the analysis of antibiotics and Dr. Becker in the operation of EMR. The operation of the Batch reactor operation and specific degradation experiments with *P. ostreatus* were performed by Dr. Palli, MNR studies for transformation products of diclofenac and ketoprofen identification were performed in collaboration with Dr. Pérez-Trujillo from the MNR service of the UAB. Olalla Borruel

operated *S. rugosoannulata* reactors. The rest of work, including the T. versicolor and continuous *P. ostreatus* reactor operation, corresponded to the author.

A manuscript called "Removal of antibiotics from hospital wastewater using alternative decontamination treatments based on *Trametes versicolor* and enzymatic membrane reactor" is prepared for submission to Biodegradation Journal.

The studies with *Pleurotus ostreatus* reactors have been reported in a journal article recently accepted in the journal of Biotechnology Progress called "*Preliminary evaluation of* Pleurotus ostreatus *for the removal of selected pharmaceuticals from hospital wastewater*". Another publication with *S. rugosoannulata* results is being prepared.

3. General methodology

3.1. Chemical compounds and reagents

Some reagents used in most of the experiments, such as glucose or dimethyl succinic acid, among others, were purchased from Sigma-Aldrich (Barcelona, Catalonia). Malt extract and high performance liquid chromatography (HPLC) solvents such as ethanol and acetonitrile were provided by Scharlau (Barcelona, Catalonia). All organic solvents used were of high purity grade. Methanol were supplied by Merck (Darmstadt, Germany). All other chemicals and reagents were of the highest available purity and purchased from Sigma-Aldrich.

PhACs used in the studies of individual degradation can be found at Table 3.1, showing their physicochemical characteristics, chemical structure and supplier.

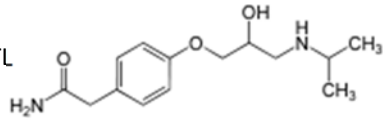
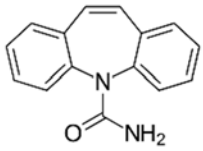
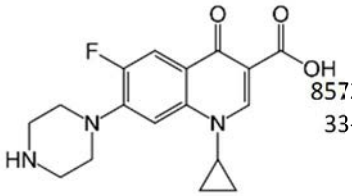
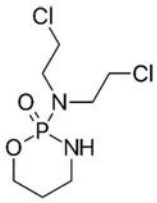
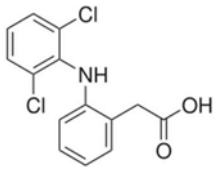
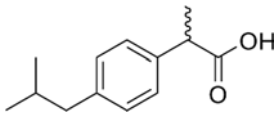
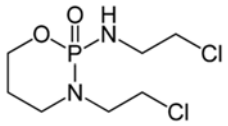
3.2. Hospital wastewater (HWW) effluents

Hospital wastewater used in the experiments was collected from the main sewer manifold of two different sites and filtered through a strainer before its use:

HWW (1): Dr. Josep Trueta Universitary Hospital (Girona, Catalonia). The main characteristics of the wastewater were: 350-500 mg/L O_2 of COD, 150-260 mg/L of total organic carbon (TOC), 48-70 mg/L of $N\_NH_4^+$, 6.1-11.1 mg/L of total suspended solids (TSS), and 8.0 – 8.4 of pH range. The pH of the wastewater was adjusted to 4.5. When needed, HWW sterilization was performed at 121ºC during 30 min.

HWW (2): Sant Joan de Déu Hospital (Barcelona, Catalonia). This HWW main characteristics were pH range of 7.8-8.7; COD: 633-1012 mg/L O_2; N_NH4^+:9.9-36 mg/L and TSS: 193-284 mg/L. The pH of the wastewater was adjusted to 4.5 before sterilization at 121ºC during 30 min.

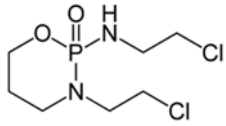
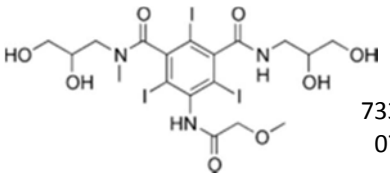
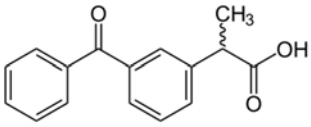
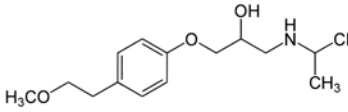
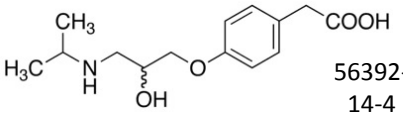
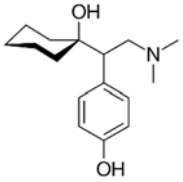
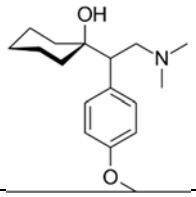
Table 3.1 Pharmaceutical active compounds (PhACs) used in this thesis.

Compound	Abbreviation	Chemical Structure	CAS number	Log Kow*	Supplier	Purity
Atenolol	ATL		29122-68-7	0.16	Sigma-Aldrich	≥98%
Carbamazepine	CBZ		298-46-4	2.45	Sigma-Aldrich	≥99%
Ciprofloxacin	CPX		85721-33-1	0.28	Sigma-Aldrich	≥98%
Cyclophosphamide	CFD		50-18-0	0.63	(EDQM**) Reference Standards	≥99.9%
Diclofenac (sodium salt)	DCF		15307-79-6	4.51	Sigma-Aldrich	99%
Ibuprofen	IBP		15687-27-1	3.97	Sigma-Aldrich	≥98%
Ifosfamide	IFD		3778-73-2	0.86	(EDQM) Reference Standards	≥99.9%

***** Log Kow data are correlated with water solubility and bioconcentration for aquatic life. Log Kow values were obtained from US Environmental Protection Agency's EPISuiteTM Database (estimates values were obtained from KowWin® software v1.67 – Syracuse Research Corporation SRC – Syracuse NY 2000).

**European Directorate for the Quality of Medicines and Healthcare (European Pharmacopoeia Reference Standards)

Table 3.1 .**(Continuation)** Pharmaceutical active compounds (PhACs) used in this thesis

Compound	Abbreviation	Chemical Structure	CAS number	Log Kow	Supplier	Purity
Ifosfamide	IFD		3778-73-2	0.86	(EDQM) Reference Standards	≥99.9%
Iopromide	IPD		73334-07-3	2.33	European Pharmacopeia	≥98%
Ketoprofen	KTP		22071-15-4	3.12	Sigma-Aldrich	≥98%
Metoprolol	MTP		51384-51-1	1.88	Sigma-Aldrich	≥98.5%
Metoprolol acid	MTPA		56392-14-4	-2.34 (estimated)	Toronto Research	98%
O-desmethyvenlafaxine	ODMVFX		93413-62-8	2.72 (estimated)	Toronto Research	≥99.8%
Venlafaxine	VFX		93413-69-5	3.28	Sigma-Aldrich	≥98%

3.3. Coagulation-Flocculation of HWW

HWW (2) from Sant Joan de Déu Hospital was coagulated-flocculated. This pretreatment involved the addition of coagulant HyflocAC50 and flocculant HimolocDR3000. Coagulant and flocculant concentration was modified depending on the characteristics of HWW batch in order to achieve an absorbance at λ_{max286} of 0±0.1, although in general, coagulant was added at a range of 150-37 mg/L during 2 min at 200 rpm, whereas flocculant concentration was 4.5-15 mg/L, and maintained for 15 min at 20 rpm (Derypol, Barcelona, Catalonia). Table 3.1 shows the characterization of HWW before and after this pretreatment.

Table 3.2 Physicochemical characterization of the hospital wastewater

HWW Sampling date		30/03/2016
	Non flocculated	Flocculated
pH	8.1	8.2
Conductivity ($mS \cdot cm^{-1}$)	2.3	2.4
Absorvance at 650 nm	0.157	0.000
Chloride ($mg\ Cl \cdot L^{-1}$)	462.6	448.0
Sulfate ($mg\ S \cdot L^{-1}$)	67.6	67.7
Nitrate ($mg\ N \cdot L^{-1}$)	6.3	3.6
Phosphate ($mg\ P \cdot L^{-1}$)	0.1	0.3
Ammonia ($mg\ N \cdot L^{-1}$)	34.1	33.8
TSS ($mg \cdot L^{-1}$)	108	16
COD ($mg\ O2 \cdot L^{-1}$)	174	87
DIC ($mg \cdot L^{-1}$)	98 ± 1	91 ± 2
DOC ($mg \cdot L^{-1}$)	25 ± 7	29 ± 3

3.4. Microorganisms

All fungi species were subcultured in Petri dishes with malt extract at 2% with agar (1.5%), corrected at pH = 4.5 and incubated at 25ºC under dark conditions. Fungal strains are represented in Table 3.2:

Table 3.3 Wood decaying fungi fungal strains used, classified according to their ecophysiological category, thus either white rot fungi (WRF), litter decomposing fungi (LDF) or both.

Species	Strain	Ecophysiological Category
Agrocybe erebia	FBCC[3]476	LDF
Ganoderma lucidum	(Leysser) Karsten FP-58537-Sp	WRF
Gymnopilus luteofolius	FBCC466	LDF/WRF
Irpex lacteus	AX1	WRF
Pleurotus ostreatus	KJ020935[4]	WRF
Pycnoporus sanguineus	CS43	WRF
Stropharia rugosoannulata	FBCC475	LDF/WRF
Trametes versicolor	ATCC[5] 42530™	WRF

Irpex lacteus AX1 strain was kindly provided by Dr. C. A. Reddy, from the Michigan State University collection (USA), *Ganoderma lucidum* (Leysser) Karsten FP-58537-Sp strain was also kindly provided by Dr. C. A. Reddy, from the United States Department of Agriculture collection, (Madison, Wis. Collection, USA). *Pleurotus ostreatus* NCBI KJ020935 strain, was kindly gived by Dr. L. Palli from the University of Florence (Italy). *Pycnoporus sanguineus* CS43 strain was ceded by the Environmental Bioprocesses Group of Tecnológico de Monterrey (Mexico).

3.5. Mycelium and pellet production

Pellet production of fungi was done following the same method for all species. As previously reported by Font et al. (2003), briefly synthesized as follows:

[3] Fungal Biotechnology Culture Collection from University of Helsinki (Finland).
[4] NCBI accession number.
[5] American Type Culture Collection (USA).

In order to produce fungal pellet, mycelium was produced first from agar fungal cultures. From the fungal growing area, four plugs of 1 cm^2 were inoculated in 150 mL of malt extract at 2% (adjusted to pH 4.5) in a 500 mL Erlenmeyer, incubated under dark conditions at 25ºC and 135 rpm. After 5-7 days, the mycelium was filtered and triturated with X10/20 homogenizer (Ystral GmbH, Dottingen, Germany) in an 8.5 g/L NaCl solution at 1:1 v/v. This suspension can be used immediately or kept at 4ºC for further use in the following 25 days.

To produce pellets from mycelium, 1 mL of mycellium suspension was taken and added to 250 mL of 2% malt extract medium in a 1 L Erlenmeyer. This culture was kept at 25ºC in the orbital shaker at 135 rpm under dark conditions, until 5-7 days when pellets were 2-3 mm in size.

Finally, pellets were strained, rinsed with milliQ water and gently pressed in order to expel any water excess. Pellets were then ready to be applied in any treatment or kept for further use instead, following the same procedure as mycelium (1:1 suspension in 8.5% NaCl solution, stored at 4ºC up to one month maximum).

3.5.1. Microorganism maintenance

All fungi cultures were subcultured on 2% malt extract agar Petri plates (pH 4.5) at 25ºC. Every 30 days (maximum) new petri plates with fresh 2% malt extract agar were reinoculated with 1 cm^2 plugs from active growing fungus area.

3.5.2. Dry weight determination

In order to measure biomass dry weight, biomass was first vacuum filtered through washed, dried, and pre-weighed Whatman GF/C glass fiber filters (Whatman, Maidstone, England), and subsequently let dry at 105ºC to a constant weight.

An amount of at least 5 g of biomass (wet weight) was taken from the initial biomass culture before the inoculation, and after desiccation at 105ºC until constant weight, a wet weight vs dry weight quotient for this biomass sample was determined in order to estimate an approximate initial dry weight for the inoculated fungal biomass.

3.6. Defined medium and growing conditions

For degradation experiments with fungus when real wastewater was not used, defined medium was applied. This medium was modified from Kirk et al. (1978), containing glucose as carbon source, and ammonium tartrate or chloride as nitrogen source. Before sterilization, medium was adjusted at pH = 4.5. Growth and experimental conditions were 25ºC in darkness, pelletization was achieved and maintained by air pulses in the fluidized bed bioreactor (FBB) or under orbital shaking (135 rpm) in Erlenmeyers. Further details can be found in previous publications (Blánquez et al., 2004). Table 3.3 and Table 3.4 describe defined medium detailed composition.

Table 3.4. Defined medium composition

Component	**Unit**	**Concentration**
Glucose	g/L	8
Ammonium Tartrate dibasic	g/L	3.3
(or **Ammonium chloride**)		1.9
2,2-Dimethyl succinic acid	g/L	1.168
Macronutrients	mL/L	10
Micronutrients	mL/L	1

Table 3.5. Macronutrients and micronutrients composition

Macronutrients	**Unit**	**Concentration**
KH_2PO_4	g/L	20
$MgSO_4 \cdot 7H_2O$	g/L	5
$CaCl_2$	g/L	1
Micronutrients	**Unit**	**Concentration**
Nitrile triacetic acid	g/L	1.5
$MgSO_4 \cdot 7H_2O$	g/L	3.0
$MnSO_4 \cdot H_2O$	g/L	0.5

NaCl	g/L	1.0
$FeSO_4 \cdot 7H_2O$	g/L	0.1
$CoSO_4$	g/L	0.1
$ZnSO_4 \cdot 7H_2O$	g/L	0.1
$CaCl_2 \cdot 2H_2O$	g/L	0.1
$CuSO_4 \cdot 5H_2O$	g/L	0.01
$AlK(SO_4)_2 \cdot 12H_2O$	g/L	0.01
H_3BO_3	g/L	0.01
Na_2MoO_4	g/L	0.01

3.7. Analytical procedures

3.7.1. Water characterization

Conductivity was determined by using a CRISON MicroCM 2100 conductivity meter and pH with pH meters CRISON MicropH 2001 and CRISON Basic 20 (Crison Instruments, Barcelona, Catalonia).

Ammonium ($N\_NH_4^+$) and chemical oxygen demand (COD) were analysed using commercial kits LCK303 (2-47 mg/L NH_4^+) or LCK314 (15-150 mg O_2) and LCK114 (150-1000 mg/L O_2), from Hach Lange (Düsseldorf, Germany).

Total suspended solids (TSS) were measured according to Standard Methods (APHA et al., 1999), whereas Total organic carbon (TOC) was measured by with a TIC/TOC Analyzer (Model 1020A, OI Analytical, USA) equipped with a non-dispersive infrared detector and a furnace maintained at 680 ºC. Chloride, sulfate, nitrate and phosphate anions were quantified by a Dionex ICS-2000 ionic chromatograph.

3.7.2. Laccase and Manganese peroxidase activity

The laccase and manganese peroxidase (MnP) activities were analysed by enzyme kinetics. Samples were filtered before the analysis with filters of either 0.22 µm nylon or PVDF syringe filters (Millex Millipore, Barcelona, Catalonia) depending on the analysis and the PhACs used. The enzymatic activities were analysed using 2,6-dimetoxyphenol (DMP) as a reductor agent, as described by Blánquez et al. (2004). The reaction mixture consisted

of 200 µL of 250 mM sodium malonate at a pH 4.5, 50 µL of 20 mM 2,6-dimethoxiphenol (DMP) and 600 µL of sample. DMP was oxidized by laccase, which causes changes in the absorbance at 468 nm that are monitored during 2 min on a Varian Cary 3 UV/visible spectrophotometer at 30ºC. One activity unit (U) was defined as the number of micromoles of DMP oxidized per minute. The molar extinction coefficient of DMP was 24.81 mM/cm (Wariishi et al., 1992).

3.7.3. Glucose concentration

Glucose concentration was measured using an YSI 2700 SELECT biochemical analyser (Yellow Spring Instruments, OH, USA). Samples were filtered before the analysis with filters of either 0.22 µm nylon or PVDF syringe filters (Millex Millipore, Barcelona, Catalonia) depending on the analysis and the PhACs used.

The quantifiable concentration ranged from 0 and 25 g/L with a precision of ±0.5 g/L. Glucose quantification by this method is based on glucose oxidation into hydrogen peroxide through glucose oxidase enzyme, immobilized onto a hydrogen peroxide perm-selective membrane (Tsuchida and Yoda, 1981). The hydrogen peroxide is, in turn, oxidized at the platinum anode, producing electrons, which can be converted into electric signal. The electron flow is linearly proportional to the steady state hydrogen peroxide concentration and, therefore, to the concentration of the substrate.

3.7.4. Toxicity assays

In cases where acute toxicity in water samples was analised, it was used a Microtox™ bioluminiscence assay. Samples were filtered before the analysis with filters of either 0.22 µm nylon or PVDF syringe filters (Millex Millipore, Barcelona, Catalonia) depending on the analysis and the PhACs used. This method relies on the decrease or inhibition in the percentage of emitted light by the bioluminescent bacterium *Aliivibrio fischeri* (former *Vibrio fischeri*) upon contact with a filtered sample at pH 7. Bioluminescent bacteria *A. fischeri* and test reagents for Microtox analyses were supplied by Strategic Diagnostics Inc. (Newark, DE, USA). The 50% effective concentration (EC50) was measured after 15 min of

exposure. Effluent toxicity was expressed in toxicity units (TU), calculated as TU=100/EC50 and an effluent was considered toxic when its TU was over 25 as it is set by local sewage disposal regulation (DOGC, 2003).

3.7.5. Analysis of PhACs

In the experiments where PhACs were spiked to an initial concentration of ≥5 mg/L, PhACs concentration was determined by HPLC-UV system. Samples were filtered before the analysis with filters of either 0.22 µm nylon or PVDF syringe filters (Millex Millipore, Barcelona, Catalonia) depending on the analysis and the PhACs used.

This equipment was a Dionex 3000 Ultimate HPLC equipped with a UV detector and a Dionex autosampler. Chromatographic separation was achieved on a GraceSmart RP 18 column (250mm x 4.6 mm, particle size 5 µm). Sampling volume was 20 µL. All determinations were performed at 30ºC. Individual programs for specific PhACs analysis were tailored for every experiment and more detailed information can be found in each corresponding chapter.

Before the analysis, all the samples were filtered with 0.22 µm PVDF or nylon (depending on the analysis and the compound) syringe filters and transferred to amber HPLC vials to avoid natural photodegradation during the analysis.

For the rest of the experiments, quantification of PhACs was performed by the chemical analytical group of Institut Català de Recerca de l'Aigua (ICRA), led by Dra. Sara Rodríguez and Dr. Damià Barceló. For the rest of experiments, a brief description of the PhACs analysis is listed below.

PhACs from chapter 5 were analysed by Daniel Lucas. The analytical procedure for the quantification of PhACs was adapted from the method of Gros et al. (Gros et al., 2012) to include mass spectrometry parameters of cytostatic compounds. PhACs concentrations were measured according to the signal detected during a specific retention time. This retention time was calculated previously through a calibration curve for each PhAC.

Chromatographic separations were conducted using a Waters Acquity Ultra-Performance™ liquid chromatograph (UPLC) system equipped with two binary pump systems using an Acquity HSS T3 column (50 mm × 2.1 mm i.d., 1.8 µm particle size; Waters Corp. Mildford, MA, USA) and positive electrospray ionization (PI). The UPLC instrument was coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. Compound dependent mass spectrometer parameters were optimized by direct infusion of individual standard solutions of each compound at 20 µg/L. Data were acquired and processed using the Analyst 2.1 software.

Venlafaxine (VFX), O-desmethylvenlafaxine (ODMVFX) and their respective transformation products (TPs) mentioned in chapter 6, were analysed by Dr. Llorca using a liquid chromatography system coupled to a hybrid linear ion trap-high resolution mass spectrometer LTQ Orbitrap (LC-LTQ Orbitrap). Aria TLX-1 chromatographic system (Thermo Fisher Scientific) was used for separation purposes. This system comprised a PAL auto sampler and two mixing quaternary pumps (eluting pump and loading pump). The entire system was controlled via Aria software, version 1.6, under the Xcalibur 2.2 software. The compounds were separated in a Hypersil GOLD analytical column (50 × 2.1; 3 µm; Thermo Fisher Scientific, Franklin, MA). To quantify the concentration of the detected TPs, the samples were analyzed by LC-MS/MS using pure standards of the target compounds. The chromatograph was coupled to a hybrid linear ion trap-Fourier Transform Mass Spectrometry Orbitrap analyzer (LTQ-OrbitrapVelosTM, Thermo Fisher Scientific) equipped with a diverter valve (used in order to divert unwanted waste portions of chromatographic runs) and an Electrospray Ionization source (ESI). The diverter valve was used with three valve positions: from 0 to 1.5 min the flow was discharged to the waste and from 1.5 to 17 min the valve was switched to injection mode. The ionization of the compounds was performed under positive and negative mode for screening purposes. Mass calibration and mass accuracy checks were performed prior to every sample run with LTQ ESI Positive and Negative Ion Calibration Solution (Thermo Fisher Scientific), where mass accuracy was always within errors of ±2 ppm. Xcalibur 2.2 software was used for data interpretation. Data processing was carried out using with SIEVE 2.0 software (Thermo Scientific) in order to perform the chromatographic peak deconvolution and

ExactFinder 2.5 software (Thermo Scientific) for quantification purposes of selected compounds.

In the case of Metoprolol (MTP), metoprolol acid (MTPA), analyses were performed by Adrián Gil. Analytical procedures and conditions were almost the same as VFX and ODMVFX analysis except for the column, a ZORBAX Eclipse XD-C18 (150 × 4.6; 5 µm; Agilent Technologies, Santa Clara, CA, USA) was used instead.

Concurrently, samples from *T. versicolor* reactor in chapter 7 corresponding to the analysis of antibiotics, they were performed by Saulo Varela following the protocol previously described by Gros et al. (2013). Briefly, chromatographic separation was carried out with a Ultra-Performance liquid chromatography system (Waters Corp. Mildford, MA, USA), using an Acquity HSS T3 column (50 mm × 2.1 mm i.d. 1.7 m particle size) for the compounds analyzed under positive electrospray ionization (PI) from Waters Corporation. The UPLC instrument was coupled to a 5500 QTRAP hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, Foster City) with a turbo Ion Spray source.

Removal parameters

Regarding PhACs removal and biodegradation in the experiments, three parameters were assessed: the removal percentage (RP) (Equation 3.1), sorption percentage (SP) (Equation. 3.2), and minimal degradation percentage (MDP) (Equation 3.3).

Removal was generally defined as the maximum difference in PhAC concentration obtained for a fungal treatment, which always corresponded to an experimental treatment because it included sorption mechanisms apart from biological ones, instead of abiotic or heat-killed controls.

$$RP = \frac{[PhAC]_0 - [Experimental]}{[PhAC]_0} \cdot 100 \qquad \textbf{(Equation 3.1)}$$

Where $[PhAC]_0$ and [Experimental] correspond to the PhAC initial concentration and to the final PhAC concentration in experimental treatment, respectively.

Besides, SP is defined in Equation 3.2, where [killed control] corresponds to the final PhAC concentration in heat-killed control treatment (Adapted from Akar et al. 2009).

$$SP = \frac{[PhAC]_0 - [Killed\ Control]}{[PhAC]_0} \cdot 100$$ **(Equation 3.2)**

Finally, MDP can be defined from previous equations (3.2 and 3.3).

$$MDP = \left(\frac{[Killed\ Control] - [Experimental]}{[PhAC]_0}\right) \cdot 100 = RP - SP$$ **(Equation 3.3)**

This parameter refers to a minimum PhACs biodegradation value, although part of the adsorbed PhACs could become also transformed, according to the degradation mechanisms proposed by Blánquez et al. (2004), who indicated that most of the biological transformation occurs at an intracellular level.

3.8. Fluidized bed bioreactors (FBB) set up and operating conditions

Two glass Fluidized Bed Bioreactors (FBB), with a working volume of 0.5 and 1.5 L were used to study the PhAC in bioreactor. Air was added from the bottom through a ceramic plate generating small bobbles and, as a result, good oxygen transfer and homogeneity were achieved with low biomass mechanical stress. Fluidised conditions were achieved by air pulses generated by an electrovalve connected to the air flow. The electrovalve was controlled by a cyclic timer, and the pulsing frequency was 0.16 s^{-1} defined as the inverse of the sum of opening and shutting times of the electrovalve: F=1/(t0+ts), t0 the opening time (1s) and ts is the shutting time (5s). The initial air flow 12L/h which was introduced to the reactor after being saturated and filtered through Polytetrafluoroethylene (PTFE) filters of 0.22 µm (Millipore, Barcelona, Catalonia). The FBB was equipped with a PO_2 probe and the air flow was manually increased if the percentage of the oxygen saturation decreased below 25%. A pH probe connected to a pH controller allowed to maintain the pH at 4.5± 0.5 by addition of HCl 1M or NaOH 1M. The probes were connected to a monitoring and register system. The bioreactor's sterilization was done empty in an autoclave at 120ªC for 30 minutes, protecting any possible connection from contamination by Hoffman clamps and aluminium foil. The filling of the reactor and the inoculation were done through suitable ports in sterile conditions. Operational modes were fed-batch or continuous. During fed-batch carbon and nitrogen sources were added from a concentrated stock through a port of the top. When FBB was switched to continuous, the feeding inlet was set in the lower lateral manifold, locating the outflow in

the upper extreme, thus avoiding the wash out of the system. An sketch of the FBB can be seen in Figure 3.1.

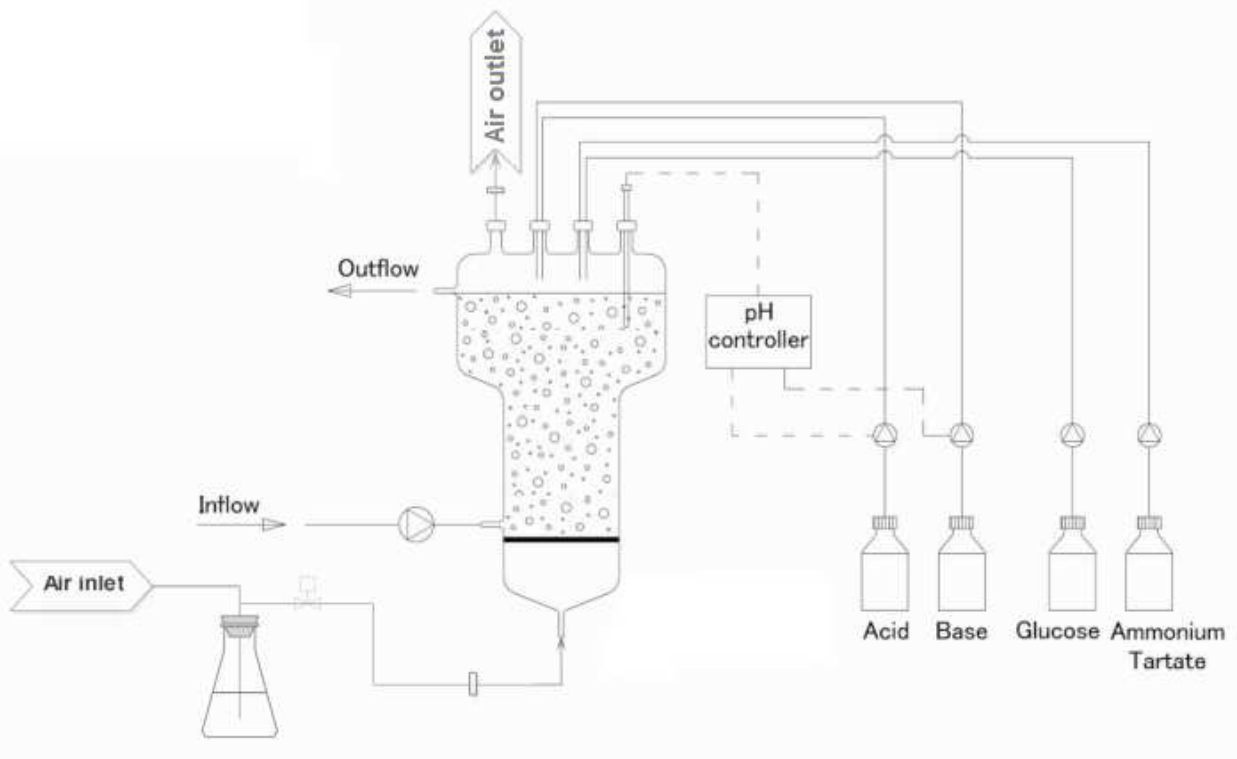


Figure 3.1 Fluidized Bed Bioreactor Sketch. Adapted from Blánquez et al. (2007).

Conditions such as sterile or non-sterile conditions, batch or continuous mode, and addition or not of extra nutrients involve different operational approaches. A brief summary of the assayed configurations can be found on table 3.5, whereas a sketch of the reactor is shown in Figure 3.1.

Experiments were performed under dark conditions within a thermostatized chamber at 25ºC, Fungal pellets were added at 2-4 g dry weight biomass/L. Fungal pellets were kept retained by placing a mesh membrane in the FBB's outlet. When performed in fed-batch mode, glucose and ammonia were added from a concentrated stock at a consumption rate depending on the fungal species and conditions. Air-pulse frequency and air flow supply was set according to the aeration and fluidization requirements of the fungal species, pH was monitored with a probe (Mettler Toledo, Spain).

Table 3.6 Operational modes and conditions for experiments with FBB.

Thesis Chapter	Days	Water[1]	Sterility	Operational mode	PhAC spiking[2]	Bioreactor	Fungus
7	32	HWW	Yes	Batch/Continuous	ATL, DCF, KTP	1.5 L	*P. ostreatus*
6	7	HWW	No	Batch	MTP and MTPA	(0.5 L)x2	*G. lucidum*
7	7	OW/HWW	No	Batch	Antibiotics	(1.5 L)x2	*T: versicolor*
7	10	HWW	Yes/No	Batch	CBZ, DCF, IPD	(0.5 L)x2	*S. rugosoannulata*

[1]OW: Osmosed water (MilliQ water); HWW: Hospital wastewater.

[2] ATL: Atenolol, DCF: Diclofenac, KTP: Ketoprofen, MTP: Metoprolol, MTPA: Metoprolol acid, CBZ: Carbamazepine, DCF: Diclofenac, IPD: Iopromide.

3.9. Solid Phase Extraction (SPE)

Mainly used when samples required to be concentrated to detect PhACs or their transformation products (TPs). Compounds were extracted from the liquid phase in one step using Oasis HLB cartridges (60 mg adsorbent, 3 mL) (Waters Corp., Milford, USA), as described elsewhere (Radjenovic et al., 2007). The cartridges were preconditioned sequentially with 5 mL of methanol and 5 mL of deionized water at the sample pH. After that, the sample was passed through the cartridge and dried under vacuum. Then the adsorbed compounds were eluted with pure methanol (volume depending on the extraction) and subsequently concentrated to dryness under a gentle nitrogen stream.

3.10. DNA extraction, PCR-DGGE and DGGE fingerprinting analysis

In Chapter 4, total DNA extraction was conducted using a PowerSoil® DNA Isolation Kit (MoBio Laboratories, USA) for both biofilm and sand samples. For bacterial analyses, a 550 bp DNA fragment in the 16S region of the small subunit ribosomal RNA gene was amplified using the primer set 341f/907r (Muyzer et al., 1993) with a GC clamp added at the 5' end of forward primer 341f (see Table 3.6). Final concentrations of the PCR

reactions consisted of 1x PCR buffer, 1.5 mM of $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 500 nM of each primer and 2.5 U of Taq DNA polymerase (Invitrogen, ThermoFisher Scientific, USA). Amplification protocol for bacteria consisted of: 94 ºC for 5 min; 18 cycles of 94 ºC for 1 min, 65 ºC for 1 min (-0.5 ºC/cycle) and 72 ºC for 3 min; 15 cycles of 94 ªC for 1 min, 56 ºC for 1 min, 72 ºC for 3 min; and a single final extension of 72 ºC for 7 min. Fungal DNA was amplified using a nested approach over a ~300 bp fragment in a second round, from the internal transcribed spacer (ITS) of fungal ribosomal encoding genes. The primer sets used were EF4/ITS4 and ITS1f-GC/ITS2 (Gardes and Bruns, 1993; White et al., 1990) for the first and second round of amplification, respectively. The GC clamp was added at the 5' end of primer ITS1f (see Table 3.6) and PCR reactions had the same final concentrations except for the addition of bovine serum albumin (0.6 µg/µL or 9 µM). PCR program for fungi was identical for both amplification rounds and consisted of: 94ºC for 5 min; 35 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ªC for 30 s; and a single final extension 72ºC for 5 min. Denaturing gradient gel electrophoresis (DGGE) was performed using the D-code Universal Mutation Detection System (Bio-Rad, Spain). 900 ng of DNA from PCR products were loaded onto 6% (w/v) polyacrylamide gels (acrylamide/bis solution 37.5:1) containing linear chemical gradients 20-60% denaturant for bacteria and 15-55% denaturant for fungi. 100% denaturing solution contained 7 M urea and 40% (v/v) deionized formamide. Gels were run in 1X Tris acetate-EDTA (TAE) for 16 h at 75 V and 60 ºC, stained with 1 mg/mL ethidium bromide solution for 25 min, washed with deionized water 25 min and photographed with Universal Hood II (Bio-Rad, Spain). DGGE images were analyzed using InfoQuest™FP software. Dice's coefficient and unweighted pair group method with arithmetic averages (UPGMA) were employed for the clustering of DGGE gel profiles. Prominent bands from the DGGE were excised, reamplified and then sequenced by Macrogen (South Korea). Obtained sequences were trimmed with FinchTV software and checked for chimeras using Mothur (Schloss et al., 2009). Each sequence was assigned to its closest neighbour according to the Basic Local Alignment Search Tool (BLAST) results (Altschul et al., 1997). DGGE images were analyzed using the InfoQuest™FP 4.5 software (Bio-Rad Laboratories, Richmond, CA, USA). Similarities of the DGGE profiles were calculated based on the Dice coefficient and dendrograms were obtained using the UPGMA clustering algorithm. A band position tolerance of 0.5% was used. Band patterns were normalized using the marker lanes as

reference, allowing the comparison among samples loaded on different DGGE gels. The number of DGGE bands in each fingerprint was used as a measure of the apparent fungal and bacterial richness (S). Shannon Index was used as a measure of genetic diversity, and was calculated as H = pi ln pi, where pi is the relative intensity of each DGGE band. Evenness (E) was calculated as E = ln (S).

Table 3.7 Oligonucleotides sequences used.

Primer	**Sequence (5' to 3')**	**Position 16S rDNA E. coli**	**Specificity**	**Reference**
341fGC	CCTACGGGAGGCAGCAG	341-357	"Universal"	(Muyzer et al., 1993)
907r	CCCCGTCAATTCCTTTGAGTTT	907-926		
Primer	**Sequence (5' to 3')**	**Position rDNA *S. cerevisiae***	**Specificity**	**Reference**
EF4f	GGAAGGGRTGTATTTATTAG	573-593	"Universal"	(Smit et al., 1999)
ITS4	TCCTCCGCTTATTGATATGC	2390-2409	"Universal"	(Gardes and Bruns, 1993)
ITS1fGC	GCACGGGGGGCTTGGTCATTTAGAG GAAGT	1731-1752	"Universal"	(Gardes and Bruns, 1993)
ITS2	GCTGCGTTCTTCATCGATGC	2024-2043	"Universal"	(White et al., 1990)

341f GC clamp: CGCCCGCCGCGCCCCGCGCCCGGCCCGCCG

ITS1f GC clamp: CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG

3.11. Statistical analysis

Means and standard deviations (SD) were calculated using Microsoft® Excel 2013 software functions (Microsoft, Ca, USA). Statistical analysis such as one-factor analysis of variance (ANOVA) were conducted using Sigmaplot 12.0 (Systat Software Inc., CA, USA). Differences were considered significant at $p<0.05$.

3.12. References

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RESEARCH WORK

4. Biosand filter: from fungal bioreactor pretreatment to PhACs removal

4.1. Introduction

During previous studies with fungal treatments for PhAC removals with real non sterile wastewater, non-favourable competition dramatically affected fungal viability in reactor due to extensive inner bacterial growth (Badia-Fabregat et al., 2016; Cruz-Morató et al., 2013a; Mir-Tutusaus et al., 2016). This competition, forced periodical fungal reinoculations and prevented the developing of an efficient treatment for real applications on an industrial scale. Hence, there was necessary to find new strategies for pretreating the effluent to decrease wastewater bacterial load in a cost-effective way that could be applied later at a larger scale. Biosand filtration, a cheap and fast method widely used around the world for the elimination of pathogens in WWTP and drinking water purification (Paraskeva and Graham, 2002), emerged as one promising solution.

Biosand filters (BSF) have been used for water disinfection (e.g. direct rapid or pulse bed sand filters). A cheap solution is the Plastic Biosand Filter (PBSF) which are applied in sub-developed countries as a cost-effective alternative to more sophisticated method (Stauber et al., 2012). In this case, PBSFs manual by Manz (2007), was used in this chapter to design, build and operate a BSF. The BSF was dimensioned considering its potential to be used as a pretreatment coupled to a lab-scale FBB fungal bioreactor (1,5 L operational volume) operating in continuous with an hydraulic retention time (HRT) of 1.84 days.

Previous evaluation studies for BSF and slow sand filters (SSF) in drinking water treatment showed that repeated charges of WW, longer operational times and HRT, and the correct ripening of the filter in case of BSF are crucial for their optimal performance of BSF (Elliott et al., 2008; Jenkins et al., 2011). Nevertheless, and despite of being the only way to purify water for many people around the world, its bacterial load reduction may not always be as efficient as previously thought. Specially during the first stages of ripening may not always provide microbiologically safe drinking water, being a source of potential biohazardousness (Elliott et al., 2008). Furthermore, one study warned about recontamination risks for BSF filtered water when stored (Stauber et al., 2012), and

another mentioned pathogenic risks concerning the manipulation of schmutzdecke or sand biofilm when BSFs are clogged (Hwang et al., 2014).

On the contrary, BSFs and SSF have shown the potential in removing PhACs from WWTP that CAS treatments are unable to eliminate (Casas et al., 2015), opening new approaches towards the implementation of this low-cost treatment in small WWTP.

Several molecular studies have already been performed to analyze the genetic profiles of BSFs (Feng et al., 2013; Hwang et al., 2014), but to the author's knowledge none has studied them according to their exposure to PhACs and its potential PhACs degradation capabilities.

In this Chapter we aimed to analyze three main aspects of BSF: i) Bacterial reduction assessment and its feasibility as potential fungal bioreactor pretreatment ii) The assessment of the BSF as a structure that hosts a wide spread of microorganisms capable of PhACs degradation and iii) The changes in the genetic profile in the BSF populations across time exposed to high concentrations of PhACs.

4.2. Materials and methods

4.2.1. Biofilter design

The BSF used in this chapter had four differentiated parts, following this order from the bottom to the top (Figure 4.1):

Underdrain layer: This layer prevented filter's outlet standpipe from collapse, allowing vertical drainage. Its thickness was sufficient to cover the standpipe's inlet. Particles of volcanic rock provided by Agroterra Tecnologías Agrarias (Alboraya, Valencia) were used in this layer, previously sieved to range between 12.5 mm and 8 mm as minimum. Total depth of this layer was 125 mm.

Separation layer was used to prevent filter media from entering the underdrain layer and the standpipe. It was the thinnest layer of the system, made from particles between 6 mm and 3 mm. In our system, separation layer had 50 mm depth and was made of granite gravel, provided by Sorigué (Barcelona, Catalonia).

Filter media was the layer where the physical filtration took place, and moreover, layer that supported, on top of it, the schmutzdecke or sand biofilm, formed at the sand - water

interface. Filter media particles were not bigger than 3 mm and its material had to be obtained from a quarry and provided by Sorigué (Barcelona, Catalonia), from recently crushed rock since this material should not be contaminated with organic matter. Organic matter percentage in our filtering media was under 0.1 % and its thickness was 400 mm.

A paused water layer (30 mm of depth) was the overtop layer of the system. Its function was to stop the water flow turbulence coming from the diffuser, preventing biofilm's potential damage and keeping it active and moisturized at the same time.

Other important elements of the system were the diffuser and the standpipe. The standpipe kept the water level over the biofilm zone, thus creating the paused water layer and allowing the outcoming water to settle down, whereas the diffuser was used to prevent the inlet water from disturbing the media when poured into the filter.

4.2.2. Biofilter set up

The three different materials were installed inside the BSF using tap water (TW), avoiding as much as possible stratification and air binding. After BSF installation, a pump was coupled to a 20L inlet tank to fed the BSF, whereas in the BSF outlet another 20L tank was connected. This system was set to continuous operating and calibrated to provide a HRT of 1.84 days for the first 174 days. After this period, HRT was set to 6.15 days until the end of the experiment (259 days in total).

The BSF was set up using TW during media installation, but after around 20 days of BSF ripening with this water, the inlet tank was fed with HWW from the main sewer manifold of Dr. Josep Trueta University Hospital. From then on, this tank was refilled with fresh HWW once every week uninterruptedly for more than 250 days.

To design our Biosand filter we combined both methacrylate and PVC materials following the measurements shown on Figure 4.1:

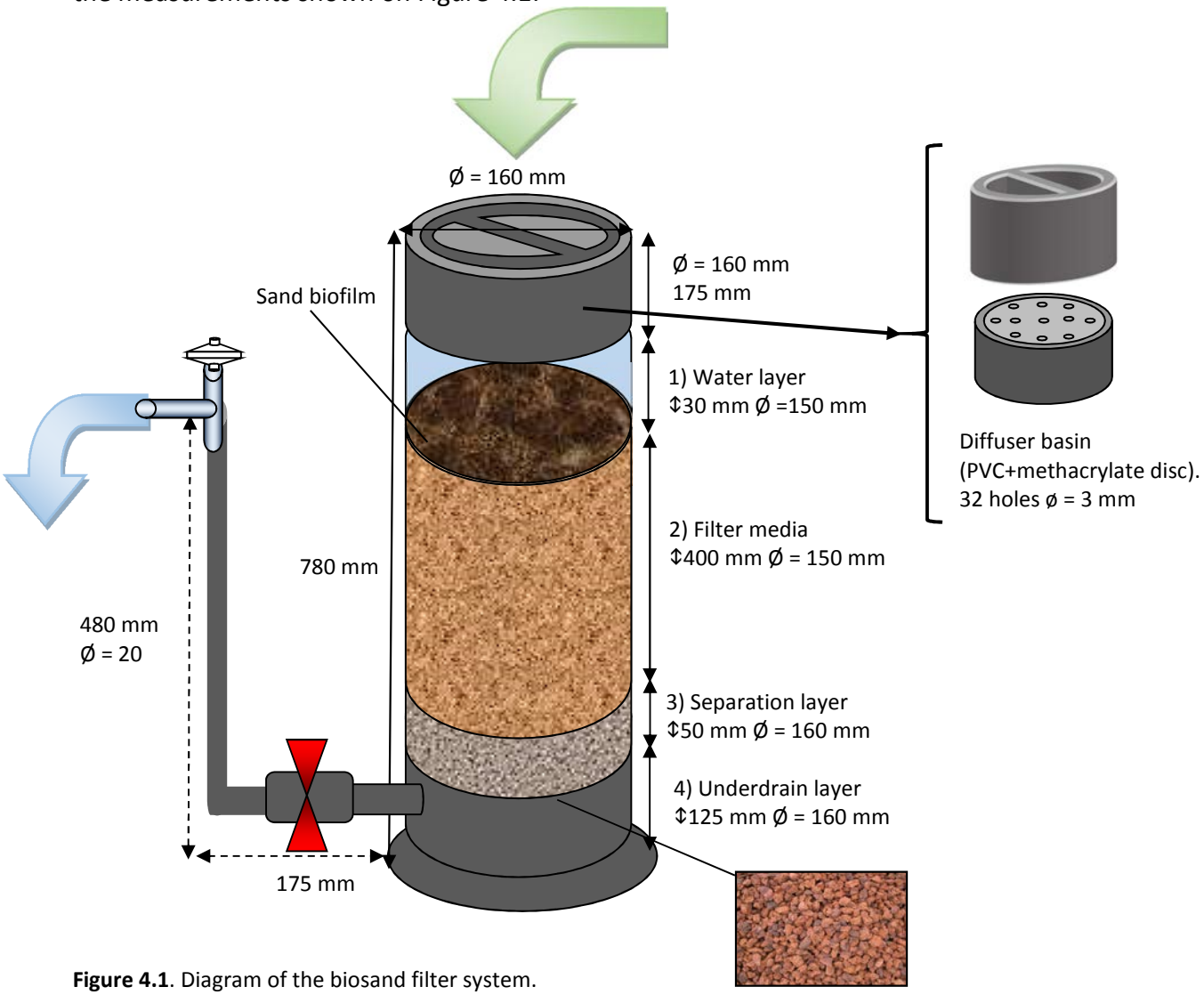

Figure 4.1. Diagram of the biosand filter system.

4.2.3. Ripening time

The development of a spontaneous biofilm cover in a BSF is reported to last usually from 2 to 3 weeks at temperatures over 20ºC (Palmateer et al., 1997), and as biofilter ripens (a process consisting in particle accumulation and biological growth in the top-most layer of the media bed), reduction in microbial concentration in water is enhanced over time (Elliott et al., 2008), with improvements starting after around 30 days from the setting up of the BSF.

In our study, BSF was considered to be ripened after more than 30 days of HWW circulation (figure 4.2), coinciding with a darker top sand color, a reduction in BSF outlet flow and an increase in bacterial concentration reduction.

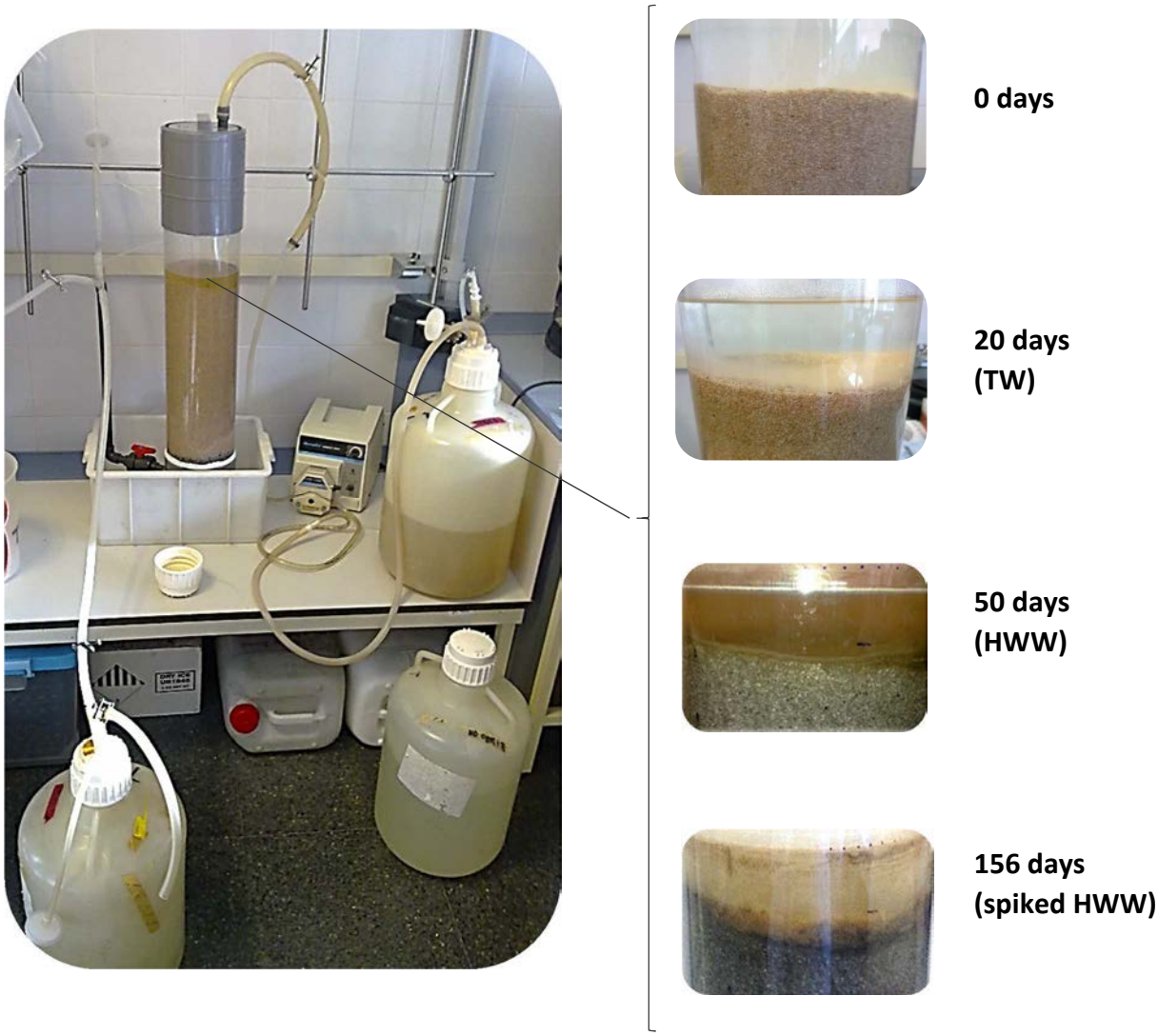

Figure 4.2. BSF system picture and zoomed pictures of the sand biofilm during the ripening process (from left to right).

4.2.4. BSF bacterial load reduction

To evaluate BSF's yield, inlet and outlet samples were collected periodically and counts for total aerobic viable bacteria (48/72 h at 37±2ºC) were perfomed with IDEXX Heterotrophic Plate Count (HPC) Quantitray kit (IDEXX Laboratorios, Madrid, Spain). A solution containing Sodium Thiosulfate ($Na_2S_2O_3$) from Sigma-Aldrich (Barcelona, Catalonia) was added to every sample to a final concentration of 100 mg/L in the sample. This addition was performed under sterile conditions before incubation, to neutralize any

residual chlorine or halogen and prevent continuation of bactericidal action during sample incubation and thus reflecting its truly bacterial concentration.

4.2.5. Analytical measurements

The BSF was exposed to room temperature during the experiment and therefore temperature was monitored using a Fourtec MicroLog Pro TEMP data logger (England, UK).

Electrical conductivity and pH were monitored during the BSF operation, whereas punctual COD and soluble ions measurements were performed comparing inlet and outlet samples.

4.2.6. Pharmaceutical spiking and detection

The recalcitrant fluoroquinolone antibiotic CPX and the analgesic/anti-inflammatory IBP were chosen as suitable PhACs for this study. High purity grade reagents of these two molecules were obtained from Sigma-Aldrich (Barcelona, Catalonia), added to an ethanol (HPLC grade) stock solution, and spiked into the HWW inlet tank of 20 L to concentrations of approximately 7 mg/L per PhAC. PhACs concentration were analyzed using Dionex 3000 Ultimate HPLC equipped with a UV detector at 230 nm. The column temperature was 30°C and a sample volume of 20 µL was injected from a Dionex autosampler. Chromatographic separation was achieved on a GraceSmart RP 18 column (250 mm x 4 mm, particle size 5 µm). In this case, for the quantification of CPX and IBP, the mobile phases (A) and (B) consisted of acetonitrile and 0.1% formic acid at pH=3.0 respectively, and were delivered at a flow rate of 0.8 mL/min in a gradient elution (starting at t=0 min with A=40%; increasing at t=20 min to A=80%; until t=30 min, where A=100% was maintained until t=35

min and then coming back to initial conditions in 5 min). The detection limit for all PhACs was 0.1 mg/L.

4.2.7. Core sampling

Biological samples of the filtration material's core of the BSF (including the sand biofilm) were taken at certain times (Table 4.1) with a sterile core probe of polycarbonate (25 mm intern diameter) and stored at -80ºC. Afterwards and under sterile conditions, two sections of around 2 cm^3 were cutted from the core probe. The first section (from now on referred as biofilm) corresponded to the top layer of the BSF and contained mainly biofilm sludge-like material, whereas the other section (from now on referred as sand) was placed 7 cm under the biofilm layer and contained mainly sand. In both cases, 12 g of samples were homogenized in 15 mL falcon tubes before taking 0.25 g (biofilm) and 0.5 g (sand) of material for the DNA extraction.

Table 4.1. Microbiological sampling schedule of the BSF

Month of the year	Time (days)	Stage	HRT (days)
3 (March)	116	Before spiking	1.84
4 (April)	156	Spiking (after 3 HRT)	1.84
5 (May)	175	Spiking stops (new HRT, after 3 HRT)	6.15
7 (July)	259	Final sample (> 80 days after spiking)	6.15

4.2.8. Molecular analysis

Total DNA extraction from each of the cores sampled was conducted using the PowerSoil® DNA Isolation Kits (MoBio Laboratories, USA) for both biofilm and sand samples as described in general methodology.

The sampling code used in this analyses contained two first letters referring to the section (BF for biofilm and S7 for sand), one number referring to the replica (1, 2, 3) and the last number referring to the state (3, 4, 5 or 7). Being in total 24 samples coded according to Table 4.2.

Table 4.2 Sampling code for molecular analysis.

Month	Stage	Biofilm section	Sand section
3	Before spiking	BF13	S713
		BF23	S723
		BF33	S733
4	Spiking (after 3 HRT)	BF14	S714
		BF24	S724
		BF34	S734
5	Spiking stops (new HRT, after 3 HRT)	BF15	S715
		BF25	S725
		BF35	S735
7	Final sample (> 80 days after spiking)	BF17	S717
		BF27	S727
		BF37	S737

4.3. Results and discussion

4.3.1. Temperature, pH and CE during ripening process

Temperature during the first 34 days of BSF ripening period was kept over 12ºC (with a maximum register of 25.3ºC, a mean of 17.88 ºC and a minimum of 12.5ºC). Afterwards temperature was maintained, from then on, over 15ºC, with a mean over 20ºC. This mild temperature registers refuse any negative influence that low temperatures could have performed into BSF ripening process and hence into BSF bacterial reduction yields.

Besides of temperature, samples of BSF's inlet (inlet tank), outlet (outlet standpipe tap) and accumulated outlet (outlet tank) were monitored for electrolytic conductivity (EC) and pH during the experiment. Especially during the first 70 days of operation with HWW (Figure 4.3A), coinciding with BSF ripening time. During this period, pH values from the inlet oscillated from 6.9 to 7.8 (with a mean of 7.18), whereas pH in the outlet remained higher and ranged between 7.2 and 8.11 (mean of 7.61). Moreover, accumulated outlet values of pH were slightly higher than direct BSF outlet (ranging from 7.37 to 8.4 and mean of 7.7). Hence, during this period BSF treatment slightly basified the filtered effluent.

Concerning EC (Figure 4.3B), high variability in samples was observed, explained by changes in ionic content caused by random variations in hospital routines where HWW come from. During this period, values of EC in the inlet ranged from 1189 to 1610 µS/cm with a mean of 1411 µS/cm. Although, despite this variability outlet values oscillated from 1121 to 1791 µS/cm, with a mean of 1419 µS/cm. These values correspond to the range of slightly saline waters equivalent to those of other HWW (Kovalova et al., 2012). On the contrary, samples from accumulated outlet tank showed higher variability, with peaks of up to 2000 µs/cm.

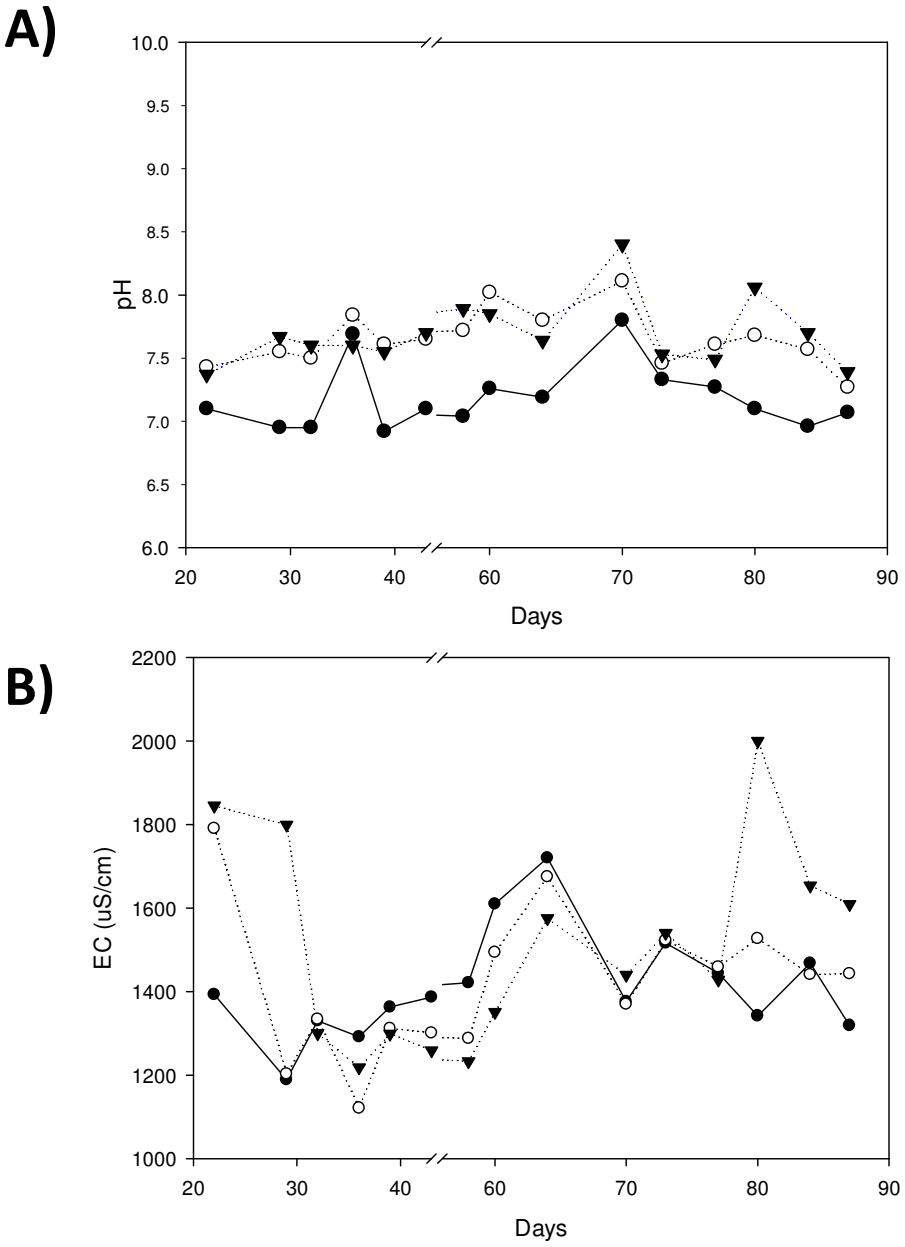

Figure 4.3. A) Evolution of pH during the first 70 days with HWW. Values for BSF inlet (●), outlet (○) and accumulated outlet (▼) are represented. B) EC values during the same period with the same sample code.

4.3.2. Bacterial load reduction

Regarding bacterial concentration, this parameter was evaluated during the first 70 days of BSF operation with HWW. However, reduction in HWW bacterial load did not show signs of improving, therefore it was decided to abandon it in spite of the advanced BSF ripening state achieved (Figure 4.4).

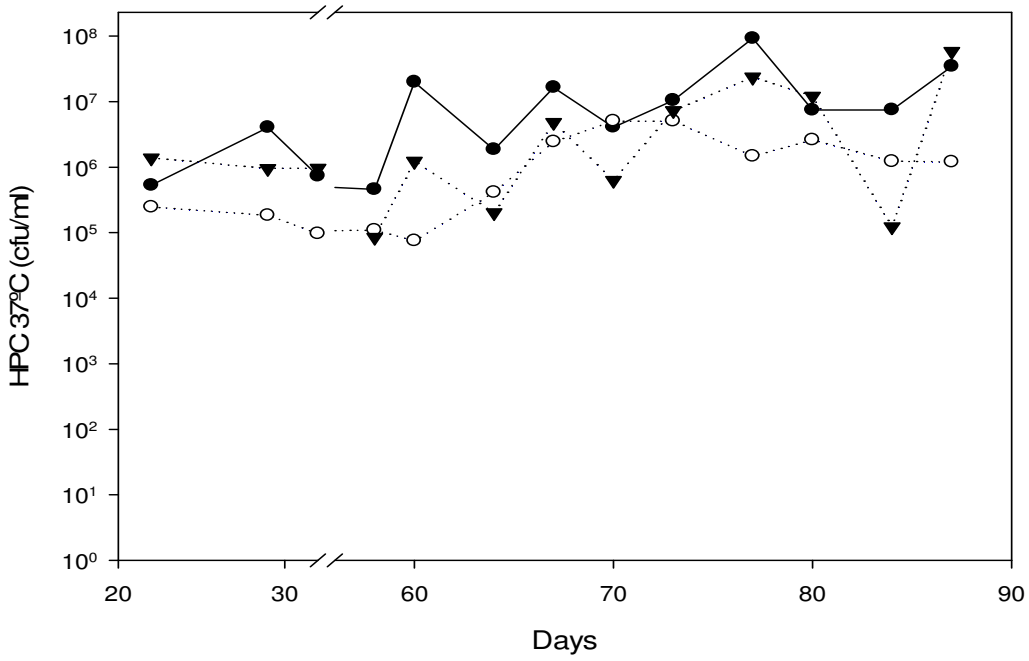

Figure 4.4. Values of Heterotrophic Plate Count monitoring (HPC) in cfu/mL. BSF inlet (●), outlet (○) and accumulated outlet (▼) are represented.

The HPC determined values in the BSF inlet between $2.5 \cdot 10^5$ and 10^7 cfu/mL, which are values within the common range of previously reported HWW analyses (Manonmani et al., 2015; Mir-Tutusaus et al., 2016). These bacterial load values, although being normal, were expected to be reduced in at least more than 10^5. However, while there was a decreasing in bacterial concentration caused by BSF application, and despite the successful BSF ripening for more than 90 days, values of HPC never achieved reductions higher than 10^2 cfu/mL, which is apparently considered an average performance in literature (Elliott et al., 2008; Fiore et al., 2010). Moreover, sampling from the accumulated BSF outlet tank revealed an oscillating behaviour, with a bacterial concentration mean slightly higher than the direct BSF outlet, and sometimes even slightly higher than the BSF inlet, confirming the biohazardous situation for BSF users of drinking

water from stored tanks, linked to recontamination phenomena reported by previous authors (Guchi, 2015; Stauber et al., 2012).

4.3.3. BSF as a novel PhAC removal HWW treatment

After rejecting BSF for an effective pretreatment strategy for fungal bioreactors, a totally different approach was set; in this case, the aim was to study the potentiality of native BSF microbial species to remove PhACs. Within the active BSF, these microorganisms could have been biostimulated throughout the exposure to HWW for several months of operation. In order to accomplish this goal, the BSF inlet was fed with the same HWW but spiked with CPX and IBP at known concentrations to evaluate its removal during 28 days.

The concentration of these two PhACs was initially spiked at 7 mg/L and could be monitored during all the PhACs removal experiment. Other PhACs were spiked at the same time, but due to difficulties in analysing their concentrations they were finally rejected from the study.

A regular monitoring of pH, EC and COD was carried out to evaluate the impact of an external PhACs addition into the BSF performance and the evolution of these parameters (Figure 4.5A). After an increase in the beginning of the experiment, EC values for both BSF inlet and outlet stayed almost constant during the experiment, although unlike the first ripening days EC was now higher in the outlet rather than in the inlet. This increase in EC could be explained by metabolic activity from microorganisms producing species with higher EC. In the same way, during the first stages of BSF ripening, the system was slightly basifying the filtered water, whereas in this experiment, in spite of having a similar pH in the inlet HWW, the resulting filtered water was clearly acidified down to 4.5. This phenomena could be explained by microorganism activity, in response to the sudden high dose of PhACs. If microbiota equilibrium had been altered by the antibiotic CPX, resilient species such as multi-resistant bacteria or WRF could have then acidified the liquid medium, as fungi had been reported to do (Tavares et al., 2006), on a metabolic feedback strategy to reduce bacterial competition).

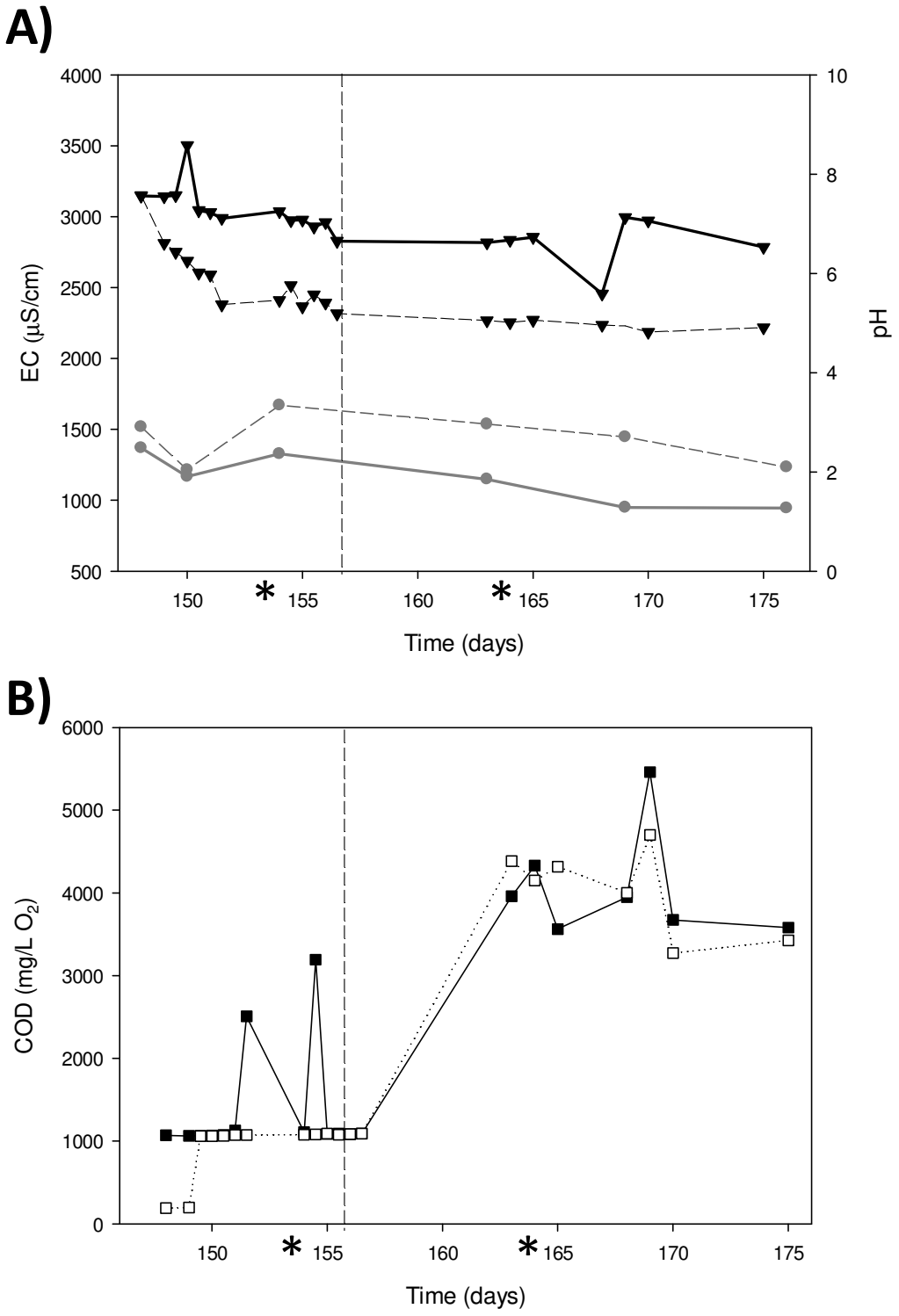

Figure 4.5. A) Evolution of EC (in grey ●) and pH (in black▼) during the HWW spiking experiment. BSF Inlet values correspond to the straight lines whereas BSF outlet values correspond to dotted lines. **Figure 4.5 B)** COD values (■) of BSF inlet (filled square and straight line) and BSF outlet (empty square and dotted line). In both 3A and 3B, the dashed line represents change in HRT (from 1.85 d to 6.15 d) and the asterisks indicate when BSF inlet tank was refilled with the fresh spiked HWW.

COD (figure 4.5B) also suffered significant changes increasing its values over 3000 mg/L O_2, in relation with COD values before the beginning of this experiment. Especially after the increase in HRT and refillments of inlet BSF tanks, COD, both in the BSF inlet and outlet had more than doubled after the experiment. COD increased in both inlet and outlet samples (being those generally lower). Anyhow, they were not necessarily related with the PhAC addition into the water. Instead, they could be attributable to changes in hospital routines, whereas in the case of being a PhAC caused effect, the exact mechanism to increase COD more than two times remains unknown. CPX and IBP achieved removal rates of more than 90% and up to 50% respectively (Figure 4.6). IBP highest removals were mainly found after the increase in HRT to 6.15 days, whereas CPX removals were almost equivalent in both HRTs. These promising results contrast with several unsuccessful attempts to biologically remove CPX from HWW, being only removed around 1.85% in CAS treatments according to US Environmental Protection Agency's EPISuite™.

Indeed, most of CPX removals recently reported in literature were obtained by physicochemical treatments (Chen et al., 2015; Jiang et al., 2013), except for certain cases such as the WRF *T. versicolor,* which achieved variable results depending on the experimental conditions, from a 35% removal under non-sterile conditions and 84 µg/L in a FBB (Cruz-Morató et al., 2013a) to over 90% removal under sterile conditions, 2 mg/L of CPX and maintenance in malt extract liquid medium (Prieto et al., 2011).

On the contrary, in the case of IBP, despite that removals over 50% are much higher than currently applied CAS technologies (with removals reported of around 28.72% according to US Environmental Protection Agency's EPISuite™) they could be considered equivalent than those achieved by other biological treatments such as certain MBRs (Hai et al., 2011); however, other biological treatments such as the WRF *T. versicolor* have shown a far better performance, being able to completely remove IBP even at higher concentrations (Marco-Urrea et al., 2009).

Parameters such as temperature, pH and hydrophobicity are deeply related to PhACs removal rates in biological treatments (Taheran et al., 2016), but, especially in non-sterile conditions, a phylogenetic analysis of the bioreactor population is crucial for understanding whether the present microorganisms are involved or uninvolved in the PhACs removal process.

All these results support the application of porous media biofilm reactors for the removal of PhACs from current wastewater effluents. Recalcitrant compounds to activated sludge and AOPs, such as IPD or DCF, were reported to be removed up to 82 and 91% respectively by Escolà-Casas and Bester (2015) with low-flow operation in porous media biofilm reactors. This opens the door for this promising technology to be implemented soon as a PhAC removal treatment in a cheap and easy way.

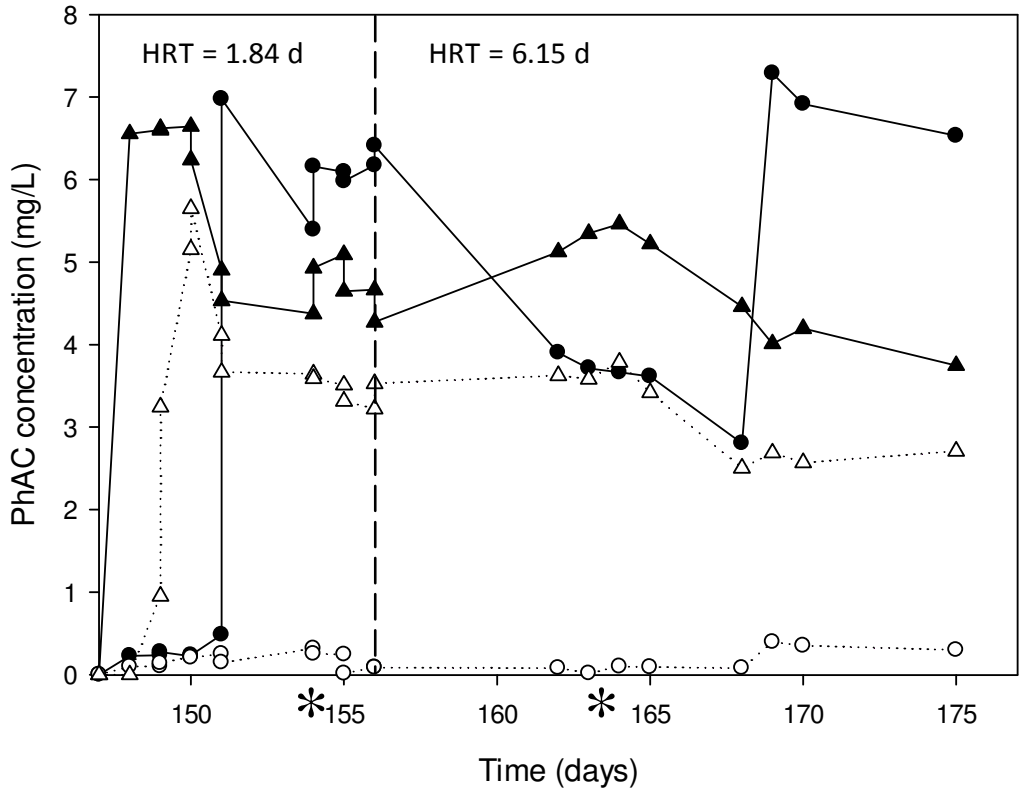

Figure 4.6. PhACs concentration of CPX (▲) and IBP (●) in the BSF inlet (straight lines and filled figures) and BSF outlet (dotted lines and empty figures). A vertical dashed line on day 156 marks the moment where HRT was set from 1.84 days to 6.15 days, and the two asterisks indicate when BSF inlet tank refill with the spiked HWW were done.

4.3.4. Molecular analysis

Band profiles obtained by the DGGE of bacterial and fungal rDNA amplified fragments and the DGGE fingerprints cluster analysis are shown in Figure 4.7 and Figure 4.8 respectively. The 16S rRNA-DGGE analysis (Figure 4.7) revealed composite banding patterns reflecting a high microbial diversity. Conversely, the ITS rDNA-DGGE analysis (Figure 4.8) showed a lower diversity in the fungal communities.

Regarding the unweighted pair group method with arithmetic mean (UPGMA) analysis of the DGGE fingerprints, two clusters of first-order could be recongized in all of the four DGGEs studied (fungal and bacterial sections of biofilm and sand). First order clusters were identified at 64.82% for biofilm bacteria, 64.04% for sand bacteria, 72.49% for biofilm fungi and 94.9% for sand fungi.

In the case of bacteria, in the biofilm section, the first-order clusters separated last stages of the experiment (spiking with the second HRT and after spiking samples) form first stages (before spiking and spiking with first HRT), and the respective second-order subclusters differentiated the samples according to their stage (except for the cases of BF35 and BF23) (Figure 4.7 A). These results suggest that the change of HRT apparently affected bacterial species present in the biofilm even more than the PhACs spiking.

On the contrary, in the sand section, first-order clusters divided the non-spiking stages from the spiking ones, (Figure 4.7B), and second-order subclusters differentiated according to stages (3 and 7 on one side, and 4 and 5 on the other). Results suggest that PhACs spiking caused an impact on the bacterial species in this section, but after spiking bacterial species were present and distributed similarly as the initial population. Therefore, this population would have been clearly affected for PhACs spiking rather than the change in HRT.

For fungal samples in the biofilm section (Figure 4.8A), first-order clusters grouped the three first stages (3, 4 and 5) separated from the last stage (7). The second-order subclusters separate, on one side replicas 1 and 2 from 3 in the stage 7, and on the other sample 3 in stage 5 from the rest of samples. This configuration suggests that after the cease on PhACs spiking, new fungal species appeared, which could be linked to irreversible changes in fungal population, promoting the substition of oportunistic species, that may

be abundant in BSF (Hwang et al., 2014) and may be non-resilient to PhACs high concentration, for other species adapted to changes in PhACs concentration. On the contrary, fungal samples for sand section (Figure 4.8B) are grouped in two first-order clusters differentiating replicas from stage 5 from the rest of stages, and concomitantly, the second-order subcluster for the rest of stages separates replicas 1 and 2 from stage 7 from replica 3, this last included with the rest of samples from stages 3 and 4, suggesting that in this case the PhACs spiking during the second HRT caused the most dramatic change for fungi present in this section.

These highly heterogeneous results in bacteria and fungi according to their position in the biofilm, and even within replicates, prevent any conclusion on whether there existed a general biostimulation effect on both bacterial and fungal populations when PhACs were spiked. Regarding these UPGMA analyses, Shannon-wiener and Evenness indexes were also calculated to study these changes in genetic profiles.

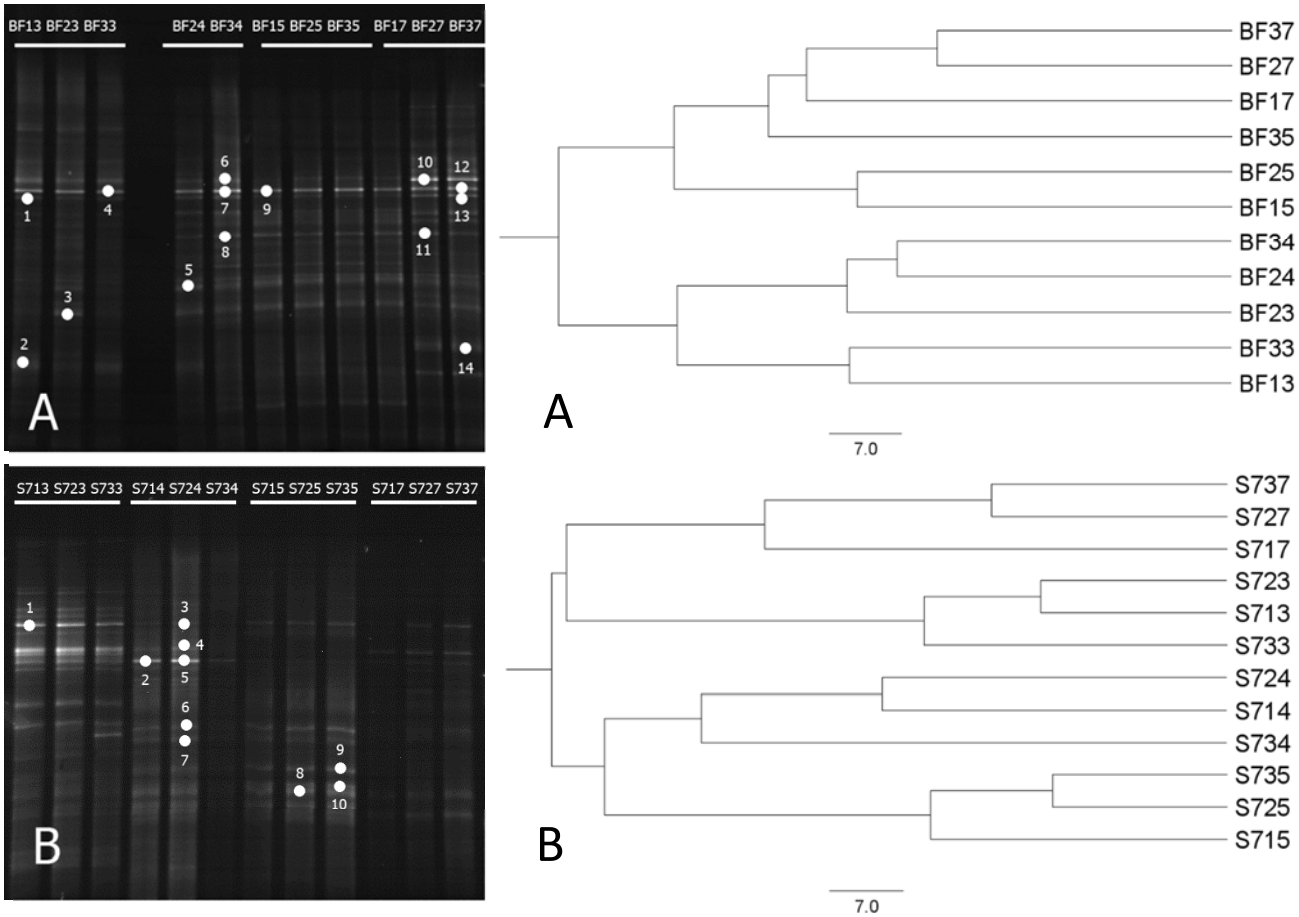

Figure 4.7 Bacterial rDNA amplified fragments and the DGGE fingerprints cluster analysis for A (biofilm part) and B (sand part). The bands marked with numbers correspond to the bands that were extracted from the

DGGE gels and sequenced. Cluster dendrograms based on UPGMA algorithm show similarity among DGGE band patterns of bacteria. Bar indicates percentage of divergence.

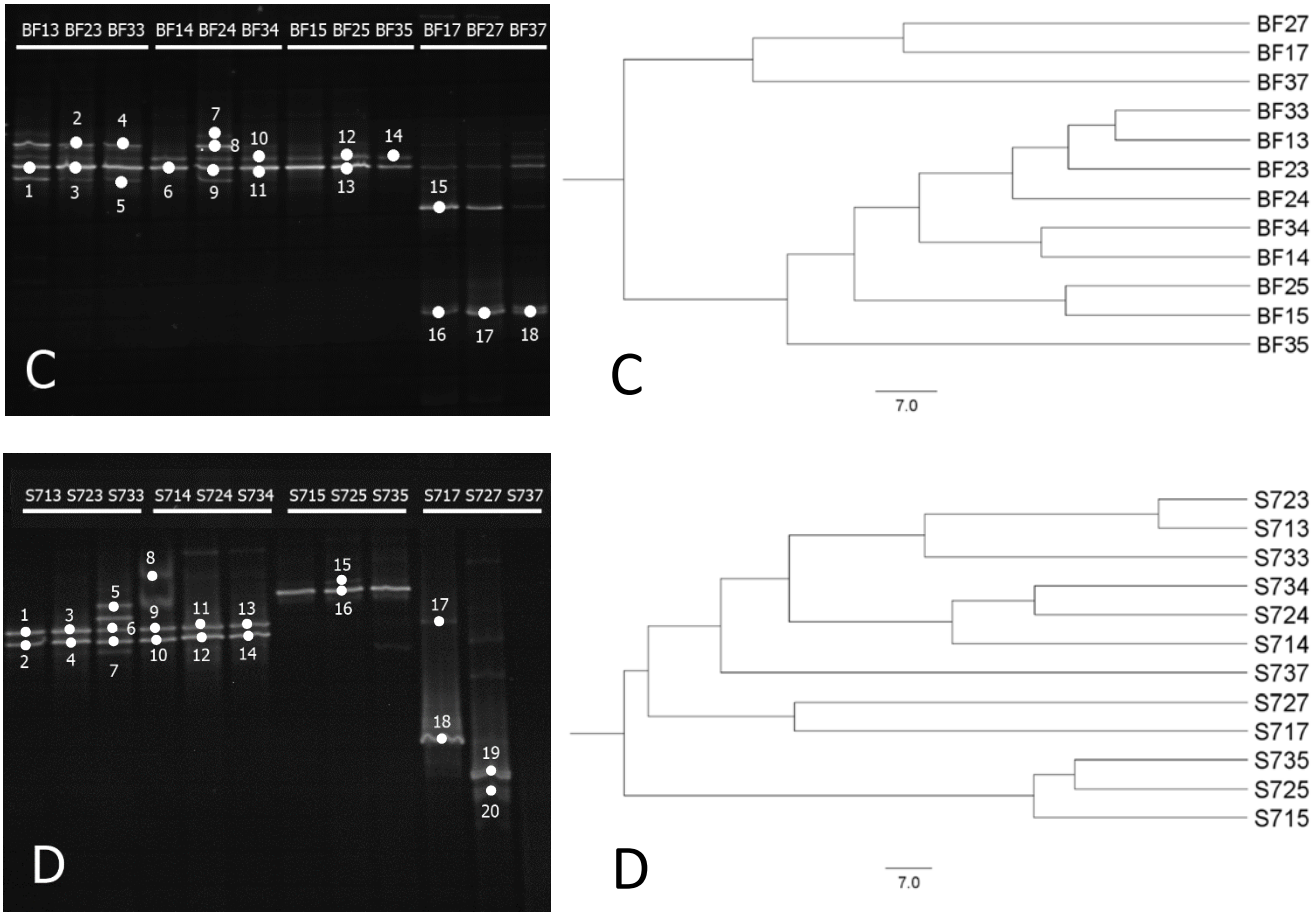

Figure 4.8 Fungal rDNA amplified fragments and the DGGE fingerprints cluster analysis for C (biofilm part) and D (sand part). The bands marked with numbers correspond to the bands that were extracted from the DGGE gels and sequenced. Cluster dendrograms based on UPGMA algorithm show similarity among DGGE band patterns of fungi. Bar indicates percentage of divergence.

Diversity variables were submitted to nonparametric analysis of variance (Tukey test) ($p<0.05$) to detect significant differences. According to Shannon-wiener index (Table 4.3), it can be stated that bacteria present in the BSF were more complex and diverse than fungi, being this difference maintained during all the experiment in spite of PhACs spiking. In the first stage without spiking, bacteria do not show differences in diversity or evenness indexs depending on the section, but biofilm fungi do present higher homogenity (evenness index) respect to the sand section.

However, when comparing this first stage results with the rest of stages, Evenness index showed opposed behaviours among bacteria and fungi. Within the same section and

microorganism, and respect to the initial values evenness in bacteria was maintained (biofilm section) or even increased (sand section), whereas in fungi decreased in both sections, dramatically in the case of biofilm section (with a high recover after the cease of PhAC spiking) and steadily in the case of sand section, hence the homogenous distribution of initial fungal species was affected by PhACs addition, whereas in the case of bacteria, PhACs performed an homogenizing effect.

Regarding species diversity accross time, bacteria present in the biofilm section had its diversity maintained during the spiking, with a significant increase after its cease, whereas in the sand section, paradoxically species diversity declined after the cease of spiking. This effect could be explained by long-term irreversible effects of PhACs on microbiota. In relation with this effect, it has been proved that certain PhACs such as antibiotics

Table 4.3 Shannon-wiener (H) and Evenness (E) indexes classified according to the sample position (biofilm or sand) and the microorganism (bacteria or fungi), for the four different sampling times, plus minus standard deviation.

				Bacteria		Fungi	
Stage	Month	Days		Biofilm section	Sand section	Biofilm section	Sand section
Before spiking	3	137	H	2.22 ± 0.25	2.34 ± 0.03	1.54 ± 0.06	1.32 ± 0.39
			E	0.81 ± 0.07	0.82 ± 0.01	0.86 ± 0.04	0.99 ± 0.00
Spiking 1st HRT (1.85 d)	4	156	H	2.55 ± 0.10	2.42 ± 0.05	1.57 ± 0.18	1.53 ± 0.06
			E	0.87 ± 0.01	0.87 ± 0.01	0.76 ± 0.09	0.92 ± 0.02
Spiking 2nd HRT (6.15 d)	5	175	H	2.50 ± 0.13	2.39 ± 0.05	1.11 ± 0.09	1.76 ± 0.42
			E	0.89 ± 0.04	0.92 ± 0.01	0.62 ± 0.05	0.92 ± 0.05
After spiking	7	259	H	2.64 ± 0.07	2.10 ± 0.08	1.71 ± 0.08	1.52 ± 0.06
			E	0.89 ± 0.02	0.92 ± 0.01	0.81 ± 0.05	0.85 ± 0.03

(ubiquitous in HWW) can cause affections on human gut microbiota from 3 months later and up to 2 years later (Jernberg et al., 2010).

For instance, the effect of amoxicillin will generally promote an increase in resistant enterobacteria and a decrease in aerobic Gram positive cocci in the gut microbiota.

On the contrary, fungal diversity was significantly affected in the biofilm section by the spiking, specially during the stage with the 2nd HRT, but it could recover and even increase after the end of spiking. However, in the case of sand section, non-significant differences were detected between stages.

On regard of these results, and considering a potential biostimulation of species able to degrade PhACs present in the BSF, the most clear case would be the fungal species present in the biofilm section during the stage with a HRT of 6.15 d, followed by bacterial species present in the sand section in the last stage after spiking.

In figures 4.7 and 4.8, the bands marked with numbers correspond to the dominant bands extracted from the DGGE gels and sequenced. Tables 4.4 and 4.5 show the sequenced bands, their similarity values compared to the closest related GenBank sequences, and their phylogenetic affiliations. Sequence similarity values compared to previously reported sequences were more than 93.5% in all cases.

Fungal and bacterial DNA sequenced from samples of biofilm and sand sections from the BSF revealed the presence of species very different from other reported BSF's genetic profiles operated with water from other sources such as rivers (Feng et al., 2013; Hwang et al., 2014).

The majority of the 24 bacterial sequences belonged to the phylum Bacteroidetes (58.3%), followed by Firmicutes (41.7%) (Table 4.4). Regarding fungi, the 38 sequences fell mostly into an unclassified taxonomic group (63.2%). However, Basidiomycota (21%) and Ascomycota (15.7%) were also present (Table 4.5). The fungal and bacterial rRNA sequences determined in this study are available at the GenBank under accession numbers MF276805 through MF276867.

On regarding to the possible bacterial candidates for the removal of the spiked PhACs (CPX, KTP and IBP), closest matches for DNA sequences present in the samples during the spiking of PhACs (stages 4 and 5) were studied. In the case of cultured bacteria present in

the NCBI database, the only match available was *Macellibacteroides fermentans* strain HH-ZS (present in biofilm during the stages 4 and 5, whereas only in the 4 stage in the case of sand section), which has been reported to be isolated from hyperalkaline contaminated soil of a lime kiln waste site.

On the other hand, concerning potential PhACs biodegraders among fungal species with matches in the analysed sequences for stages 4 and 5, one species is specially frequent in the analysis, sequences had matches of 100% with an uncultured *Trichosporon (clone CHiv91*), which was present in both biofilm and sand sections. According to the NCBI database, this fungus would have been isolated from gut samples of VIH-patients, which would fit an environment with a high concentration of PhACs, and thus offering promising potentiality to degrade them.

On the other hand, *Apiotrichum scarabaeorum* has also been matched abundantly in the biofilm. There is not abundant literature describing this species, but some species of the same genus have been reported to be involved in the methylation of metalloids (Bentley and Chasteen, 2002). Apart from the uncultured *Trichosporon*, one match (100%) of the yeast *Williopsis sp.* was also reported at stage 4.

TABLE 4.4 Bacterial DGGE bands sequenced, band length, associated GenBank accession numbers, and phylogenetic affiliation from profiles of bacterial populations.

Phylotype			Phylogenetic affiliation			
Band code[6]	**Sequence length**	**Accession code**	**Taxonomic linage**[7] **(Phylum, Class, Order, Family, Genus)**	**Closest match**[8]	**Covery (%)**	**Similarity**[9] **(%)**
A1	423	MF276846	Bacteria(100), Bacteroidetes(100), Bacteroidia(100), Bacteroidales(100), Rikenellaceae(99), vadinBC27(99), unclassified	Uncultured anaerobic bacterium clone P-1	100	96
A2	406	MF276844	Bacteria(100), Firmicutes(100), Clostridia(100), Clostridiales(100), Ruminococcaceae(99), uncultured(80), unclassified	Uncultured bacterium gene	100	99
A3	387	MF276845	Bacteria(100), Firmicutes(93), Clostridia(85), Clostridiales(85), Clostridiaceae(41), Oxobacter(36), unclassified	Uncultured bacterium clone SP3_F05	99	95
A4	421	MF276847	Bacteria(100), Bacteroidetes(100), Bacteroidia(100), Bacteroidales(100), Porphyromonadaceae(100), Parabacteroides(100), unclassified	Macellibacteroides fermentans strain HH-ZS	100	100
A5	406	MF276850	Bacteria(100), Firmicutes(97), Clostridia(97), Clostridiales(97), Ruminococcaceae(91), Acetanaerobacterium(80), unclassified	Uncultured bacterium clone OTU631	95	99
A6	424	MF276848	Bacteria(100), Bacteroidetes(100), Bacteroidia(88), Bacteroidales(88), Porphyromonadaceae(72), Parabacteroides(64), unclassified	Uncultured bacterium clone Porp1	100	94
A7	423	MF276849	Bacteria(100), Bacteroidetes(100), Bacteroidia(100), Bacteroidales(100), Porphyromonadaceae(100), Parabacteroides(100), unclassified	Macellibacteroides fermentans strain HH-ZS	97	99
A8	423	MF276856	Bacteria(100), Firmicutes(99), Erysipelotrichi(92), Erysipelotrichales(92), Erysipelotrichaceae(92), Erysipelothrix(91), unclassified	Uncultured bacterium clone SuLd1_H13	99	97
A9	462	MF276855	Bacteria(100), Bacteroidetes(100), Bacteroidia(100), Bacteroidales(100), Porphyromonadaceae(100), Parabacteroides(100), unclassified	Macellibacteroides fermentans strain HH-ZS	91	100
A10	425	MF276852	Bacteria(100), Bacteroidetes(100), Sphingobacteria(95), Sphingobacteriales(95), WCHB1-69(83), unclassified	Uncultured bacterium clone MedPSep07RW42	100	96
A11	400	MF276851	Bacteria(100), Firmicutes(98), Clostridia(97), Clostridiales(97), Clostridiaceae(94), Anaerobacter(65), unclassified	Uncultured organism clone ELU0130-T340-S-NI_000207	90	95

[6] *Band numbers correspond to those presented in **Figure 4.7** for bacterial samples (A corresponds to biofilm, B to sand section.)*

[7] *Taxonomic string with boot strap values (in parentheses), generated in mothur using SILVA data base reference file release 119.*

[8] *Closest relative according to INSA (International Nucleotide Sequence Database).*

[9] *Percentage sequence covery and similarity with closest INSA using BLAST tool.*

TABLE 4.4 (Continuation) Bacterial DGGE bands sequenced, band length, associated GenBank accession numbers, and phylogenetic affiliation from profiles of bacterial populations.

Band code[10]	Phylotype Sequence length	Accession code	Phylogenetic affiliation Taxonomic linage[11] (Phylum, Class, Order, Family, Genus)	Closest match[12]	Covery (%)	Similarity[13] (%)
A12	419	MF276853	Bacteria(100), Bacteroidetes(100), Bacteroidia(93), Bacteroidales(93), Porphyromonadaceae(55), Parabacteroides(40), unclassified	Uncultured Parabacteroides sp. clone JXSH-34	100	94
A13	427	MF276854	Bacteria(100), Bacteroidetes(100), Bacteroidia(100), Bacteroidales(100), Rikenellaceae(100), vadinBC27(100), unclassified	Uncultured anaerobic bacterium clone P-1	100	99
A14	407	MF276857	Bacteria(100), Firmicutes(87), Clostridia(87), Clostridiales(82), Lachnospiraceae(58), Catabacter(52), unclassified	Uncultured bacterium clone SHUX538	99	97
B1	412	MF276864	Bacteria(100), Bacteroidetes(100), Bacteroidia(100), Bacteroidales(100), Porphyromonadaceae(100), Petrimonas(100), unclassified	Uncultured bacterium clone SuSb2_G02	100	99
B2	427	MF276865	Bacteria(100), Bacteroidetes(100), Bacteroidia(100), Bacteroidales(100), Porphyromonadaceae(100), Parabacteroides(100), unclassified	Uncultured Bacteroidetes bacterium	99	99
B3	416	MF276866	Bacteria(100), Bacteroidetes(100), Bacteroidia(99), Bacteroidales(99), Bacteroidaceae(82), Bacteroides(82), unclassified	Uncultured bacterium clone Bact1	100	95
B4	420	MF276867	Bacteria(100), Bacteroidetes(100), Bacteroidia(100), Bacteroidales(100), Porphyromonadaceae(100), Parabacteroides(100), unclassified	Macellibacteroides fermentans strain HH-ZS	100	99
B5	414	MF276861	Bacteria(100), Bacteroidetes(100), Bacteroidia(100), Bacteroidales(100), Porphyromonadaceae(100), Parabacteroides(100), unclassified	Macellibacteroides fermentans strain HH-ZS	100	99
B6	399	MF276862	Bacteria(100), Firmicutes(100), Clostridia(100), Clostridiales(100), Clostridiaceae(100), Clostridium(99), unclassified	Uncultured bacterium clone GP_2aaa01e04	100	99
B7	405	MF276863	Bacteria(100), Bacteroidetes(100), Bacteroidia(96), Bacteroidales(96), Rikenellaceae(95), Alistipes(92), unclassified	Uncultured bacterium clone LFS2_17	100	98

[10] *Band numbers correspond to those presented in **Figure 4.8** for fungal samples (C refers to biofilm section and D to sand section).*

[11] *Taxonomic string with boot strap values (in parentheses), generated in mothur using SILVA data base reference file release 119.*

[12] *Closest relative according to INSA (International Nucleotide Sequence Database).*

[13] *Percentage sequence covery and similarity with closest INSA using BLAST tool.*

TABLE 4.4 (Continuation) Bacterial DGGE bands sequenced, band length, associated GenBank accession numbers, and phylogenetic affiliation from profiles of bacterial populations.

Phylotype			Phylogenetic affiliation			
Band code[14]	**Sequence length**	**Accession code**	**Taxonomic linage**[15] **(Phylum, Class, Order, Family, Genus)**	**Closest match**[16]	**Covery (%)**	**Similarity**[17] **(%)**
B8	402	MF276860	Bacteria(100), Firmicutes(100), Clostridia(100), Clostridiales(100), Peptostreptococcaceae(100), Incertae_Sedis(99), unclassified	Uncultured bacterium clone ncd629e02c1 1	100	98
B9	392	MF276859	Bacteria(100), Firmicutes(63), Clostridia(62), Clostridiales(62), Clostridiaceae(46), Anaerobacter(41), unclassified	Uncultured bacterium clone 384H04	97	90
B10	403	MF276858	Bacteria(100), Firmicutes(100), Clostridia(100), Clostridiales(100), Peptostreptococcaceae(98), Incertae_Sedis(51), unclassified	Uncultured bacterium clone 3N21hH35	100	98

[14] *Band numbers correspond to those presented in **Figure 4.8** for fungal samples (C refers to biofilm section and D to sand section).*

[15] *Taxonomic string with boot strap values (in parentheses), generated in mothur using SILVA data base reference file release 119.*

[16] *Closest relative according to INSA (International Nucleotide Sequence Database).*

[17] *Percentage sequence covery and similarity with closest INSA using BLAST tool.*

TABLE 4.5 Fungal DGGE bands sequenced, band length, associated GenBank accession numbers, and phylogenetic affiliation from profiles of fungal populations.

Phylotype			Phylogenetic affiliation			
Band code[18]	**Sequence length**	**Accession code**	**Taxonomic linage**[19] **(Phylum, Class, Order, Family, Genus)**	**Closest match**[20]	**Covery (%)**	**Similarity**[21] **(%)**
C1	89	MF276820	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
C2	86	MF276818	Unclassified(100)	Uncultured fungus clone 36F	95	99
C3	127	MF276819	Basidiomycota(85), Unclassified(85)	Uncultured Trichosporon clone CHiv91	93	98
C4	133	MF276816	Basidiomycota(86), Unclassified(86)	Apiotrichum scarabaeorum culture-collection CBS:5601	92	98
C5	92	MF276817	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
C6	91	MF276822	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
C7	130	MF276815	Basidiomycota(84), Unclassified(84)	Apiotrichum scarabaeorum culture-collection CBS:5601	92	98
C8	99	MF276821	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
C9	126	MF276823	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
C10	130	MF276812	Basidiomycota(81). Unclassified(81)	Apiotrichum scarabaeorum culture-collection CBS:5601	92	98
C11	130	MF276813	Unclassified(100)	Apiotrichum scarabaeorum culture-collection CBS:5601	92	98
C12	89	MF276810	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
C13	155	MF276811	Fungi(99), Basidiomycota(91), Tremellomycetes(84), Trichosporonales(82), Trichosporonaceae(82), Unclassified(82)	Apiotrichum scarabaeorum culture-collection CBS:5601	91	96

[18] *Band numbers correspond to those presented in **Figure 4.8** for fungal samples (C refers to biofilm section and D to sand section).*

[19] *Taxonomic string with boot strap values (in parentheses), generated in mothur using SILVA data base reference file release 119.*

[20] *Closest relative according to INSA (International Nucleotide Sequence Database).*

[21] *Percentage sequence covery and similarity with closest INSA using BLAST tool.*

TABLE 4.5 (continuation) Fungal DGGE bands sequenced, band length, associated GenBank accession numbers, and phylogenetic affiliation from profiles of fungal populations.

Phylotype			Phylogenetic affiliation			
Band code[22]	Sequence length	Accession code	Taxonomic linage[23] (Phylum, Class, Order, Family, Genus)	Closest match[24]	Covery (%)	Similarity[25] (%)
C14	131	MF276809	Basidiomycota(83), Unclassified(83)	Apiotrichum scarabaeorum culture-collection CBS:5601	92	98
C15	175	MF276807	Unclassified(100)	Amanita muscaria isolate d17	47	100
C16	125	MF276808	Ascomycota(100), Sordariomycetes(88), Unclassified(88)	Uncultured fungus clone 037A33534	93	99
C17	168	MF276806	Ascomycota(100), Sordariomycetes(95), Unclassified(95)	Uncultured fungus clone 035A8833	94	98
C18	166	MF276805	Ascomycota(100), Sordariomycetes(96), Unclassified(96)	Uncultured fungus clone 035A8833	94	97
D1	90	MF276825	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
D2	93	MF276824	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
D3	88	MF276827	Unclassified(100)	Uncultured Trichosporon clone CHiv91	90	100
D4	87	MF276826	Unclassified(100)	Uncultured fungus clone 36F	94	99
D5	99	MF276829	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
D6	93	MF276828	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
D7	93	MF276830	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
D8	68	MF276833	Unclassified(100)	Uncultured fungus clone 36F	100	97
D9	92	MF276832	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
D10	92	MF276831	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100

[22] *Band numbers correspond to those presented in **Figure 4.8** for fungal samples (C refers to biofilm section and D to sand section).*

[23] *Taxonomic string with boot strap values (in parentheses), generated in mothur using SILVA data base reference file release 119.*

[24] *Closest relative according to INSA (International Nucleotide Sequence Database).*

[25] *Percentage sequence covery and similarity with closest INSA using BLAST tool.*

TABLE 4.5 (continuation) Fungal DGGE bands sequenced, band length, associated GenBank accession numbers, and phylogenetic affiliation from profiles of fungal populations.

Phylotype			Phylogenetic affiliation			
Band code[26]	**Sequence length**	**Accession code**	**Taxonomic linage**[27] **(Phylum, Class, Order, Family, Genus)**	**Closest match**[28]	**Covery (%)**	**Similarity**[29] **(%)**
D11	92	MF276835	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
D12	92	MF276834	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
D13	93	MF276837	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
D14	93	MF276836	Unclassified(100)	Uncultured Trichosporon clone CHiv91	91	100
D15	67	MF276838	Unclassified(100)	Uncultured fungus clone 36F	100	100
D16	158	MF276839	Ascomycota(90), Saccharomycetes(89), Saccharomycetales(89), Saccharomycetaceae(84), Williopsis(84),Williopsis(84), Williopsis(84)	Williopsis sp. JHL	93	100
D17	201	MF276843	Basidiomycota(100), Agaricomycetes(100), Polyporales(100), Polyporaceae(92), Trametes(91), Unclassified(91)	Trametes versicolor	95	100
D18	181	MF276840	Basidiomycota(100), Agaricomycetes(99), Polyporales(98), Polyporaceae(95), Trametes(83), Unclassified(83)	Trametes versicolor	94	99
D19	144	MF276841	Ascomycota(100), Sordariomycetes(100), Microascales(96), Microascaceae(96), Pseudallescheria(96), Pseudallescheria ellipsoidea(96), Pseudallescheria ellipsoidea(96)	Pseudallescheria ellipsoidea isolate 35-CL	93	99
D20	170	MF276842	Ascomycota(100), Sordariomycetes(99), Unclassified(99)	Uncultured fungus clone 035A8833	94	98

[26] *Band numbers correspond to those presented in **Figure 4.8** for fungal samples (C refers to biofilm section and D to sand section).*

[27] *Taxonomic string with boot strap values (in parentheses), generated in mothur using SILVA data base reference file release 119.*

[28] *Closest relative according to INSA (International Nucleotide Sequence Database).*

[29] *Percentage sequence covery and similarity with closest INSA using BLAST tool.*

4.4. Conclusions

Although having shown great success and low cost in purifying drinking water, and in spite of being operated for more than 90 days, BSF were only able to reduce bacterial concentrations to a maximum of 2 log units cfu/mL. Having discarded BSF as a pretreatment to decrease bacterial load for fungal bioreactors, the aim of the study was to investigate the possibility of the BSF's biostimulated microbiota from HWW to degrade recalcitrant PhAC.

Known concentrations of CPX and IBP were spiked to the water, obtaining removals of up to more than 90% for CPX and 50% for IBP.

A molecular biology study for core BSF samples was performed. Regarding the changes in genetic profile from samples before, during and after the PhACs spiking in the BSF's microbiota, DGGE fingerprinting and band characterization analyses showed that fungal population diversity had been specially affected during PhACs spiking, mainly with a longer HRT and in the biofilm section. Band characterization allowed to identify at least two fungal and one bacterial species (an uncultured *Trichosporon*, *Apiotrichum scarabaeorum* and *Macellibacteroides fermentans,* respectively) which might be involved in degradation of CPX and IBP.

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5. Promising fungal species for PhACs removal in contaminated water

5.1. Introduction

Fungal biodegradation has become a promising approach among wastewater treatment technologies to remove emerging micropollutants (including endocrine disruptors, drugs or recalcitrant PhACs) due to the nonspecific ligninolytic enzymatic system of fungi, which targets the degradation of both biological and xenobiotic compounds (Harms et al., 2011). As mentioned in the general introduction, promising results have been achieved in the biodegradation of many PhACs (Cruz-Morató et al., 2013a; Marco-Urrea et al., 2009; Vasiliadou et al., 2016). Nonetheless, most of the fungal species used entail some compounds to remain recalcitrant (Ferrando-Climent et al., 2015), which propels further research into finding new biodegradation candidates.

Ligninolytic fungi rely on two main mechanisms for the biodegradation of compounds: on the one hand, extracellular oxidation occurs by laccase or other manganese and lignin peroxidase enzymes; on the other hand, intracellular degradation involving cytochrome P450 monooxigenases and nitroreductases occurs via either oxidation and/or reduction reactions or by the conjugate formation by transferases. However, these enzymatic processes do not always end with a desirable or complete mineralization of the compounds but can instead result in intermediate transformation products or just the sorption of parent compounds into the biomass (Cruz-Morató et al., 2013b; Prieto et al., 2011).

The performance of six different ligninolytic fungi (*Trametes versicolor, Ganoderma lucidum, Irpex lacteus, Stropharia rugosoannulata, Gymnopilus luteofolius* and *Agrocybe erebia*) was explored as an alternative biological treatment to eliminate selected PhACs.

From an ecophysiological point of view, all 6 fungi can degrade all components of plant cell walls, including lignin, thus they could be considered white-rot fungi (WRF). However, in the current classification in wood decaying fungi (Riley et al., 2014a), *G. luteofolius* and *S. rugosoannulata* are either considered WRF (Anasonye et al., 2015; Galletti, 1992), or litter decomposing fungi (LDF) (Harms et al., 2011; Kabiersch et al., 2011; Kähkönen et al.,

2008). Nonetheless, *Agrocybe* species always appears as LDF in the literature (Kähkönen et al., 2008; Suhara et al., 2011).

T. versicolor, *G. lucidum* and *I. lacteus* have been commonly used for biodegrading contaminants of emerging concern in water and soil (Asgher et al., 2008b; Badia-Fabregat et al., 2012a; Marco-Urrea et al., 2010b; Moon and Song, 2012; Novotný et al., 2009; Stella et al., 2016). In contrast, *S. rugosoannulata*, *G. luteofolius* and *A. erebia* have been mainly studied as hydrolytic enzyme producers (Kähkönen and Hakulinen, 2011). However, there are few examples of its use in micropollutants removal. For instance, *S. rugosoannulata* is able to degrade industrial dyes such as Basic Blue 22 and Acid Red 183 in liquid cultures (Jarosz-Wilkołazka et al., 2002) and other toxic compounds in soil such as polychlorinated dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF) (Anasonye et al., 2014). *G. luteofolius* has been reported to degrade trinitrotoluene (TNT) in soil (Anasonye et al., 2015), and not specifically *A. erebia* (but other species in the *Agrocybe* genus) showed potential biodegradability for chlorinated dichlorodiphenyltrichloroethane (DDT) pesticide and polycyclic aromatic hydrocarbons (Steffen et al., 2003; Suhara et al., 2011).

These three species share many enzymes such as versatile peroxidase or manganese peroxidase, with *T. versicolor*, *G. lucidum* and *I. lacteus*, but may also have exclusive enzymes such as aromatic peroxygenases (heme-thiolate peroxidases) (Harms et al., 2011; Hofrichter et al., 2010).

In relation with PhACs removal, promising results for *T. versicolor*, *G. lucidum* and *I. lacteus* have been already reported in several publications (Bernats and Juhna, 2015; Marco-Urrea et al., 2009; Vasiliadou et al., 2016). On the contrary, and to the author's best knowledge, this is the first time that *S. rugosoannulata, G. luteofolius* and *A. erebia* have been used for pharmaceutical removal in water.

In this chapter, a screening of novel fungal candidates for PhACs removal and its degradation was performed. This study involved the combination of 6 fungi and 6 PhACs, analyzing PhACs removals and its biodegradation rates. Target PhACs were selected based on their ubiquity in hospital wastewater effluents and poor biodegradability in WWTP.

Poor biodegradability of PhACs is related to its complex structure (Figure 5.1) (Taheran et al., 2016). However, in spite of sharing structural complexity, these PhACs belong to

different chemical and pharmaceutical therapeutic families: carbamazepine (CBZ) is used as an antiepileptic drug, cyclophosphamide (CFD) and ifosfamide (IFD) as cytostatics, diclofenac (DCF) as an analgesic drug, iopromide (IPD) as a contrast media agent, and finally venlafaxine (VFX) as an antidepressant. Table 5.1 in section 2.5.2.1 shows several chemical properties of these PhACs.

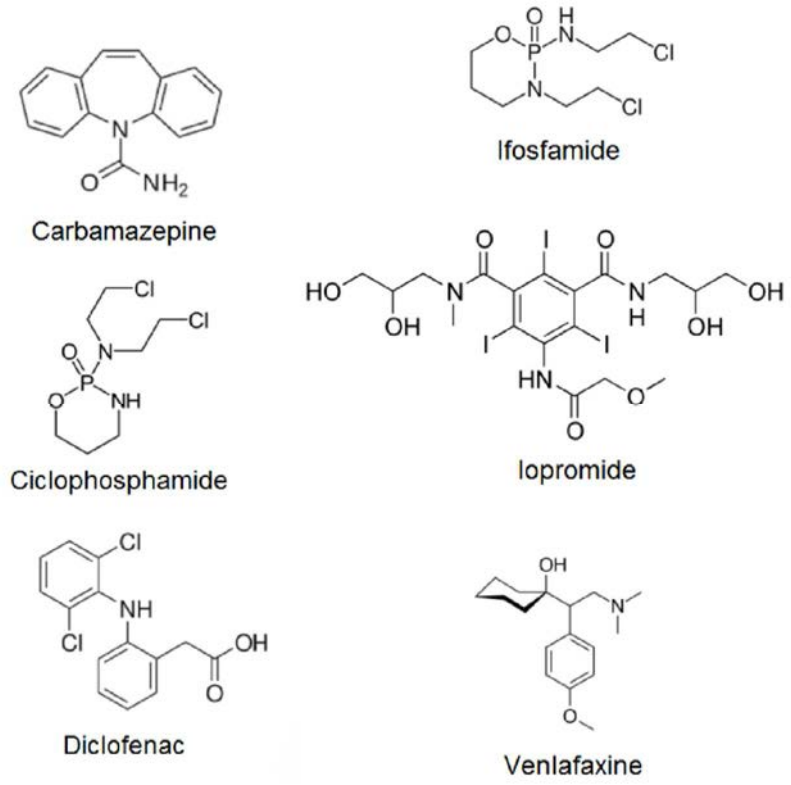

Figure 5.1 Chemical structure of the 6 different PhACs used in this study.

Besides, the sorption of four of these PhACs (CBZ, DCF, IPD and VFX) onto fungal biomass in comparison to the concentration of these PhACs in spiked water was evaluated. Concentration of PhACs in both liquid and solid (biomass) phases involved in the fungal treatment was measured, allowing to compare these direct measurements of PhACs concentration (and its corresponding degradation) with the indirect measurements such as assumed minimal degradation rates calculated from removal differences between experimental and killed control treatments. Finally, a mass balance of PhACs was performed to estimate PhAC final fate and occurrence.

The goals of this chapter, hence, were three: i) the investigation of the potential removal of 6 recalcitrant PhACs by 6 different fungal species, ii) the determination in every case the contribution of each mechanism involved in PhACs removal such as sorption or degradation, and finally iii) the purification of potentially involved laccase extracts for every fungi in order to perform a characterization of these enzymes and detect potential PhACs transformation pathways.

5.2. Materials and Methods

5.2.1. Fungi and chemicals

Six different species of fungi from different collections were used in this experiment: *Trametes versicolor* (WRF) (American Type Culture Collection 42530^{TM} strain), *Irpex lacteus* (WRF) (AX1 strain, Michigan State University collection), *Ganoderma lucidum* (WRF) (Leysser) (Karsten FP-58537-Sp, United States Department of Agriculture, Madison, Wis. Collection); cultures from *Stropharia rugosoannulata* (LDF/WRF), *Gymnopilus luteofolius* (LDF/WRF) and *Agrocybe erebia* (LDF) strains number 475, 466 and 476 respectively, came from the Finish Fungal Biotechnology Culture Collection (University of Helsinki).

Pellet immobilization was achieved for all of the fungi following the same procedure described by Blánquez et al. (2004). The pellets obtained by this process were washed with sterile deionized water and kept (if needed) in a 0.8% NaCl solution at 4ºC. The subculture and pellet formation methodology are described in section 1.3.4. Biomass was added at an approximated concentration of 4.5 g/L of initial biomass dry cell weight (DCW) (table 5A.5 in the annex).

A stock solution containing 6 different PhACs (CBZ, CFD, IFD, DCF, IPD and VFX) was used to spike a defined medium for the PhACs removal experiments. High purity grade ethanol from Sigma Aldrich (Barcelona, Catalonia) was used as a solvent for the stock solution. All of the standards purity grades for PhACs were high (>90%) and were all purchased from Sigma Aldrich (Barcelona, Catalonia), except for iopromide (purchased in European Pharmacopeia) and cytostatics cyclophosphamide and ifosfamide, purchased by European Directorate for the Quality of Medicines and Healthcare (EDQM) Reference Standards (Strasbourg, France)

Table 5.1 Chemical properties of the six PhACs used in the study.

Pharmaceutical compound	Abbreviation	Formula	Molecular Weight	Application	Log Kow[a]	Total Removal in WWTP (%)[b]	Total Biodegradation in WWTP (%)[b]
Carbamazepine	CBZ	$C_{15}H_{12}N_2O$	236.269 g·mol^{-1}	Antiepileptic	2.45	2.96	0.1
Venlafaxine	VFX	$C_{17}H_{27}NO_2$	277.402 g·mol^{-1}	Antidepressant	3.28*	8.91	0.15
Iopromide	IPD	$C_{18}H_{24}I_3N_3O_8$	791.11187 g·mol^{-1}	Contrast Media Agent	2.33[c]	-	-
Diclofenac	DCF	$C_{14}H_{11}Cl_2NO_2$	296.148 g·mol^{-1}	Analgesic	4.51	56.55	0.53
Cyclophosphamide	CFD	$C_7H_{15}Cl_2N_2O_2P$	261.086 g·mol^{-1}	Cytostatic	0.63	1.86	0.09
Ifosfamide	IFD	$C_7H_{15}Cl_2N_2O_2P$	261.1 g·mol^{-1}	Cytostatic	0.86	1.88	0.09

[a] Log Kow data are correlated with water solubility and bioconcentration for aquatic life. Log Kow values were obtained from US Environmental Protection Agency's EPISuite™ Database.

[b] Obtained from biological WWTP from the US Environmental Protection Agency's EPISuite™ Database

[c](Gabarrón et al., 2016)

*Estimated values (non-experimental)

5.2.2. Degradation experiments

Degradation experiments were performed in batches of 250 mL Erlenmeyer flasks, filled with 100 mL of PhAC spiked defined medium from a stock solution containing the 6 PhACs, achieving an initial concentration (IC) ranging from 40 to 184.7 µg/L per PhAC among treatments, being this difference attributable to divergences between batches.

Apart from the experimental treatment, abiotic controls (where the matrix contained target compounds exposed to the same experimental conditions but without fungi) were used to assess potential physicochemical degradation. Moreover, an alive or biotic control (including fungal biomass but free from PhACs) and heat-killed (killed control) fungal biomass controls were also included. This last killed control, was autoclaved and set-up under identical conditions to those of the experimental cultures in order to analyze PhACs sorption processes into the fungal biomass, whereas the biotic control was set to assess the potential toxicological effects of PhACs on the biomass.

The experiment lasted 6 days, and the fungal biomass was added in pellet morphology. All controls and experimental treatments were run in triplicate. Therefore, the experimental set-up included 24 Erlenmeyer flasks per fungus, and considered three initial and three final replicates per treatment.

After inoculation and PhACs addition (when applicable), the flasks were incubated under orbital shaking (135 rpm) at 25ºC for six days under dark conditions to prevent the possible photodegradation of the compounds. The entire content of the flasks was sacrificed at the initial and final times. All samples were filtered through a 1 µm glass fibre filter to remove biomass from the liquid phase and collect aliquots for glucose, laccase and MnP activity analyses afterwards.

5.2.3. Pharmaceutical analysis

For the analysis of the samples, 900 µL of the water samples was collected from each sampling time, and 100 µL of methanol was then added to create 1 mL of extract in methanol-water (10:90 v/v). Some samples were diluted (1:10) when necessary before their analysis. Finally, 10 µL of an internal standard mix at 1 ng/µL was added to all of the

samples before direct injection into the LC-MS/MS system for internal standard quantification.

The analytical procedure for the quantification of PhACs was adapted from the method of Gros et al. (2012) to include mass spectrometry parameters of CFD and IFD (Table 5A.1 in the Annex). PhACs concentrations were measured according to the signal detected during a specific retention time. This retention time was calculated previously through a calibration curve for each PhAC. Chromatographic separations were conducted using a Waters Acquity Ultra-Performance^{TM} liquid chromatograph system equipped with two binary pump systems using an Acquity HSS T3 column (50 mm × 2.1 mm i.d., 1.8 µm particle size; Waters Corp. Mildford, MA, USA) and positive electrospray ionization (PI). The optimized separation conditions were as follows: solvent A was methanol, and solvent B was 10 mM formic acid/ammonium formate (pH 3.2) at a flow rate of 0.5 mL/min. The gradient elution was as follows: initial conditions 5% A; 0–4.5 min, 5–95% A; 4.5–4.6 min, 100% A; 4.6–6.0 min, 100% A; from 6.0 to 6.1 to return to the initial conditions; and 6.1–6.7, equilibration of the column. The injected sample volume was 5 µL. The UPLC instrument was coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. Compound dependent MS parameters (declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP)) were optimized by direct infusion of individual standard solutions of each compound at 20 µg/L. A summary of the optimum values and the MRM transitions is available in the supporting information (Table 5A.1 in the Annex). All transitions were recorded using the Scheduled MRMTM algorithm, and the data were acquired and processed using the Analyst 2.1 software. Analytical limits of detection and quantification for this method can be consulted in Table 5A.2 in the Annex.

5.2.4. Biomass samples pre-treatment for PhACs detection

For the analysis of micropollutants in the fungal biomass samples, the biomass was freeze dried and then homogenized using a Robot Coupe Blixer food processor (Robot Coupe USA, Jackson, MS) and a mortar. For each sample, 1 g of biomass was used; then 4 mL of MeOH-Na_2EDTA (50:1.5 v/v) were added and vortexed for 30 s. Later on, samples were sonicated for 3 min and centrifuged at 1500 rpm for 5 min at 5 ºC. The supernatant was

kept together with the pellet, and the procedure was repeated twice more using 3 mL of MeOH- Na_2EDTA each time. The total resulting supernatant was centrifuged at 3200 rpm for 20 min, decanted, filtered with polyvinylidene difluoride (PVDF) filters (Sterlitech, WA, USA), evaporated under nitrogen stream using a Reacti-Therm 18824 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} Na_2EDTA were added since it has been observed that it considerably improves extraction efficiency of antibiotics as well as other pharmaceuticals (Hernández et al., 2007; Yang et al., 2005). 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 (Gros et al., 2012).

5.2.5. Sorption and alternative biodegradation calculation

Aiming to determine the role of biodegradation and sorption processes in the elimination of PhACs by fungi, a determination of PhACs concentration both in solid (fungal biomass) and liquid phase (water) was performed and the following parameters were calculated with these data for the 4 PhACs available (CBZ, DCF, IPD and VFX):

Total elimination for each PhAC was first calculated as (Equation **5.1**:

$$\mathrm{E} = (\mathrm{C_{wi}} - \mathrm{C_{wf}}) \cdot \mathrm{V} \qquad \textbf{(Equation 5.1)}$$

where E is total elimination (ng), C_{wi} and C_{wf} are the concentration (ng mL^{-1}) of each PhAC measured in the water samples (Table 5A.3 in the Annex) at the beginning and at the end of the experiment respectively, and V is the volume of the water in each flask (mL). PhACs sorpted for each compound can be expressed as Equation 5.2

$$\mathrm{A} = \mathrm{C_{bf}} \cdot \mathrm{B_f} \qquad \textbf{(Equation 5.2)}$$

where A is the mass of PhAC sorpted (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 according to (Equation **5.3**:

$$B_d = \mathrm{E} - \mathrm{A} \qquad \textbf{(Equation 5.3)}$$

where B_d is the mass of PhAC (ng) eliminated by biodegradation, E is the total mass of PhAC eliminated (ng), and A is the mass of PhAC (ng) eliminated by sorption processes.

Another parameter used to analyse the data is the solid–water partition coefficients (K_d) (Equation **5.4**), (Ternes et al., 2004) which defines the distribution of a compound between water and biomass. This coefficient takes into account both absorption and adsorption and is used when evaluating the 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 by then:

$$K_d = \frac{C_{sorpted}}{C_{soluble}} \qquad \textbf{(Equation 5.4)}$$

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

5.2.6. Laccase purification

Extracts of laccases from each fungal species used in this study, plus *Pycnoporus sanguineus* CS43 strain and *Pleurotus ostreatus* NCBI KJ020935 strain[30], were produced with the ultimate objective to characterize these enzymes and investigate potential PhACs degradation pathways.(Golan-Rozen et al., 2015; Rodríguez-Delgado et al., 2016)

Fungal mycelium was produced following adapted previous protocols from Font et al. (2003) as previously reported in general methodology (section 1.3.4).

Before inoculation, pellets were strained, rinsed and weighted and finally added to 500 mL Erlenmeyer (10 g of wet weight per Erlenmeyer flask, approximately at a concentration of 1 g/L of dry weight per Erlenmeyer). The experiment was performed in duplicate; briefly 200 mL of defined liquid medium were added, and the experiment was run for 7 days at 28 °C in a rotatory-shaker (130 rpm). An abiotic control was also performed to analyse possible changes in liquid medium in the absence of fungi.

An induction cocktail was added to improve laccase production after confirming low levels of laccase activity for certain species. The induction cocktail contained ethanol, $CuSO_4$ and

[30] Another two species of potential interest in PhACs biodegradation.

the dyer grey Lanaset G. This cocktail was added in the liquid medium to a final concentration of 0.75%(v/v), 79.8 mg/L and 120 mg/L respectively, since concentrations of these compounds showed high laccase induction for several species in previous reports (Casas et al., 2013; Dhillon et al., 2012; Fonseca et al., 2010; Kumar et al., 2015; Manavalan et al., 2013; Palmieri et al., 2003; Tavares et al., 2005) and previous laboratory assays with this cocktail confirmed induction effects on most of studied species. In order to monitor the laccase production, samples were taken on 0, 2, 3, 4 and 7 days. Total reducing sugars (glucose in the second experiment), pH and laccase activity were analysed. After 7 days the three fungal Erlenmeyer flasks were sacrificed and liquid broth containing the fungal extract was separated from biomass by vacuum filtering, through paper filters (2.5 µm filters grade number 5, Whatman, Maidstone, England). Before ultrafiltration, liquid extract was filtered again with Whatman cellulose acetate filters (0.45 µm cellulose acetate filter, Whatman, Maidstone, England). Ultrafiltration was then performed to concentrate the enzymatic extract. In the first attempt of purification, ultrafiltration was performed using Millipore (EMD Millipore, USA) ultracel regenerated cellulose ultrafiltration membrane of 10 kDa in a stirred cell under nitrogen gas pressure, whereas in the second, a Labscale TFF system coupled to a 10 kDa Pellicon XL 50 casette (Millipore, Barcelona, Catalonia) was used.

After ultrafiltration, samples were ultracentrifugated at 9000 g, and supernatant was concentrated with Amicon Ultra centrifugal filters of 15 mL, PLGC membrane Ultracel-PL, 10 kDa (Merck Millipore, Mexico). This purified protein extract was separated by ÄKTA fast protein liquid chromatograpy (FPLC) system (GE Healthcare Pharmacia, Mexico) with a DEAE Sepharose FF 16/10 column and Superdex 200 gel filtration column (GE Healthcase Life Sciences, Mexico) until achieving purified samples. Purification process was monitored by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS PAGE) and enzymatic activity assays.

5.3. Results and discussion

5.3.1. Preliminary pelletization assay

Before starting any PhAC removal experiment, fungal species where previously grown under orbital shaking conditions to achieve their growth in a pellet shape. Not all ligninolytic fungal species are able to grow in this type of morphology, therefore it was important to achieve pellets for all fungi in order to exclude changes in morphology or growth as responsible for differences in removals or laccase production.

Growth in pellet morphology was achieved for all species, and this was the first time of pelletization reported for *S. rugosoannulata*, *A. erebia* or *G. luteofolius* (Figure 5.2). The success in this culture methodology promises suitable growth and operational advantages for these species when applied in fluidized bed bioreactors (FBBs).

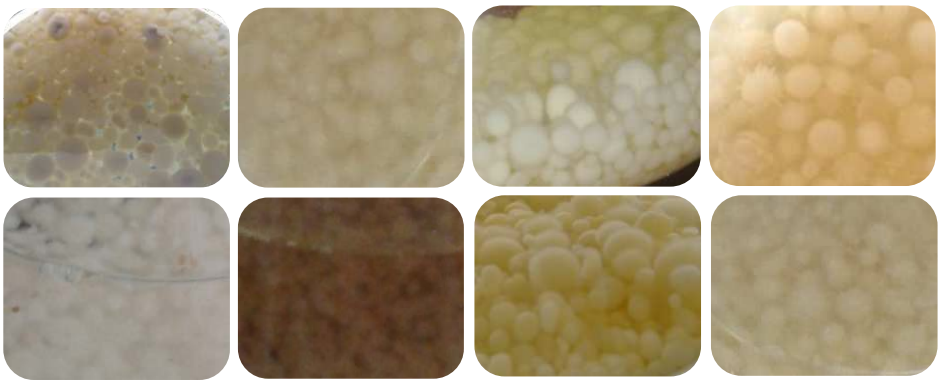

Figure 5.2 Pellet morphology appearance of the fungal species used in this thesis. From up left to down right: *A. erebia*, *G. lucidum*, *G. luteofolius*, *I. lacteus*, *P. ostreatus*, *P. sanguineus*, *S. rugosoannulata* and *T. versicolor.* (Pictures credit: Francesc Castellet-Rovira CC-BY 2.0.)

5.3.2. Abiotic PhAC stability assessment

On the contrary, regarding PhACs tested, the majority of them showed a high chemical stability when tested in abiotic controls during the 6 days of incubation (Figure 5.3). Hence, any PhAC removal in experimental treatments must be exclusively attributed to fungal sorption and biodegradation mechanisms. Only the cyclophosphamide (CFD) concentration decreased 29% compared to the initial values because of the physicochemical processes. According to Česen et al. (2015), CFD can be transformed by

advanced oxidation processes such as UV irradiation, ozone and hydrogen peroxide oxidation or hydrodynamic cavitation; however, it is strongly unlikely that any of these phenomena occurred spontaneously during the development of this experiment. However, according to Trissel and Bethesda (1996), spontaneous hydrolysis resulting in <1.5% of CFD decomposition can occur in constituted solutions within 8 hours at 24-27ºC or even within 6 days at the storage temperature (5ºC).

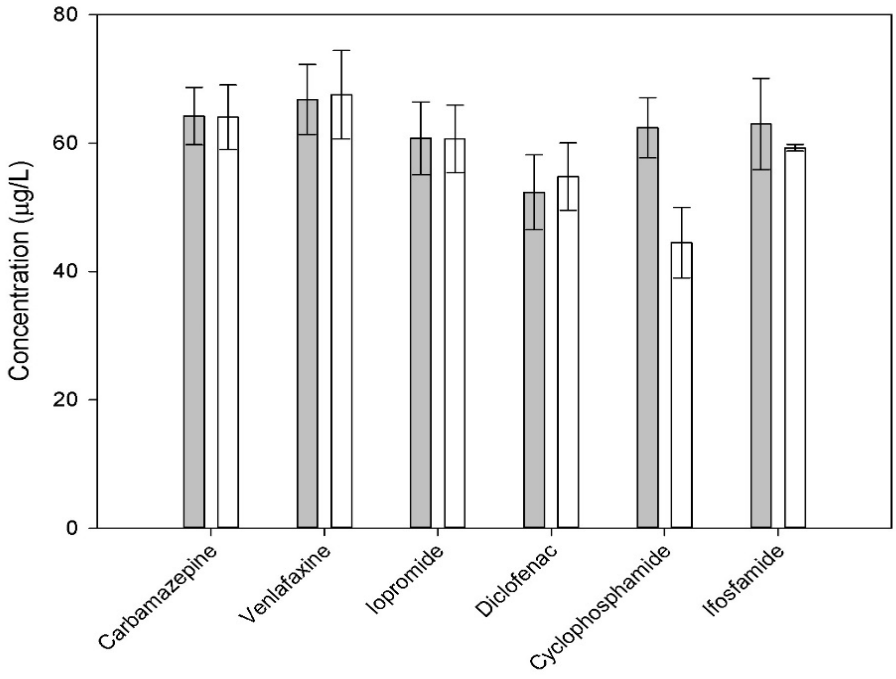

Figure 5.3.

Initial (grey bars) and final (empty bars) pharmaceutical concentrations (expressed in µg/L) for the abiotic control. The error bars contain the standard deviation.

5.3.3. Total Accumulated Removal and Degradation

The screening was performed in consecutive batch experiments (one batch experiment per fungus including the experimental and control treatments). Therefore, the initial concentration (IC) of PhACs may vary between batches (Table 5A.4 in the Annex). Considering these changes in PhAC concentrations, the biodegradation performances for the different fungi must be taken into perspective regarding the concentration of the PhAC exposed in each case. Therefore, there were differences in the summation of the total PhAC concentrations between the fungi; the highest total value measured was 679 ± 55 µg/L for *A. erebia*, whereas the minimum total concentration detected was 300 ± 16 µg/L for *G. lucidum*, as shown in Figure 5.4 (A).

When looking at the total PhACs concentrations at the end of the experiments for each fungus assayed, statistically significant differences (p-values below 0.05 in Tukey test) were observed between experimental treatment (which includes degradation and sorption processes) and the killed control experiment (which only accounts for sorption processes) in all cases. This confirms that the main cause of total PhACs removal was degradation rather than sorption phenomena.

Only *T. versicolor* and *S. rugosoannulata* were able to achieve more than 50% of removal percentage (RP) and reached (in both cases) a minimal degradation percentage (MDP) of 38% from the total accumulated concentration. These results indicate that these two fungi are versatile PhACs biodegrading candidates for general wastewater treatment, although the removals obtained with them for some PhACs were not always the highest; e.g. *G. luteofolius* achieved better results in the removal and degradation of IPD (Figure 5.5)

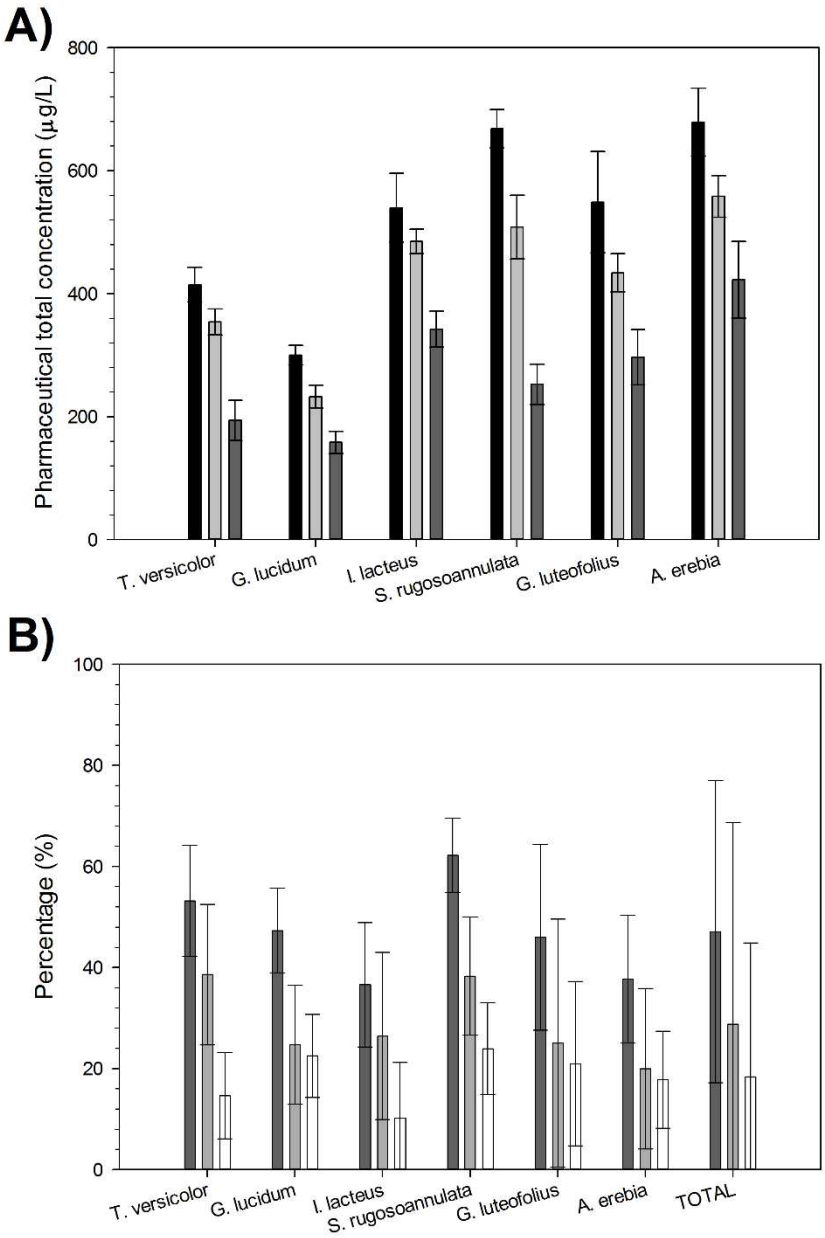

Figure 5.4

5.4 A) For the 6 different fungal species, summations of PhACs concentrations from left to right: initial concentrations (black bars, representing total equivalent concentration); final concentration for killed control treatment (light grey bars, representing sorption) and final values for experimental treatment (dark grey bars, representing biodegradation). Error bars express the standard deviation. *G. lucidum* values are significantly lower than the mean. (Mean= 486.97±57.49 µg/L; $p<0.05$).

5.4 B) For all PhACs used and from left to right, summation of the removals (TR, dark bars), degradation (MDP, grey bars) and sorption (SP, white bars) for the 6 fungal species studied in this experiment plus the total. Error bars express the standard deviation.

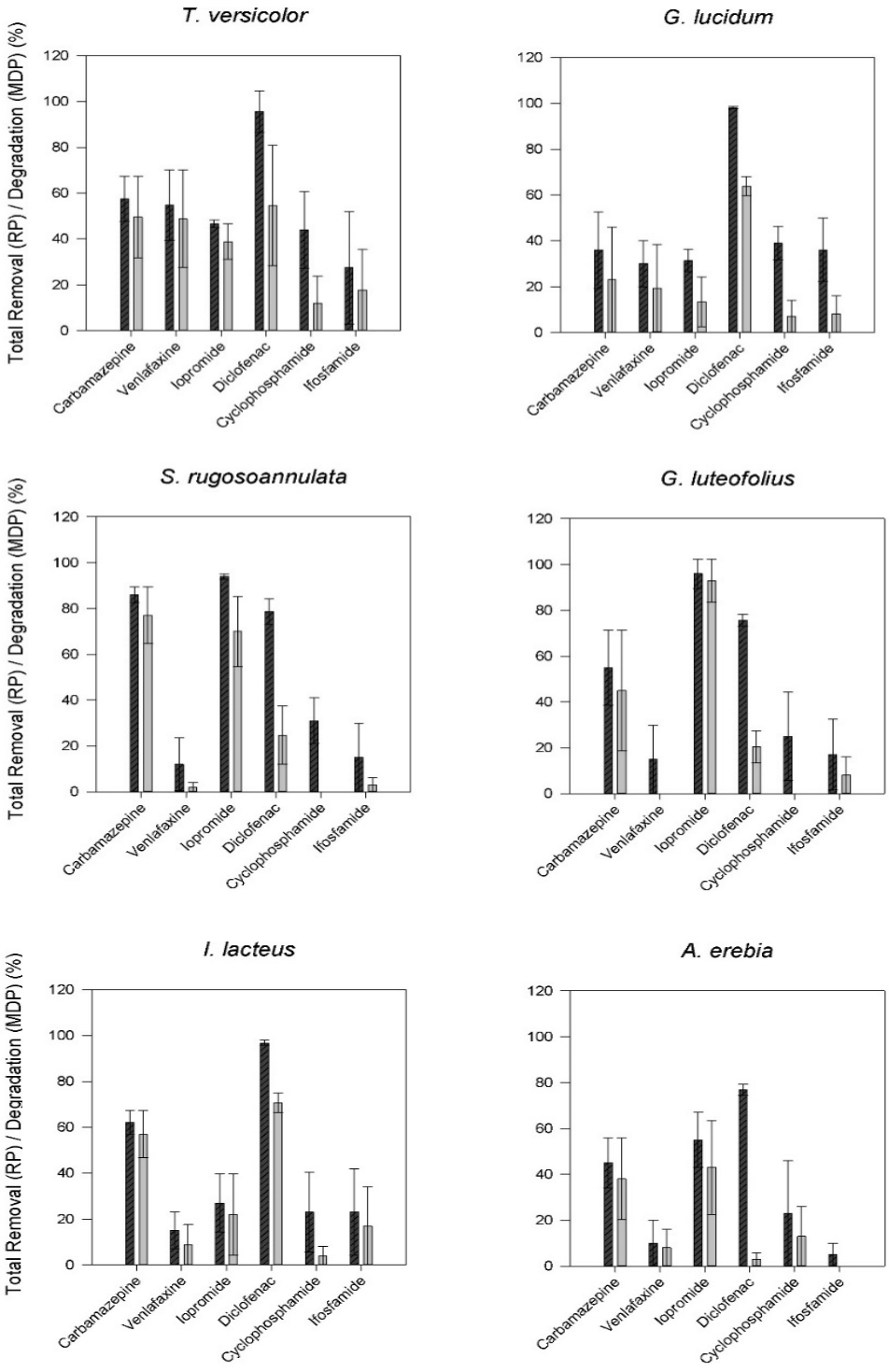

Figure 5.5.

RPs (striped dark bars) and MDPs (empty grey bars) for the different treatments with *T. versicolor, G. lucidum, S. rugosoannulata, G. luteofolius, I. lacteus and A. erebia*. The difference between RP and MDP corresponds to SP values obtained in the heat-killed control treatment. The error bars contain the standard deviation in percentage.

5.3.4. Removal and Biodegradation of each PhAC by fungi

The total removal and biodegradation percentages for PhACs in each of the fungal experiments are shown in Figure 5.5.

On an individual basis, *T. versicolor* achieved almost complete removal (96%) for DCF (55 µg/L of IC), although only 55% can be attributed to MDP, which is probably due to a high sorption to fungal biomass, as reported in previous studies (Badia-Fabregat et al., 2014). For CBZ, IPD and VFX removals were also quite significant, with RPs of approximately 50%, being SPs less than 15% in all cases. Jelic et al. (2012) reported higher CBZ degradation by *T. versicolor* (94% at 9 mg/L of IC after 6d), although this percentage was reduced to 61% when only 50 µg/L was added, similarly to the RP obtained in this study (50±18%) at comparable concentrations (47±3 µg/L of IC). Vasiliadou et al. (2016) achieved lower removals of this pharmaceutical (spiked at 50 µg/L) using *T. versicolor* and *G. lucidum* individually, but the removals reached 40% (and its degradation up to 95%) by combining both species simultaneously. For IPD, the degradation reached 85% of the total removal (47% from 174.4 µg/L IC), which was again slightly lower than the values obtained by Gros et al. (2014) with a 60% removal after longer treatment (7 d) but at a higher concentration (12 mg/L). Concerning VFX, other authors have achieved similar removals (Cruz-Morató et al., 2014), but to the author's knowledge, our work is the first one to report specific biodegradation by *T. versicolor* as the main cause of removal (89% from a total removal of 55% at 53.57 µg/L IC). On the contrary, cytostatic drugs presented the lowest PhACs removals (barely superior than 25%); however, these results can be considered notable when compared to previous literature concerning fungal degradation (Ferrando-Climent et al., 2015) where removals of CFD and IFD with *T. versicolor* were negligible, although they were performed at higher concentrations (10 mg/L IC). Besides, compared to other biological treatment such as conventional WWTP, these results are considerably higher, regarding removal rates below 2% (as seen above in Table 5.1) for both PhACs. Removals of cytostatics with T*. versicolor* together with *G. lucidum*, were the highest observed in the study. However, for such recalcitrant PhACs, biological degradation does not explain CFD main removal mechanism, but adsorption, and only IFD removal could be potentially attributed to fungal biodegradation (although the standard deviation prevents this affirmation).

G. lucidum also achieved very high RP for DCF (98% at 61 µg/L IC) and, in this case, showed a biological degradation up to 64%. The results for DCF are comparable with recent studies (Vasiliadou et al., 2016) that showed similar removals with this fungus (up to complete removal at 50 µg/L IC) but lower sorption in biomass (biodegradation contribution to total removal of 95%). For the other PhACs, the RPs were lower than 40%, but only in CBZ (at 54.8 µg/L IC) and VFX (at 50.5 µg/L IC) did biodegradation achieve percentages over 60% of the total RP, which contrasts the same study by Vasiliadou et al. (2016). In this study, CBZ (at an IC of 50 µg/L) biodegradation contribution was almost zero for a 15% RP. As previously said, compared to *T. versicolor* it is also significant that the CFD (43.5 µg/L IC) and IFD (39.1 µg/L IC) removals were higher for IFD, and almost the same for CFD. On the contrary, the biodegradation yields represent lower fractions in the RPs compared to *T. versicolor*. *G. lucidum* was reported to be able to degrade PhACs after 7 days of treatment, ibuprofen (100 %) and CBZ (47%) added at an IC of 10 mg/L but unable to degrade clofibric acid (Marco-Urrea et al., 2009).

I. lacteus also achieved high RPs of DCF (97% at 61µg/L IC), and this removal was caused mainly by degradation. *I. lacteus* also showed a high RP of CBZ (62% at 83.4 µg/L IC), and 92% of it was attributable to biodegradation processes. Contrasting previous studies at higher ICs (10 mg/L) reported lower CBZ removals than 20% (Marco-Urrea et al., 2009). For the rest of the PhACs, also low RPs (of approximately 20%) could be explained mainly with biodegradation, except for CFD, in which, from a total RP of 23%, a small fraction (17%) was due to biodegradation. In the case of studies with other PhACs, low degradation percentages were obtained with clofibric acid, whereas *I. lacteus* was able to degrade ibuprofen (100%) (Marco-Urrea et al., 2009).

S. rugosoannulata achieved RPs over 75% for CBZ (123 µg/L IC), DCF (113 µg/L IC) and, in particular, for IPD (184.7 µg/L IC), with a RP of 94%. However, for the rest of the PhACs, the removals were less than 40%. Nonetheless, regarding degradation in VFX, DCF, CFD and IFD, it represented far less than 35% of their RPs, but for CBZ and IPD, the biodegradation rate was the main cause of removal and achieved over 75%.

In previous literature, *S. rugosoannulata* was tested for decolorizing a dye with defined medium in a static and an agitated system (Jarosz-Wilkołazka et al., 2002), whereas recently reported applications have focused on soil bioremediation for other emerging

pollutants (Anasonye et al., 2014; Valentín et al., 2013), however this is the first evidence for its application in PhACs removal of contaminated waters.

G. luteofolius showed a very high removal yield in IPD (96% at 82 µg/L IC) with a MDP of 93%, much higher than that obtained with *T. versicolor* for this compound. Moreover, CBZ and DCF elimination is also remarkable, with a RP of 55% (71 µg/L IC) and 76% (106 µg/L IC), respectively. In contrast, CBZ biodegradation represents 81% of its elimination, whereas DCF degradation only explains 27% of the removal. For the rest of the PhACs, the removal yields ranged from 15 to 25%, and degradation represented less than 5%, except for IFD, which reached 50% of the removal (17% of RP at 94 µg/L IC). This is the first time that *G. luteofolius* has been applied in the elimination of pollutants in liquid phase, although previous studies did investigate the removal of chlorophenols and other pollutants for soil bioremediation applications (Anasonye et al., 2015; Winquist et al., 2009).

Last but not least, *A. erebia* achieved a high RP in DCF (77% at 104 µg/L IC), although its MDP is minimal (3%). Good performances of this fungus are again found for IPD (at 159 µg/L IC) and CBZ (at 114 µg/L IC), achieving 55% removal for IPD, with degradation being the main cause (78%), and 45% removal for CBZ, with an even more important degradation factor (84%).

In the case of cytostatic PhACs, CFD RP achieved 23% (at 95 µg/L IC) with MDP contributing to more than half of the removal. For VFX and IFD, the removals were lower than 10%, which was considered negligible.

As for the other two basidiomycetes, this is the first work where *A. erebia* has been tested as a PhACs biodegrader.

Once compared all fungi on an individual basis, the highest RP was obtained by *G. lucidum* for DCF (95%), whereas the lowest RP was obtained by *A. erebia* for IFD (5%).

Regarding the influence of sorption mechanisms, the highest SP (coinciding with the lowest MDPs of 0%) were obtained for CFD with *S. rugosoannulata* and *G. luteofolius* (100%), for IFD (100%) with *A. erebia,* and for VFX with *G. luteofolius* (100%), highlighting cytostatics sorption phenomena. However, in relation with biodegradation yields, the

highest MDP was detected in *G. luteofolius* with a 93% for IPD, which also coincides with the lowest SP obtained (3%).

5.3.5. Pharmaceutical concentration and enzymatic activity

In an attempt to monitor the activation of fungal biodegradation mechanisms, the laccase and manganese peroxidase (MnP) extracellular activities were analysed during the experiments. In the present study, only *T. versicolor*, *S. rugosoannulata* and *A. erebia* (Figure5.6) were reported to synthetize laccase and MnP, whereas poor or non-existent activity was detected for the rest of the fungi. Neither pH (stable at 4.5±0.5) nor glucose concentration (Table 5A.5 in the Annex) seemed to explain such enzymatic activity performances among the fungi (there were no significant differences in neither pH nor glucose among treatments), but other factors, such as a lack of inducers or changes in carbon, nitrogen or xenobiotics concentration, may be responsible (Janusz et al., 2013). As shown in Figure, in most cases the enzymatic activity of experimental and (non-spiked) biotic control treatments showed similar values (or even higher for experimental treatment), which indeed suggests the pharmaceutical stimulation of fungal activity. The fungi with higher enzymatic activity were *T. versicolor* (lac) and *S. rugosoannulata* (MnP), in both cases corresponding both to the experimental treatment.

Laccases promote the removal of a hydrogen atom in hydroxyl groups of phenolic substrates and aromatic amines using molecular oxygen as a primary electron acceptor (Martínez et al., 2005). In this experiment, laccase activity could have catalysed DCF transformation into 4-(2,6-dichlorophenylamino)-1,3-benzenedimethanol (Marco-Urrea et al., 2010b).

CBZ transformation has been reported to be catalysed by cytochrome P450 systems (Marco-Urrea et al. ,2009), however some authors (Hata et al., 2010) reported laccase degradation through mediators by adding pulses of laccase and the mediator HBT to a CBZ spiked malonate buffer solution every 8 hours (achieving up to 60% removal in 48 h) generating 10,11-dihydro-10,11-epoxycarbamazepine and 9(10H)-acridone as transformations products (TPs). Moreover, MnP inhibition results in a reduction up to 30% CBZ removal levels, suggesting MnP plays a role in CBZ transformation process (Golan-Rozen et al., 2011). Rode and Müller (1998) proved that triodinated benzoates such as IPD

were transformed by an extracellular enzyme concentrate of *T. versicolor* in the presence of $MnSO_4$ and malonate, whereas Haroune et al. (2014) suggested extracellular laccase and MnP may play a key role.

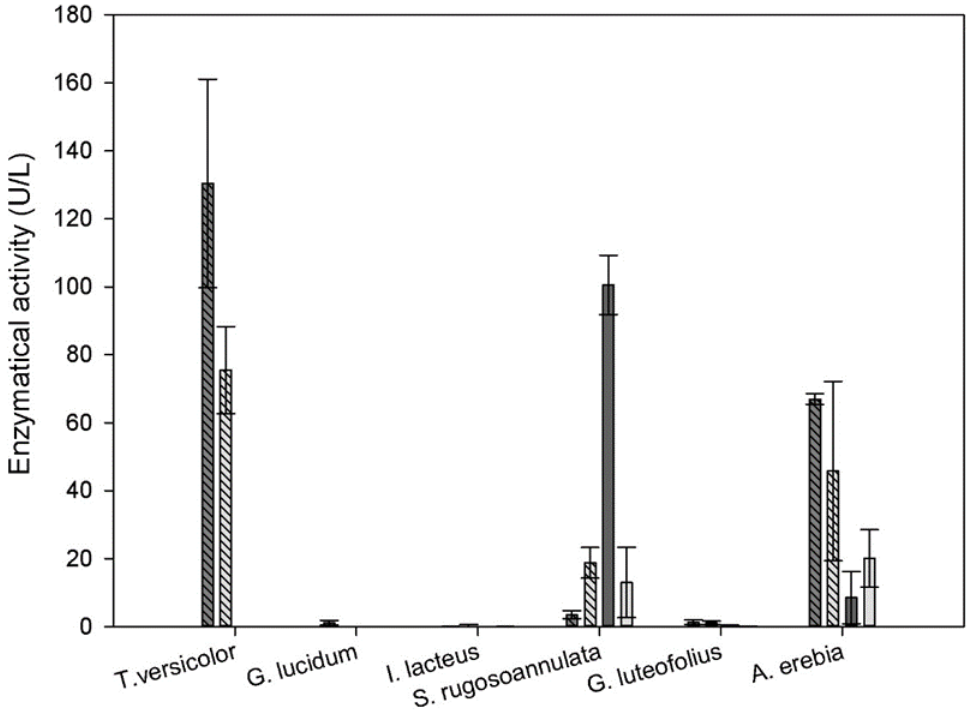

Figure 5.6

Laccase (striped bars) and manganese peroxidase (MnP) (empty bars) enzymatic activity (in U/L) for the final samples of the experimental (dark bars) and non-spiked alive control treatments (light bars).

VFX was hypothesized to be degraded by fungal enzymes by Cruz-Morató et al. (2014), being O-Desmethylvenlafaxine (ODMVFX) one of its main TPs (Kern et al., 2009).

On the contrary, cytostatics can apparently inhibit or modify enzymatic system of WRF according to Ferrando-Climent et al. (2015), but in terms of PhAC biodegradability, a direct correlation between WRF extracellular enzymes and cytostatic transformation cannot be established. Hence, the exact transformation pathways remain unknown for most of the PhACs studied, and especially in the case of cytostatics, further research needs to be performed in the detection of transformation products and secondary metabolites (Kosjek and Heath, 2011; Salman et al., 2016).

Regarding toxicity, Microtox assays were performed for the final samples of the experiments and most of them showed decreasing or stable values. None of them showed significant toxicity levels under 20% of EC_{50}.

Additional experiments may also be needed to assess whether there is a threshold in pharmaceutical concentration that could affect fungal metabolism and PhAC removal yields; if the PhAC concentrations are higher than a set threshold value, the result would either activate fungal metabolism or induce toxicity in these fungal species. However, considering the available measured parameters (glucose consumption, pH, and laccase and MnP enzymatic activity), the fungal toxic threshold was apparently not reached in this experiment, which could avoid fungal toxicity problems when scaling up in real conditions because the common PhAC concentrations in real wastewater might be equal or even lower. Nonetheless, it would be interesting to have other metabolic parameters available to determine the toxic effects on fungal biomass (such as the acetate-in-ergosterol incorporation rate or respiration for instance (Rajapaksha et al., 2004; Rath et al., 2016)) to refute or confirm this hypothesis.

Considering the potential for biodegradation shown by *T. versicolor* and *S. rugosoannulata* in this study, another studies for process optimization should be performed. Regarding *T. versicolor*, many studies have already been performed to scale up PhAC biodegradation treatment with this fungus (Cruz-Morató et al., 2013a; Mir-Tutusaus et al., 2016), but at this moment, there is still a lack of literature for *S. rugosoannulata* as a PhACs biodegrader. Thus, deeper research is needed to assess the optimal biodegradation conditions for this candidate.

5.3.6. Sorption contribution in PhACs elimination

The role of sorption was specifically determined for CBZ, DCF, IPD and VFX based on the results obtained from the measurements of PhACs in liquid and fungal phases after 6 days of treatment (Table 5A.3 in the Annex). The sorption contribution in the PhACs elimination ranged from 3% to 13% (Figure 5.7). According to these data, *S. rugosoannulata* was the most effective fungus in terms of elimination of the 4 selected PhACs (an overall removal of 75%), but also in terms of biodegradation (being the main factor of removal); followed

in this order by *G. luteofolius*, *T. versicolor*, *G. lucidum*, *A. erebia* and *I. lacteus*; the latest exhibiting the lowest elimination value, 44% (mainly attributed to biodegradation).

On regard of these results and 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 a minimal fraction of total elimination can be attributed to sorption processes while most of the elimination is due to biodegradation. In contrast*;* the relevance of the sorption processes achieved the highest values in the case of *G. lucidum*. For this fungus, sorption processes can be attributed to less than 30% of the total removal being biodegradation the main attributable reason of PhACs elimination.

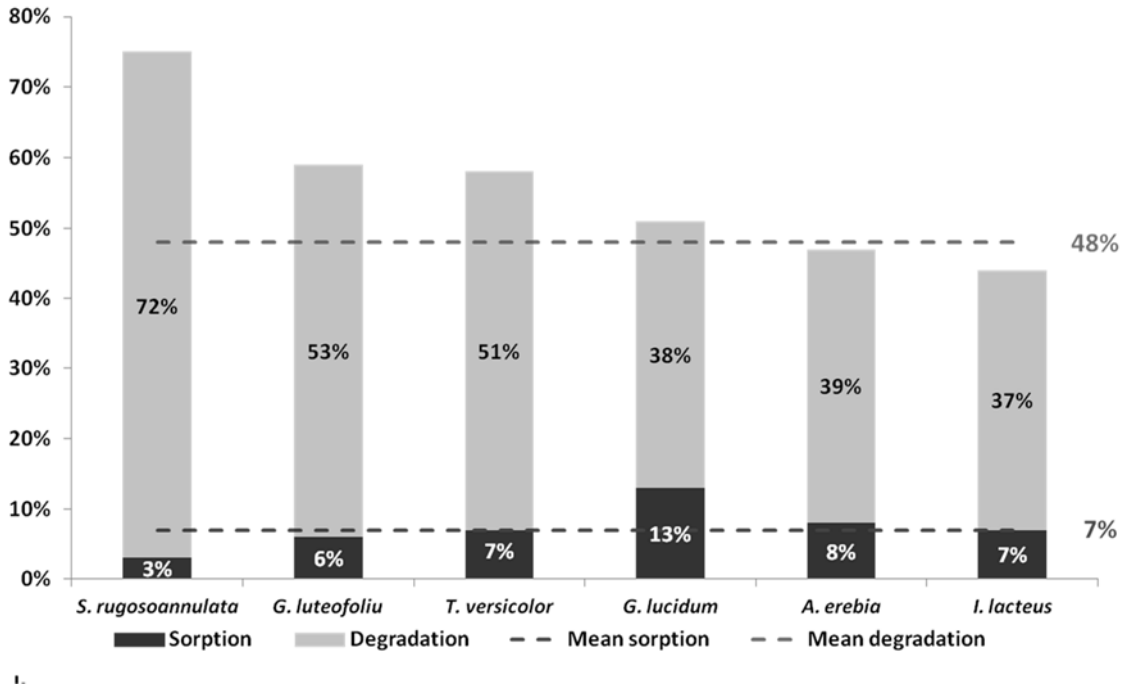

Figure 5.7 Contribution of sorption and biodegradation (%) to the total removal of the 4 PhACs, for each fungus from experimental culture studied in the batch experiments with fungi and spiked synthetic medium. (Data obtained from direct measurement method).

The behaviour of each compound in the six different fungal experiments was also evaluated (

Figure 5.8 and Table 5A.4 in the Annex). DCF 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 DCF, considering all fungi, 5213.2 ngg^{-1}(25% of initial amount). These high DCF sorption values are in agreement with those exhibited in previous fungal and conventional activated sludge (CAS) treatments (Marco-Urrea et al., 2010b; Radjenović et al., 2009).

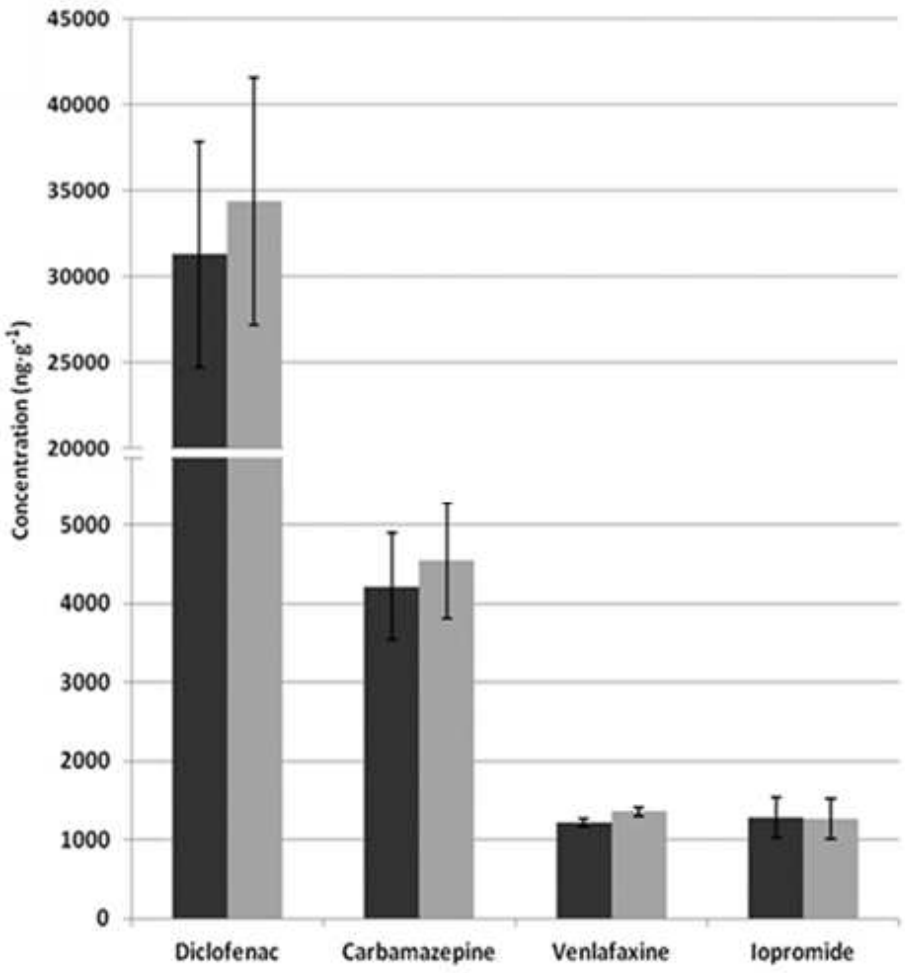

Figure 5.8. Mean concentration of each PhACs detected in the fungal biomass at the end of the experimental (dark bars) and killed control (grey bars) treatments.

The great sorption of DCF in the present experiment might be due to its high partition coefficient (log P at pH 4.5 = 3.6) indicating its hydrophobic character (Mannhold et al., 2009). In contrast, IPD and VFX 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, which is in agreement with the low sorption shown by these compounds in sludge, reported in studies performed with CAS (Batt et al., 2006; Joss et al., 2008). Low hydrophobicity of IPD (log P at pH 4.5 = -0.44) and VFX (log P at pH 4.5 = -0.69) may explain the low

concentrations of these compounds in the fungal biomass. Moreover, sorption values obtained in the experimental treatments were compared with sorption values of killed control treatments, and differences between direct and indirect calculation of the sorption contribution in the total removal of PhACs were also evaluated. Heat killed treatments are a common strategy for control treatment in biodegradation experiments, being used to evaluate contribution of the sorption processes in the elimination of the pollutants. It is thus assumed that sorption of the active biomass is equivalent to the sorption of the inactive (killed control) biomass. Therefore, in indirect methods, the difference in elimination between treatments is attributed to biodegradation processes (killed control PhAC removal is subtracted to the removal of experimental PhAC treatment in the liquid phase).

For most of the compounds, sorption percentages calculated with direct and indirect strategies were similar. However, for some compounds the differences were quite remarkable, like in the case of the degradation of DCF by *I. lacteus* (Table 5.2), with 42% degradation calculated through the direct method and 71% of MDP with the indirect method, or the degradation of VFX by *G. luteofolius* (15% of degradation calculated with the direct method and no MDP measured with the indirect method). These differences could be partially attributed to the different sorption values between the active and the inactive biomass; however, biodegradation processes of absorbed compounds can occur in the active biomass due to intracellular enzymes, whereas no degradation occur in inactivated biomass. In spite of this biomass sorption capacities from the killed control may change according to the inactivation mechanism. Specifically, it has been reported for certain fungal cells that being exposed to heat treatments alter the physicochemical properties of their surfaces leading to a greater, equivalent or less bioadsorptive capacities than that of living cells depending on the pollutant (Arica et al., 2003; Bayramoğlu and Yakup Arica, 2007). Besides, active transport, lacking in killed control biomass, may play an important role in the sorption processes of biotic cultures.

Alternatively, calculation of biodegradation by direct methods avoids the use of killed controls. Removal by biodegradation is considered the difference between initial PhAC concentration and the final experimental PhAC concentration, corrected by adding the sorpted PhAC concentration detected in the biomass from the experimental treatment.

However, this method requires the analyses of PhACs concentration in both liquid and biomass samples, which is not always technically possible.

Nevertheless, there is still another method for calculating total degradation based on PhAC mass balance considering available data from both direct and indirect methods, and hence being generally more accurated. Experimental and killed control liquid and biomass concentrations. In this case, the initial liquid PhAC content (free from biomass) and the summation of final killed control PhAC content (both in biomass and liquid phases) should be the same. This means the amount of pollutant is conserved and it constitutes a way to validate the recovery yields of the PhACs biomass extraction method. If this statement is fulfilled, the degradation can be considered as the difference between the final PhACs content in killed control (considered the initial PhAC mass reference) and the final experimental PhACs content.

Following this strategy, PhAC mass balance (Table 5.2) was calculated to compare the PhAC content of initial liquid samples with the final killed control liquid and fungal samples. Since PhAC's content was generally equivalent, degradation could be calculated with PhAC content in experimental treatment.

It should be expected that biodegradation values obtained by this method should be higher than direct/indirect calculated degradation. No significant differences were observed in most of the cases except for IPD and DCF in *S. rugosoannulata*, where killed control PhACs mass values represented around 75% and 50% of the initial liquid PhACs content respectively. In these two cases the obtained biodegradation could not be not considered accurate, being the differences in the mass balance possibly explained by low recoveries from PhACs extraction method. Concomitantly, direct method degradation would be also less accurate in this case.

For the rest of cases were PhAC mass balance was fulfilled, it provided accurate degradation percentages. Degradation values calculated by the indirect method were generally closer than the ones obtained by the direct method, and this pointed out that, in spite of being considered more conservative, in these conditions degradation calculated by the indirect method (considered MDP), was generally more accurated than the direct method (which tended to overestimate biodegradation rates).

If degradation percentage from the mass balance is similar to the one calculated for the indirect method, evidences the lack of degradation of the absorbed PhACs in 6 days. This means that longer periods are necessary to degrade absorbed PhACs.

This argument contrasts with studies claiming degradation of sorpted contaminants in active fungal biomass by fungal intracellullar metabolism (Blánquez et al., 2004).

Table 5.2 For each fungus, approximation of PhAC mass balance for initial and final values of PhAC killed control and experimental treatments for the 4 PhACs analyzed in the biomass, plus minus standard deviation. Biodegradation calculations are represented in the three columns on the right, comparing percentages obtained with the mass balance, direct and indirect methods, plus minus respective standard deviations.

T. versicolor	Mass balance			Biodegradation		
	Initial PhAC amount (ng)	Final KC PhAC amount (ng)	Final Exp PhAC amount (ng)	Mass Balance Degradation (%)	Indirect Degradation (%)	Direct Degradation (%)
Carbamazepine	4767 ± 553	4700 ± 446	2333 ± 518	50 ± 15	50 ± 7	51 ± 7
Diclofenac	5500 ± 1609	5077 ± 742	1981 ± 772	61 ± 22	54 ± 15	64 ± 16
Iopromide	17440 ± 1093	16200 ± 1729	9401 ± 668	42 ± 12	39 ± 3	46 ± 7
Venlafaxine	5357 ± 556	5132 ± 597	2512 ± 911	51 ± 23	49 ± 9	53 ± 8
G. lucidum	Mass balance			Biodegradation		
	Initial PhAC amount (ng)	Final KC PhAC amount(ng)	Final Exp PhAC amount (ng)	Mass balance Degradation (%)	Indirect Degradation (%)	Direct Degradation (%)
Carbamazepine	5480 ± 957	4962 ± 661	3802 ± 835	23 ± 22	23 ± 2	30 ± 6
Diclofenac	6100 ± 473	6018 ± 536	2552 ± 254	58 ± 22	64 ± 6	51 ± 8
Iopromide	5110 ± 492	4229 ± 358	3581 ± 295	15 ± 11	13 ± 1	30 ± 7
Venlafaxine	5050 ± 337	4541 ± 706	3599 ± 660	21 ± 11	19 ± 1	28 ± 5
I. lacteus	Mass balance			Biodegradation		
	Initial PhAC amount (ng)	Final KC PhAC amount (ng)	Final Exp PhAC amount(ng)	Mass balance Degradation (%)	Indirect Degradation (%)	Direct Degradation (%)
Carbamazepine	8340 ± 1299	8424 ± 778	3480 ± 385	59 ± 12	57 ± 7	56 ± 5
Diclofenac	6100 ± 588.5	6725 ± 755	2110 ± 391	69 ± 7	71 ± 9	42 ± 6
Iopromide	10500 ± 1381.8	9983 ± 942	7666 ± 1265	23 ± 16	22 ± 3	24 ± 11
Venlafaxine	10400 ± 1675	10024 ± 551	8991 ± 467	1 ± 15	9 ± 1	12 ± 3

Table 5.2 (continuation) For the 4 PhACs analyzed in the biomass, approximation of PhAC mass balance for initial values and at the end of killed control and experimental treatments, plus minus standard deviation. The two columns on the right compare degradation percentages obtained with the mass balance and indirect methods.

G. luteofolius	Mass balance			Biodegradation		
	Initial PhAC amount(ng)	Final KC PhAC amount (ng)	Final Exp PhAC amount (ng)	Mass balance Degradation (%)	Indirect Degradation (%)	Direct Degradation (%)
Carbamazepine	7100 ± 1510	6977 ± 1119	3380 ± 1101	52 ± 24	45 ± 10	52 ± 11
Diclofenac	10600 ± 1580	9235 ± 716	4396 ± 646	52 ± 28	20 ± 2	53 ± 9
Iopromide	8200 ± 2510	8118 ± 671	354 ± 626	96 ± 14	93 ± 4	96 ± 25
Venlafaxine	10600 ± 2210	9176 ± 1690	9028 ± 1902	2 ± 11	0 ± 0	15 ± 3
A. erebia	Mass balance			Biodegradation		
	Initial PhAC amount (ng)	Final KC PhAC amount (ng)	Final Exp PhAC amount (ng)	Mass balance Degradation (%)	Indirect Degradation (%)	Direct Degradation (%)
Carbamazepine	11400 ± 780	11021 ± 1300	6599 ± 357	40 ± 13	38 ± 3	41 ± 9
Diclofenac	10400 ± 1476	8754 ± 957	5878 ± 578	33 ± 9	3 ± 0	31 ± 8
Iopromide	15900 ± 1320	14034 ± 1707	7212 ± 365	49 ± 14	43 ± 4	55 ± 20
Venlafaxine	10600 ± 1475	10463 ± 751	961 ± 486	8 ± 13	8 ± 1	9 ± 3
S. rugosoannulata	Mass balance			Biodegradation		
	Initial PhAC amount (ng)	Final KC PhAC amount (ng)	Final Exp PhAC amount (ng)	Mass balance Degradation (%)	Indirect Degradation (%)	Direct Degradation (%)
Carbamazepine	12300 ± 1140	11383 ± 1373	2017 ± 533	82 ± 16	77 ± 12	84 ± 7
Diclofenac	11300 ± 943	5240 ± 980	3577 ± 946	32 ± 22	25 ± 5	68 ± 6
Iopromide	18470 ± 1879	14073 ± 2842	1207 ± 269	91 ± 27	70 ± 13	93 ± 8
Venlafaxine	8270 ± 697	7456 ± 1156	7359 ± 1162	1 ± 27	2 ± 0	11 ± 7

Values of sorpted PhACs in killed control and experimental biomass were generally similar and particularly high in the case of DCF (as mentioned before consequence of its partition coefficient). In spite of lower values for the rest of PhACs, significant concentrations over 100 ng/g indicate that fungal biomass can absorb and accumulate PhAC concentrations up to those equivalents from CAS in WWTP; or even much higher in the case of DCF, considering a range of 100-200 ng/g of this PhAC reported by Radjenović et al. (2009) in sludge from a WWTP in Terrassa (Catalonia), 50 times less than the concentration of the fungal biomass in this study. According to these results, fungal biomass used in PhACs removal treatment should be managed in the same way as sludge from CAS in WWTP.

Table 5.3 Mean final PhAC's concentration in biomass of experimental and killed control treatments with standard deviations.

PhAC	Killed control Mean fungal PhAC concentration value (ng/g)	Experimental Mean fungal PhAC concentration value (ng/g)
Carbamazepine	757 ± 341	703 ± 211
Venlafaxine	226 ± 219	202 ± 155
Iopromide	125 ± 100	124 ± 74
Diclofenac	5735 ± 4332	5213 ± 2422

5.3.7. Laccase characterization

There is an increasing interest worldwide in the purification and characterization of laccases in order to fit and optimize the wide range of applications for this enzyme (Mayer and Staples, 2002) and in particular for micropollutants removal (Macellaro et al., 2014; Majeau et al., 2010). In this chapter our interest in the purification and characterization of the fungal laccases involved several objectives: from identifying possible degradation pathways for PhACs to laccase affinity for certain PhAC molecules and even to predict the formation of their subsequent TPs. However, finding a propper laccase purification method compatible for such a wide group of fungal species resulted complex enough to be completely finished. Attempts in obtaining purified laccase from fungal samples under the same experimental conditions for PhAC removal resulted in scarce laccase production, therefore, an inducer cocktail containing up to three reported inducers ($CuSO_4$, ethanol and the dye Grey Lanaset G) was added to the laccase production assays.

Figure 5.9 shows the effect of the inducer cocktail from day four. However, the main induced laccases were only two, *T. versicolor* and *P. sanguineus*. For the rest of fungi, laccase production only experimented a slight growth.

This strategy caused some positive effects in laccase production during the experiments, however on the final days of the experiment and during the purification process this increase in laccase activity dropped to lower values, conditioning the subsequent downstream process (Table 5.4). The main limiting factor of this purification was the low laccase concentration in fungal extracts, but also the last downstream step with FPLC revealed crucial to avoid potential enzymatic inactivation and a successful separation without sample dilution. The need for tailoring each laccase separation and purification protocol from their corresponding fungal extract (with particular differences in molecular weight and isoelectric point) emerged as an extra difficulty to reach an ultimate purification.

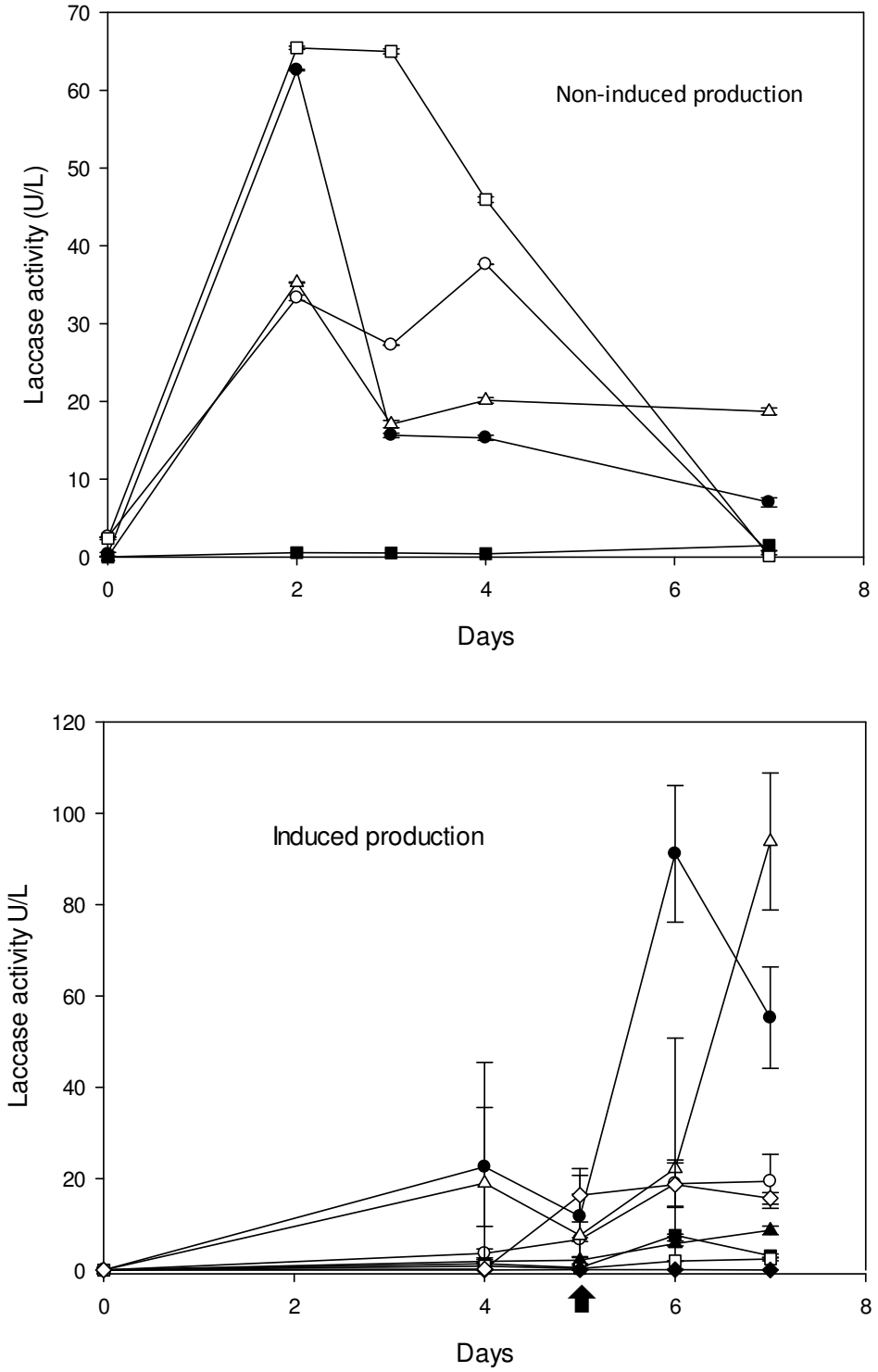

Figure 5.9

Laccase activity comparison between first and second batch experiments. Fungal species used were *T. versicolor* (●), *S. rugosoannulata* (○), *A. erebia* (▲), *G. lucidum* (□), *P. ostreatus* (■), *G. luteofolius* (◇); *P. sanguineus* (△) and *I. lacteus* (◆). The arrow indicates the induction at day four.

Table 5.4. Laccase enzymatic activities for induced fungal purified extracts

Fungus	Original Activity (U/L)[31]	Ultracentrif. (U/L)[32]	Total units	[Protein] (mg/L)	Total protein (mg)	Total activity (U/mg)
A. erebia	33.7	99.0	0.69	339.33	2.38	0.29
G. lucidum	0.0	173.06	1.12	592	4.14	0.29
G. luteofolius	13.2	40.5	0.28	898.67	6.29	0.05
I. lacteus	0.4	109.56	0.77	814	5.7	0.13
P. ostreatus	11.7	37.44	0.26	588	4.12	0.06
P. sanguineus	70.2	78.61	0.55	2140	14.98	0.04
S. rugosoannulata	94.9	926.61	6.49	1304.67	9.13	0.71
T. versicolor	6.9	133.89.	0.94	661.33	4.63	0.20

Several laccases and corresponding isoforms from *T. versicolor* have been previously characterized with molecular weights (MWs) between 60 and 100 kDa (Han et al., 2005; Martínez-Morales et al., 2014; Que et al., 2014); however after FPLC, *T. versicolor* extract (lane 10 of

Figure **5.**10) did not present any significant band, which could be attributed to a low protein concentration. On the contrary, *G. luteofolius* (lane 9) did present a band around 37 kDa position, but in spite of being a laccase producer species (Anasonye et al., 2015), studies of laccase identification for this fungus are still missing in current literature. *G. lucidum* has been reported to produce laccases with MW from 38 to 67 kDa (Baldrian, 2006; Manavalan et al., 2013) and several isoforms (24-66 kDa) (Kumar et al., 2015). In our study, *G. lucidum* fungal extract contained one band (lane 8) of around 24 kDa, that could potentially be attributed to a laccase isoenzyme IV identified by Kumar et al. (2015) for *G. lucidum* MDU-7.

The fungal extract of *P. ostreatus* (lane 7) did not contain any protein, although several reports for multiple strains of this fungus have claimed to produce at least six laccase isoforms (59-85 kDa) (Giardina et al., 2007; Palmieri et al., 2003, 1997). The lack of bands

[31] Original laccase activity after fungal extract ultrafiltration.
[32] Laccase activity after ultracentrifugation (up to 7 ml).

as well as enzymatic activity could be caused by a low laccase production in spite of the inducer.

On lane 6, *P. sanguineus* fungal extract showed up to 3 bands (with an approximated MW of 37, 20 and 10 kDa). Apparently these MW did not match with laccases reported by previous authors (61-68 kDa) (Lu et al., 2007; Ramírez-Cavazos et al., 2014). On the contrary, when compared to the two first lanes of the SDS PAGE gel, which corresponded to laccases produced by the same fungal strain (characterized as isoforms of 68 and 66 kDa), it can be assumed that a lower MW of the bands in lane 6 and a very low laccase activity in the purified extracts could be explained by an enzymatic denaturalization during the purification process, splitting the enzyme of 66-68 kDa into 3 subunits of approximately 10, 20 and 37 kDa.

Laccase activity has also been reported for *I. lacteus,* although in lower concentrations than the rest of fungi and mainly mycelium-associated (Shin, 2004; Svobodová et al., 2008). However, to the author's knowledge, there is still a lack of laccase characterization studies for this fungus. In this experiment, in lane 5, corresponding to this fungus extract, there are 3 bands of approximately 35, 22 and 15 kDa, and although it could have happened a similar denaturalization of laccases as in *P.sanguineus* case, the lack of published information for *I. lacteus* (and a higher laccase activity than P. sanguineus) prevents any conclusion.

On the contrary, and despite of having considerable laccase activity, *A. erebia* did not present any band in SDS PAGE (lane 4), which may be explained by presenting the lowest protein concentration of all fungi studied.

Specifically for *S. rugosoannulata*, a laccase of 66 kDa was reported by Schlosser and Höffer (2002), possibly coinciding with one or both bands in lane 3 of appearing between 50 and 75 kDa ladder marks. However, the main band of this extract is approximately 35 kDa; whether this band belongs to a laccase or not remains unknown.

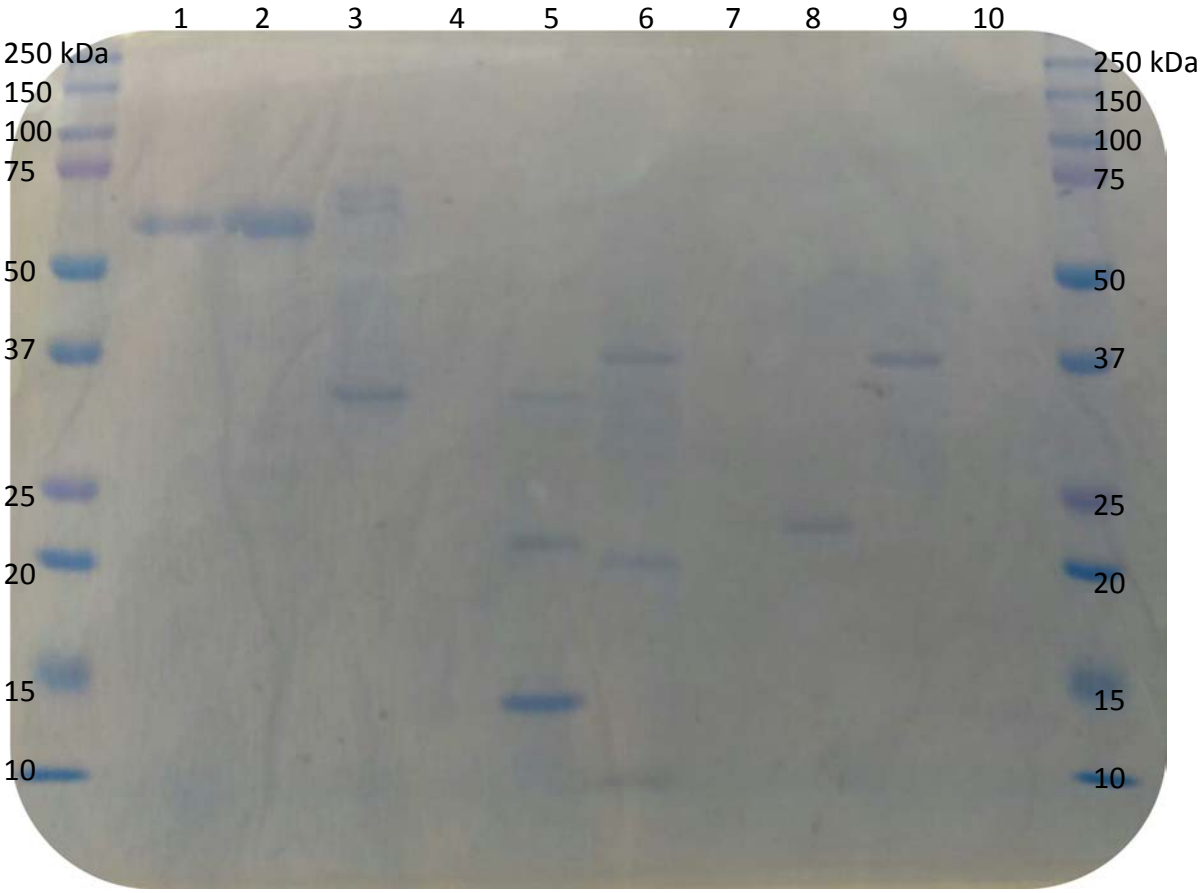

Figure 5.10

SDS PAGE gel, from left to right:
1. Standard sample of laccase I from P. sanguineus CS43 (Ramírez-Cavazos et al., 2014); 2. Standard sample of laccase II from P. sanguineus CS43 (Ramírez-Cavazos et al., 2014); and fungal extracts of 3. *S. rugosoannulata;* 4. *A. erebia;* 5. *I. lacteus;* 6. *P. sanguineus CS43;* 7. *P. ostreatus;* 8. *G. lucidum;* 9. *G. luteofolius* and 10. *T. versicolor.*

5.4. Conclusions

Mycelium from *S. rugosoannulata*, *G. luteofolius* and *A. erebia* was self-immobilized in pellet morphology and maintained in submerged cultures to remove the PhACs from water.

The promising results obtained suggest new suitable fungal candidates for the biodegradation of recalcitrant compounds. *S. rugosoannulata* was the most outstanding candidate among them, with PhAC removal percentages similar to those obtained with *T. versicolor,* a well-known fungus in PhACs biodegradation treatments.

The applicability of fungal treatment technology is already being studied for T. versicolor, but in the case of S. rugosoannulata, an optimization study of its culturing conditions could enhance its biodegradation performance.

When specifically comparing the total biodegradation yields per PhAC, the highest rate was achieved by *G. luteofolius* for IPD (97% from a 96% total removal) and by *S. rugosoannulata* for CBZ (77% from a 86% total removal). Cytostatics still showed recalcitrance to biodegradation for most of the fungi used; however, for CFD and IFD removals, over 25% of the biodegradation was achieved by *T. versicolor* and *I. lacteus*.

In the evaluation of PhACs removal, it might be worthwhile to measure target compounds in both liquid and solid (biomass) phases in order to determine the role of sorption and biodegradation mechanisms,

Sorption processes accounted for a mean of 7.3% of the PhACs elimination, however, the contribution of the specific sorption mechanisms depends on the fungus and the PhAC.

Degradation percentages calculated from the PhACs concentration measured in the liquid phase provide enough accuracy in most of cases due to the lack of degradationof the adsorbed PhACs within the 6 days.

Sorption of PhACs on inoculated biomass can be considered similar to that observed into the sludge from CAS treatments and therefore, it must be managed in the same way as the sludge from CAS treatments.

Finally, in spite of being able to stablish biodegradation as the main removal mechanism in all fungi, laccase mediated biodegradation could not be proven to take part in any

specific PhAC degradation, showing the need of further research on PhACs biodegradation mechanisms and potential transformation products.

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6. Identification of transformation products of recalcitrant pharmaceuticals

6.1. Introduction

In this chapter the fungal degradation of two recalcitrant PhACs, metaprolol (MTP) and venlafaxine (VFX), and their main respective transformation products, metoprolol acid (MTPA) and O-desmethylvenlafaxine (ODMVFX) was assessed.

MTP is one of the most consumed β-blockers (group of pharmaceuticals used to treat hypertension as well as patients recovering from heart attacks) around the world (Dong et al., 2013) and can be detected in wastewater up to 2 µg/l (Maurer et al., 2007). Low elimination rates have been described both for conventional wastewater treatments (Scheurer et al., 2010) but also for certain tertiary treatments (Maurer et al., 2007). In conventional WWTP, its removal is attributed to biological degradation since adsorption on activated sludge can be considered negligible (Alder et al., 2010), but biological degradation is only partial and inefficient, causing the occurrence of MTP and its metabolites into the water environment, where direct photodegradation is considered negligible without the presence of photosensitizers (Filipe et al., 2017), thus becoming a potential biohazard. Being one of the most consumed β-blockers, MTP is also highly detected in wastewaters, in a range of 160-2000 ng/L (Germany and Canada) (Maurer et al., 2007; Scheurer et al., 2010). Besides, MTP has shown potential ecotoxicity at mg/L level for several microorganisms (Cleuvers, 2005), and beyond the parental compound, the toxicity of metabolites (generated both from human and microbial metabolism) needs to be considered for a proper understanding of ecotoxicity, because they can significantly contribute to it (Escher et al., 2006). Nonetheless, in general very few data are available on the toxicity of MTP metabolites with any kind of test.

MTPA is known to be the major human metabolite of MTP (renal elimination of MTP reported to be 60-65% as MTPA) (Escher et al., 2006) whereas other metabolites such as O-desmethylmetoprolol (O-DMTP), α-hydroxymetoprolol (a-HMTP) and deaminated metoprolol can also be present in urine but at much lower concentration. MTPA, has also been described to be formed from the parent compound in biodegradation batch studies and generated as biodegradation product from atenolol, known as atenolol acid (Kern et

al., 2010). Very few studies have focused on the presence of MTP metabolites in wastewaters and surface waters, being MTPA the main transformation product that has been detected in WWTPs (Kern et al., 2010; Rubirola et al., 2014).

Venlafaxine (VFX) is an antidepressant drug that insides in the serotonin-norepinephrine reuptake inhibition (SNaRI or SNRI) (García-Galán et al., 2016). This antidepressant is widely used for the treatment of major depressive disorders, generalized anxiety disorder, panic disorder and social phobia. During the last years, *O*-desmethylvenlafaxine (ODMVFX), one of its main human metabolites, has also been commercialized as a new SNaRI (CIMA, 2016; García-Galán et al., 2016). The presence of these compounds in the environment is related to their low removal percentages in conventional wastewater treatment plants (WWTPs) but also to the transformation of VFX into ODMVFX during degradation processes both in the WWTP and in the aquatic environment. For example, venlafaxine has been detected at concentrations of 13 – 1914 ng/L in hospital effluents (Portugal) (Santos et al., 2013), between 68 and 268 ng/L in urban WWTP influents (Portugal) (Santos et al., 2013), and ranging from 175.9 to 214.6 ng/L in effluents in Montreal WWTP (Lajeunesse et al., 2008), 2190 ng/L in Denton (USA) (Schultz and Furlong, 2008) or between 184 and 322 ng/L in urban effluents from Portugal WWTPs (Santos et al., 2013), while the concentrations that have been detected in river waters are much lower, ranging from 12.9 to 45.9 ng/L in Montreal (Lajeunesse et al., 2008) or 1310 ng/L in Denton in USA (Schultz and Furlong, 2008). The elimination of VFX by conventional WWTP treatments has been detected inefficient. Moreover at least in one case it was reported a higher VFX concentration in effluent WWTPs than in influent. (Santos et al., 2013) (Portugal).

However, Gros et al. (2012) detected much higher concentrations of VFX after conventional activated sludge treatment in a WWTP that received industrial and hospital wastewaters in Catalonia. This high concentration in WWTP should be seriously considered as an alarm on PhACs increasing concentration in wastewater systems, but later on, Collado et al. (2014) evaluated the efficiency of the same WWTP for the elimination of some pharmaceuticals including VFX. The authors detected VFX at 2.6 µg/L

in the WWTP effluent, but could only be remove by 27% after UV tertiary treatment (Collado et al., 2014).

These results evidenced the low elimination efficiency of conventional water treatment for some pharmaceuticals, including VFX and MTP. In addition, in the case of VFX TPs, due to its use as antidepressant, as well as because of its generation during treatments with VFX as human metabolite, ODMVFX is detected in influent and effluent WWTPs at concentrations ranging from 222.5 to 330.0 ng/L and in rivers between 21.0 and 68.7 ng/L (Lajeunesse et al., 2008).

Therefore, new advanced wastewater treatment technologies need to enhance the removal of such recalcitrant compounds. Some authors studied the removal of VFX and ODMVFX by advanced oxidation processes (AOPs)(García-Galán et al., 2016; Giannakis et al., 2017; Lambropoulou et al., 2017), commonly applied as tertiary treatments (Oller et al., 2011). Regarding MTP and MTPA, multiple alternative processes to Conventional Activated Sludge (CAS) systems in WWTP have shown complete removal for metoprolol: from several advanced oxidative processes (AOPs) (Li et al., 2017; Romero et al., 2016) to hybrid magnetical nanoadsordents (Soares et al., 2016) and even combinative biological activated carbon (BAC) both by sorption or biological methods (Abromaitis et al., 2017). However, all these processes (being AOPs the most implemented) usually imply initial expensive investments and sometimes also expensive operational costs, such as high chemical dosages or high energy consumption (Pérez-González et al., 2012) and thus they are not considered sustainable cost-effective technologies.

Hence, an environmental friendly alternative treatment based on the potential of fungal biodegradation is proposed in this work for the removal of these PhACs from contaminated waters. Within this study, three white rot fungi (WRF) are assessed as potential PhACs biodegradation candidates, including *Trametes versicolor*, *Ganoderma lucidum* and *Pleurotus ostreatus*. *T. versicolor* is one of the most common used species for fungal biodegradation treatments, and has already been successfully used for the elimination of PhACs (Badia-Fabregat et al., 2015; Cruz-Morató et al., 2014; García-Galán et al., 2011; Marco-Urrea et al., 2010b, 2009; Carlos E. Rodríguez-Rodríguez et al., 2012). *Pleurotus ostreatus* has also been deeply studied in many applications, including PhACs removal in contaminated waters such as carbamazepine (Golan-Rozen et al., 2011), and

in spite of being less popular in PhACs degradation, previous studies reported certain degradation of *G. lucidum* for ibuprofene, clofibric acid and carbamazepine experiments (Marco-Urrea et al., 2009). Laccase, among other extracellular enzymes, is one of the most characteristic WRF extracellular enzymes and has been proved able to degrade many pharmaceuticals, such as antibiotics or DCF among others (Becker et al., 2016; Nguyen et al., 2014). The activity of this enzyme was analyzed in this study as an indicator of fungal activity.

Generation of any possible transformation product (TP) from target pollutants during treatment processes should be assessed since these byproducts can sometimes be more toxic than parent compounds, specially in the case of VFX and ODMVFX (Lambropoulou et al., 2017). Furthermore, VFX and ODMVFX were pointed out as precursors of potential carcinogenous such as the disinfection byproduct *N*-nitrosodimethylamine (NDMA). However, there are no previous studies investigating the potential of green biodegradation technologies such as the ones based on fungi with capability to degrade VFX, ODMVFX and their respective TPs.

In this context, the main objectives of the present work were I) the evaluation of three fungal species for the elimination of VFX, ODMVFX, MTP and MTPA; II) the characterization of TPs generated during the biodegradation process (in the case of VFX and ODMVFX); and III) the implementation of *G. lucidum* bioreactor treatment for MTP and MTPA.

6.2. Materials and methods

6.2.1. Chemicals and fungal biomass

Venlafaxine standard (VFX) and Metoprolol (MTP) were purchased from Sigma-Aldrich (Barcelona, Catalonia) while Metoprolol acid (MTPA), *N*-desmethylvenlafaxine (NDMVFX) and *O*-desmethylvenlafaxine (ODMVFX) were purchased from Toronto Research Chemicals TRC (Ontario, Canada). Labelled sulfamethoxazole for VFX and ODMVFX and atenolol-d7 for MTP and MTPA, were used as respective internal standards, being

purchased at Fluka (Buchs, Switzerland) and CDN isotopes (Quebec, Canada). The calibration mixture used for high resolution mass spectrometry purposes was supplied by Thermo Fisher Scientific (LTQ ESI Positive Ion Calibration Solution and ESI Negative Ion Calibration Solution).

All the solvents used during the studies were of high purity grade. High-performance-liquid-chromatography (HPLC) grade methanol, acetonitrile and water (Lichrosolv) were supplied by Merck (Darmstadt, Germany). Formic acid 98% was provided by Merck (Darmstadt, Germany). HPLC-high resolution mass spectrometry grade acetonitrile and water (Lichrosolv) were supplied by Thermo Fisher Scientific.

Three different white rot fungi (WRF) were used in this work: *Trametes versicolor* (ATCC 42530^{TM}), *Ganoderma lucidum* (Leysser) Karsten FP-58537-Sp, and *Pleurotus ostreatus* (NCBI KJ020935). Fungal biomass was obtained as explained in section 1.3.4.1 from General Methodology.

6.2.2. Degradation experiments (in synthetic medium)

Degradation experiments were performed in triplicate in 250 mL Erlenmeyer flasks filled with 100 mL of defined medium spiked with MTP, MTPA, ODMVFX or VFX respectively at a concentration of 5 ± 3 mg/L. Sterile conditions were obtained by sterilizing the medium at 121ºC for 30 min. The PhACs were spiked with an appropriate volume according to the PhAC concentration in the methanol stock solution. Each flask was inoculated with mycelial pellets approximately equivalent to 3.5 ± 0.8 g/L dry cell weight (DCW). Samples were taken at 0, 3, 7, 10 and 15 days and further centrifuged in glass vials to remove any biomass fragments or suspended solids.

Abiotic (same conditions as described above but without biomass), live or biotic (same conditions but without PhACs) and killed control (same conditions but with heat-killed biomass) controls were performed in triplicate to measure the potential effects of physicochemical processes in pharmaceutical concentration, their toxicological effects on fungal biomass, and pharmaceutical sorption processes on biomass, respectively.

6.2.3. Degradation experiments in bioreactor

In an attempt to reproduce real conditions, HWW was used in the bioreactor experiments, spiked to a concentration of 2 ± 0.5 μg/L for both MTP and MTPA.

Hospital wastewater (HWW) used in bioreactor experiments was collected from the main sewer manifold of Sant Joan de Déu Hospital (Barcelona, Catalonia). HWW samples were collected and frozen until used. Concerning bioreactor operation, electrovalve was maintained with 1 second of air pulse every 3 seconds. The aeration rate was 0.8 L/min. Glucose and ammonium chloride from Sigma-Aldrich (Barcelona, Catalonia) were supplied at 7.5 C/N molar ratio, from concentrated stock solutions in fed-batch operation mode at consumption rate (0.8 g $C_6H_{12}0_6$ g DCW^{-1} and 0.19 g NH_4Cl g DCW^{-1}). HWW was pretreated by coagulation-flocculation but not sterilized, working under non-sterile conditions for 7 days. Coagulation-flocculation pretreatment involved the addition of coagulant HyflocAC50 at 43 mg/L during 2 min at 200 rpm and flocculant HimolocDR3000 at 4.8 mg/L for 15 min at 20 rpm (Derypol, Barcelona, Catalonia). *G. lucidum* was inoculated in mycelial pellets with approximately 2.5 ± 0.3 g/L dry weight. Water samples were taken at 0, 3 and 7 days and further centrifuged in glass vials to remove any biomass fragments or suspended solids.

6.2.4. Instrumental analyses

Analysis of VFX, ODMVFX and related TPs

The quantification of target compounds and the identification of any possible transformation product was carried out using a liquid chromatography system coupled to a hybrid linear ion trap – high resolution mass spectrometer LTQ Orbitrap (LC-LTQ Orbitrap). Aria TLX-1 chromatographic system (Thermo Fisher Scientific) was used for separation purposes. This system comprised a PAL auto sampler and two mixing quaternary pumps (eluting pump and loading pump). The entire system was controlled via Aria software, version 1.6, under the Xcalibur 2.2 software. The compounds were separated in a Hypersil GOLD analytical column (50 × 2.1; 3 µm; Thermo Fisher Scientific, Franklin, MA). The separation was achieved working under gradient conditions with water (ammonium formiate/formic acid buffer, pH 4) (phase A) and acetonitrile (phase B). The gradient was as follows: 5% of B for 1 min, then the gradient reaches 95% of B after 10 min and 100% during the next minute. The percentage of B was maintained for 5 minutes and then returned to initial conditions in 1 min. These percentages were maintained for one more minute. The total run time for each injection was of 17 min, with an injection volume of 10 µL.

To quantify the concentration of the detected TPs, the samples were analyzed by LC-MS/MS using pure standards of VFX, ODMVFX and NDMVFX. The chromatograph was coupled to a hybrid linear ion trap-Fourier Transform Mass Spectrometry Orbitrap analyzer (LTQ-OrbitrapVelos^{TM}, Thermo Fisher Scientific) equipped with a diverter valve (used in order to divert to waste unwanted portions of chromatographic runs) and an Electrospray Ionization source (ESI). The diverter valve was used with three valve positions: from 0 to 1.5 min the flow was discharged to the waste and from 1.5 to 17 min the valve was switched to injection mode. The ionization of the compounds was performed under positive and negative mode for screening purposes. Mass calibration and mass accuracy checks were performed prior to every sample run with LTQ ESI Positive and Negative Ion Calibration Solution (Thermo Fisher Scientific), where mass accuracy was always within errors of ± 2 ppm. More detailed information can be seen elsewhere (Llorca, M. et al., 2017). The samples were acquired using three different acquisition methods in

parallel: 1) the first method was triggered through full scan within a mass-to-charge (m/z) range of 100 to 1000 m/z at a resolving power of 60,000 FWHM; 2) the second experiment was performed with data-dependent analysis through the MS fragmentation of the 5th most intensive ions obtained in the 1st experiment at each scan time, isolated in the ion trap with a width of 2.0 Da, a collision induced dissociation activation type (Q = 0.250 and an activation time of 30 ms) and normalized collision energy (35); and 3) the third experiment was also with data-dependent analysis thorough the fragmentation of the molecular ion of VFX (m/z = 278.2114) and (O or N)DMVFX (m/z = 264.1958) for confirmation purposes with the same instrumental parameters as describe in point (2). Xcalibur 2.2 software was used for data interpretation.

Data processing was carried out using with SIEVE 2.0 software (Thermo Scientific) in order to perform the chromatographic peak deconvolution and ExactFinder 2.5 software (Thermo Scientific) for quantification purposes of selected compounds.

Chemical analysis of MTP and MTPA

The quantification of target compounds and the identification of any possible transformation product in MTP and MTPA experiments was performed with the same liquid chromatography system coupled to a hybrid linear ion trap – high resolution mass spectrometer LTQ Orbitrap (LC-LTQ Orbitrap) used in VFX and ODMVFX experiments. Again, the same Aria TLX-1 chromatographic system (Thermo Fisher Scientific) was used for separation purposes, but, unlike VFX and ODMVFX analysis, the compounds of interest were separated in a ZORBAX Eclipse XD-C18 (150 × 4.6; 5 µm; Agilent Technologies, Santa Clara, CA, USA). The separation was achieved working under gradient conditions with water (ammonium formiate/formic acid buffer, pH 3) (as A phase) and acetonitrile (B phase). The gradient was as follows: 5% of B for 1 min, then the gradient reaches 95% of B after 10 min and 100% during the next minute. The percentage of B was maintained for 5 minutes and then returned to initial conditions in 1 min. These percentages were maintained for one more minute. The total run time for each injection was of 17 min, with an injection volume of 20 µl.

Samples were also analyzed by LC-MS/MS using pure standards of MTP and MTPA to determine the concentration of PhACs. The chromatograph detection system was the

abovementioned hybrid linear ion trap-Fourier Transform Mass Spectrometry Orbitrap analyzer and the same conditions for diverter valve. The ionization of the compounds was also performed under positive and negative mode for screening purposes. Mass calibration and mass accuracy checks were performed as well, prior to every sample run with LTQ ESI Positive and Negative Ion Calibration Solution (Thermo Fisher Scientific), where mass accuracy was always within errors of ± 2 ppm. More detailed information can be seen elsewhere (Llorca, M. et al., 2017). The samples were acquired using two different acquisition methods in parallel: 1) the first method was triggered through full scan within a mass-to-charge (m/z) range of 100 to 1000 m/z at a resolving power of 60,000 FWHM; 2) the second method was also with data-dependent analysis through the fragmentation of the molecular ion of MTP and MTPA with a width of 2.0 Da, a collision induced dissociation activation type (Q = 0.250 and an activation time of 30 ms) and normalized collision energy (30). Xcalibur 2.2 software was used for data interpretation.

For bioreactor liquid samples, 70 mL of initial and final samples were taken and filtered with Durapore® membrane, PVDF, hydrophylic, 0.45 µm, 47 mm filters) prior to chemical analyses.

6.3. Results and discussion

6.3.1. Biomass, glucose consumption and laccase activity

On the first part of this study, Erlenmeyer flasks experiments were set to analyze fungal removals and TPs generation for the four PhACs studied. In this studies, biomass concentration was maintained or slightly increased in most of the cases. An exception was found for *P. ostreatus* in the ODMVFX experiment where biomass increased to approximately 60% (dry weight, data shown on figure 6.1).

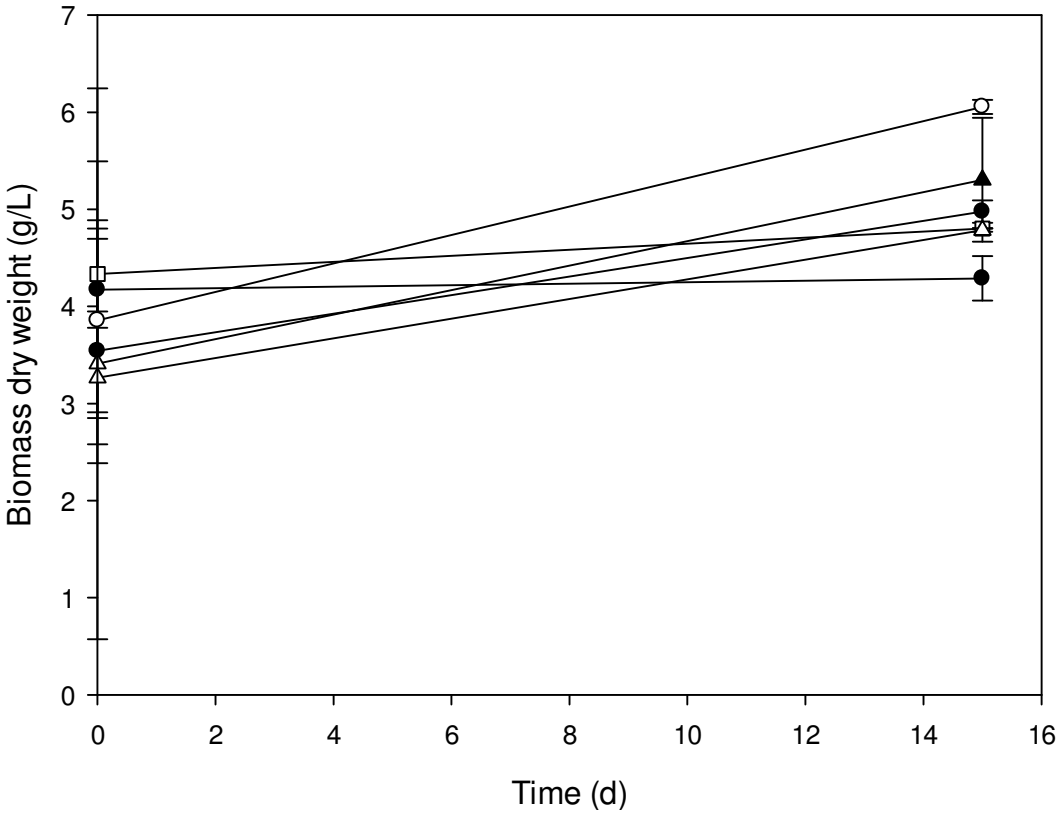

Figure 6.1 Initial biomass dry weight estimation versus final biomass dry weight real measurements. Filled dots correspond to VFX and empty ones to ODMVFX, for the three selected fungi: T. versicolor (■), G. lucidum (▲) and P. ostreatus (●).

These results are related with the decrease in glucose concentration in all experiments (Figure 6.2). Glucose was the only carbon source available and it was totally consumed after 10 days of experiment for all species in MTP, MTPA and ODMVFX experiments. On the contrary, in VFX experiment, *P. ostreatus* presented a slower consumption of glucose and no less than 7.6 g/L glucose remained at the end of VFX experiment (from an initial concentration of 12 g/L). This slower decrease in carbon source could be explained by a lower metabolism which could have impacted its capability to remove VFX. Nonetheless, *T. versicolor* and *G. lucidum* consumed glucose almost totally before the end of the experiment with VFX.

Laccase activity showed great differences among assayed fungi (Figure 6.2). *T. versicolor* was the species with higher activity, considerably high for VFX and ODMVFX (a maximum of 167 U/L was detected in the case of VFX both at day 3 and at day 15), whereas less than half of these values were obtained in MTP and MTPA experiments, with the highest values being registered at the first days of the experiment.

In the case of ODMVFX, although activity was slightly lower than VFX maximum, laccase peaks could be observed during the first and last days of the experiment and could be explained by changes in carbon and carbon/nitrogen ratio concentration as pointed out by previous studies where certain carbon/nitrogen ratio or a depletion of the carbon source could promote laccase production (Hailei et al., 2009; Mikiashvili et al., 2005).

For the rest of species lower laccase activity levels were detected in all experiments (with a range of 0-5 U/L of lacase activty.

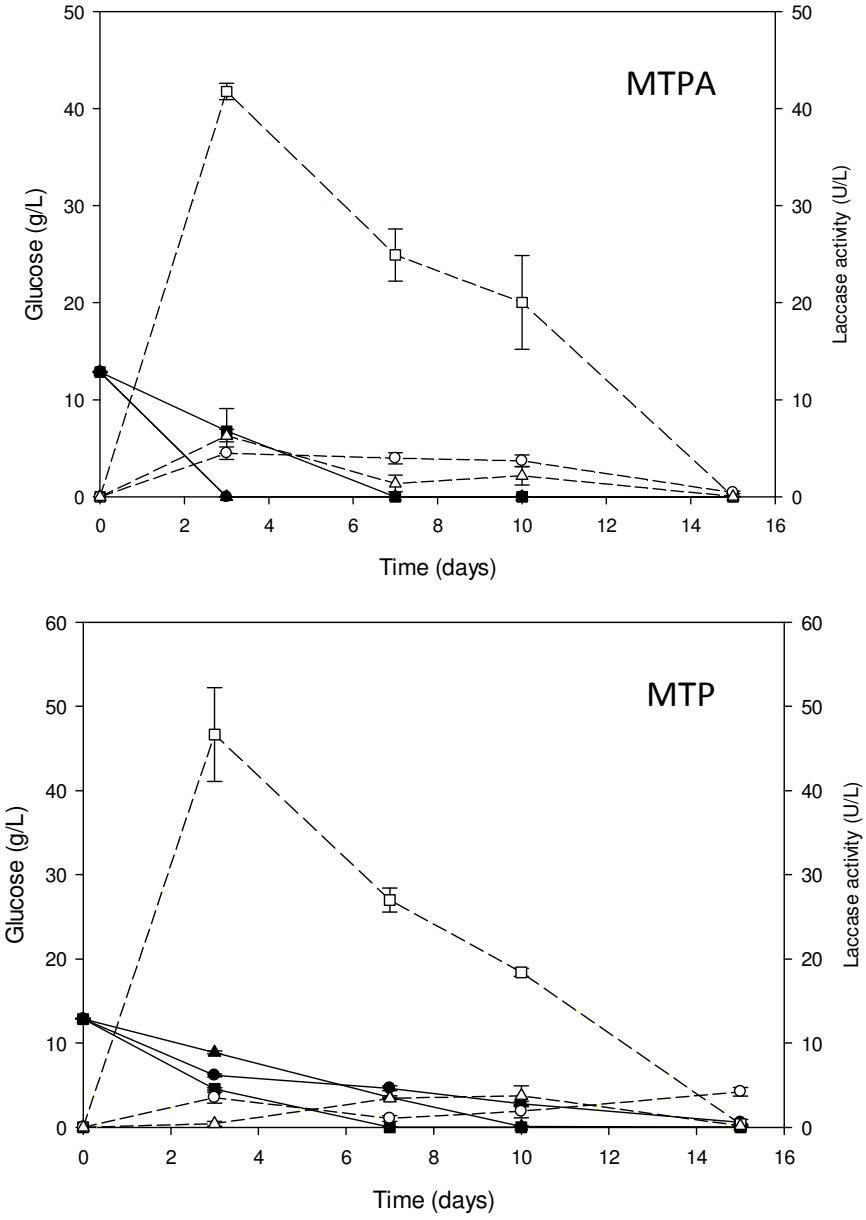

Figure 6.2A Results for MTP and MTPA: glucose consumption (straight lines, filled symbols) and laccase enzymatic activity (dashed lines, empty symbols), for the three selected fungi: T. versicolor (■), G. lucidum (▲) and P. ostreatus (●) and their respective experiment of PhAC degradation.

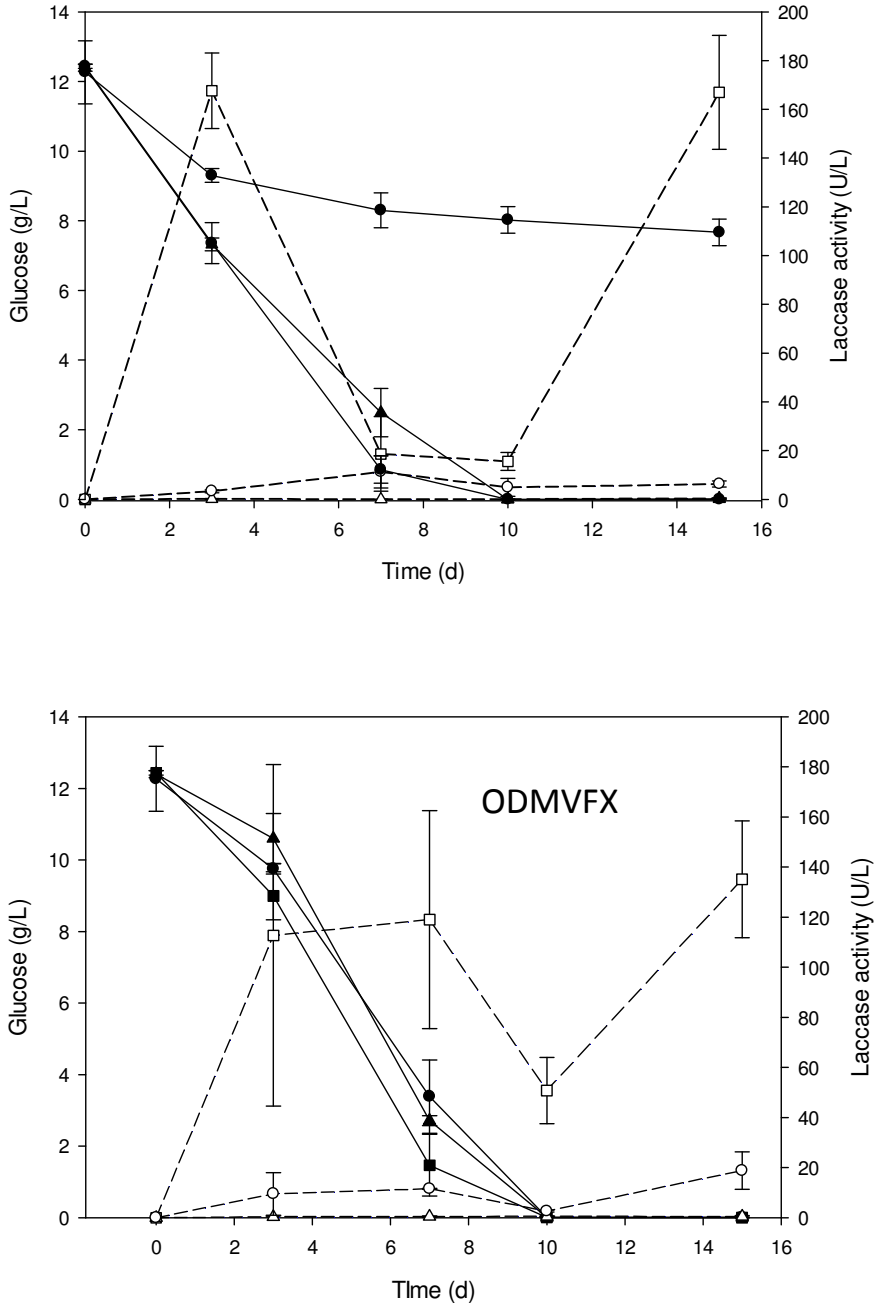

Figure 6.2B Results for VFX and ODMVFX: glucose consumption (straight lines, filled symbols) and laccase enzymatic activity (dashed lines, empty symbols), for the t-hree selected fungi: T. versicolor (■), G. lucidum (▲) and P. ostreatus (●) and their respective experiment of PhAC degradation.

The second highest laccase activity was reached by *P. ostreatus* where a maximum of 19 U/L in day 15 of the ODMVFX removal experiment was observed. *G. lucidum* laccase activity was negligible for all experiments instead. Hence, any potential removal or biodegradation phenomena in the experiments with *G. lucidum*, is less probable to be caused by laccase oxidation mechanisms . On the contrary, and considering these results, a possible laccase induction mechanism for *T. versicolor* and *P. ostreatus* such as the ones described by Tinoco et al. with other compounds, (2011) could be activated in the presence of VFX and ODMVFX, although any correlation between its activity and PhACs degradation could not be demonstrated.

6.3.2. PhACs removal

Abiotic control experiments showed that removal by physicochemical processes was negligible for all PhACs in these conditions. No sorption onto bottle experiments was observed while sorption onto fungi was generally low in the cases of VFX and ODMVFX, but it represented the main fraction of removal for MTP in some species (Figure 6.3). In particular, for *T. versicolor* and *P.ostreatus* removals of over 25% were achieved in MTP, but sorption onto biomass was responsible for over 90% of it. Although MTPA sorption onto fungi was less important, it was not negligible for some species, reaching up to 15% of *G. lucidum* MTPA removal.

Sorption mechanisms at the end of the VFX experiment were only noticeable in *T. versicolor* and *P. ostreatus,* being responsible for 25% of removal or even more. On the other hand, the removal percentages for VFX were *c.a.* 70% for *G. lucidum* and *T. versicolor,* while just a 25% removal was observed for *P.ostreatus* after 15 days. Despite the different VFX removal percentages observed between species, two different trends could be appreciated for all of them during the 15 days of experimental time.

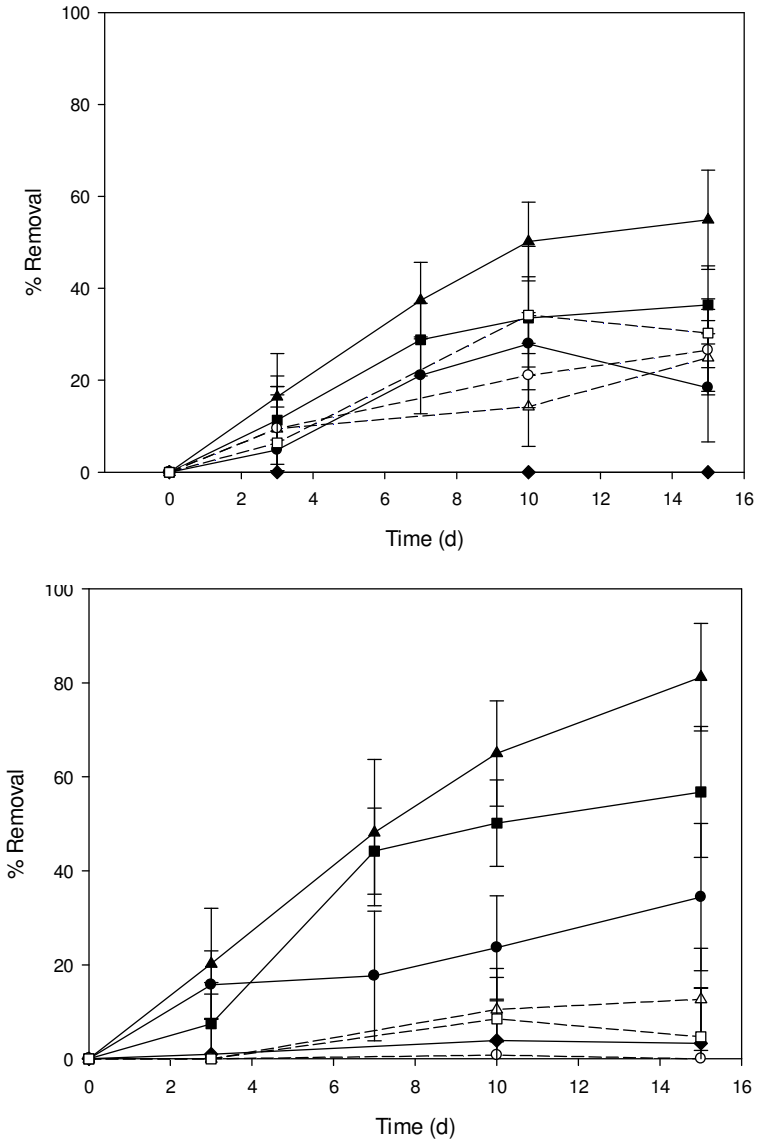

Figure 6.3 Results for MTP and MTPA removals. Fungal treatment are represented in continuous lines and filled symbols (▲ for *G.lucidum*, ■ for *T. versicolor*, ● for *P.ostreatus* and ◆ for the abiotic treatment), whereas killed control treatments appear in dashed lines and empty symbols.

VFX removal during the first 7 days was faster (near to 20% removal for *P. ostreatus* and near to 55% for the other two species), coinciding with the period were glucose was still available in solution. After this time, the removal rate decreased until the end of the experiment as it can be appreciated in Figure 6.4. Previous works related to the biodegradation of VFX showed an elimination c.a. 50% after 90 days of aerobic activated sludge treatment spiked at concentrations of 30 µg/L (Gasser et al., 2012). The same study

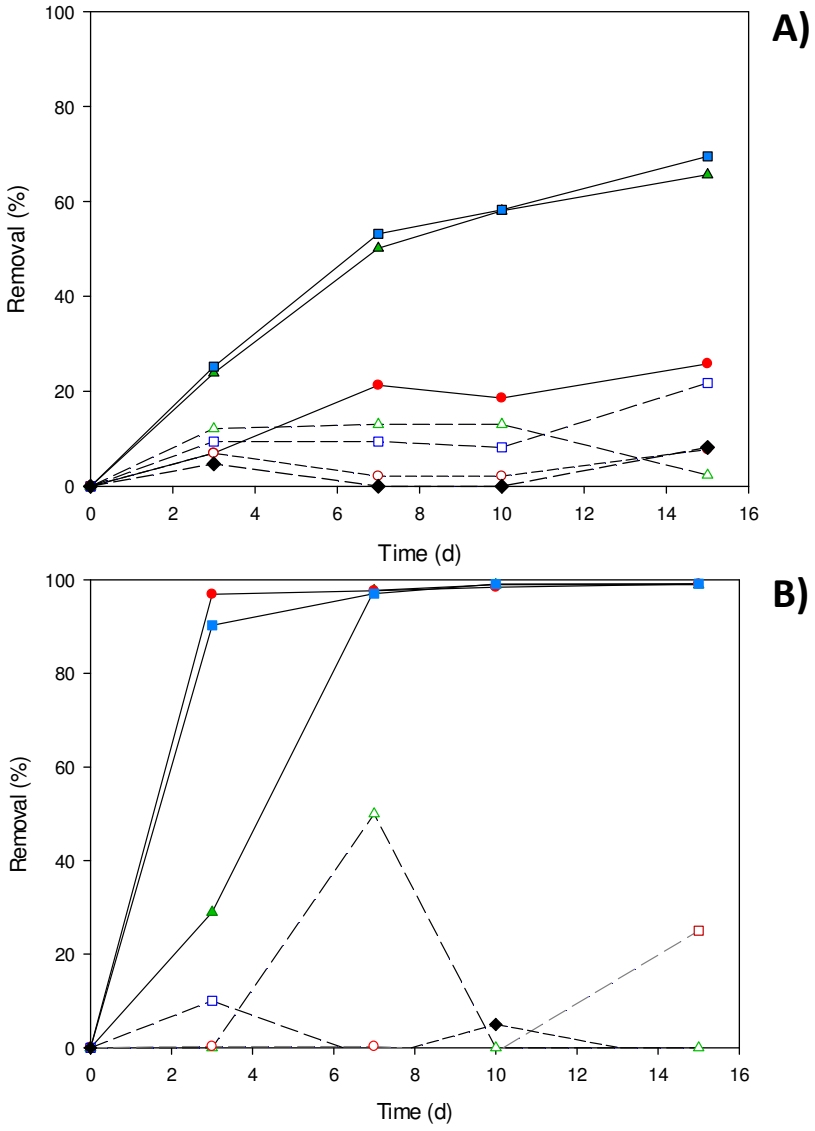

Figure 6.4: Removal percentage of (A) venlafaxine and (B) *O*-desmethylvenlafaxine with *P.ostreatus* (▼), *T. versicolor* (●) and *G. lucidum* (■). Abiotic control (◆) and killed control (KC) (in dashed lines ▽, ○ and □ respectively).

showed that under anaerobic conditions, even though VFX levels remained unaffected during the first 40 days, 100% VFX removal was reached after 90 days (Gasser et al., 2012).

O-desmethylvenlafaxine was completely removed after 3 days in the case of *P. ostreatus* and *T. versicolor* and after 7 days in the case of *G. lucidum* (Figure 6.4B). In this sense, the three species can be considered highly effective for the removal of ODMVFX. The elimination of ODMVFX can be entirely attributed to biological processes since no physicochemical processes were detected during the control experiments. Gasser et al.

(2012) investigated the removal of ODMVFX by activated sludge under aerobic and anaerobic conditions at 25 µg/L and observed 100% removal after 60 days of experiment under aerobic conditions, while this percentage was almost negligible under anaerobic conditions (Gasser et al., 2012). The authors suggested that fungal biodegradation treatment could be an effective and faster solution to remove ODMVFX from wastewaters.

In MTP and MTPA fungal removal in Erlenmeyer flasks, maximum elimination was achieved by *G. lucidum*, which reached removals up to 55% for MTP and 81% for MTPA after 15 days of operation. Minimum degradation percentage (MDP) was slightly higher than 50% of total MTP removal and almost 85% of MTPA total removal, proving that degradation was the main removal mechanism in both cases for this fungus.

On the contrary, removals of less than 40% were detected for *T. versicolor* and *P.ostreatus* for MTP. This last species stopped its removal activity on day 10 (27.9%) and decreased in its removal at the end of the experiment (18.4%), being almost equivalent to their sorption removal.

Although biodegradation capacity for both species was almost negligible in MTP experiments, MDP was higher than 30%, and *T. versicolor* achieved removals of 56% (52% of MDP).

6.3.3. Identification of venlafaxine and O-desmethylvenlafaxine transformation products during fungal biodegradation

A summary of the main TPs detected during the degradation experiments of VFX and ODMVFX can be consulted below (Table 6.1):

Table 6.1: Main transformation products identified during biodegradation of venlafaxine with *T. versicolor*, *G. lucidum* and *P. ostreatus*.

Proposed TPs	Biodegradation process detected
Venlafaxine	
O-desmethylvenlafaxine	*T. versicolor*
	G. lucidum
N-desmethylvenlafaxine	*T. versicolor*
	G. lucidum
N,N-didesmethylvenlafaxine	*T. versicolor*
	G. lucidum
O-desmethylvenlafaxine	
N,O-didesmethylvenlafaxine	*P. ostreatus*
	T. versicolor
	G. lucidum
Venlafaxine	*P. ostreatus*
	T. versicolor
	G. lucidum

Figure 6.5 and 6.6 show VFX and ODMVF levels respectively along with their corresponding TPs during the experimental time. For VFX, better results for the identification of TPs were obtained with samples from *T. versicolor* and *G. lucidum* than those from *P. ostreatus* (elimination c.a. 25%). Therefore, the TP study focused in the evaluation of byproducts identified in *T. versicolor* and *G. lucidum* experiments. The main TPs postulated for venlafaxine were ODMVFX and *N*-desmethylvenlafaxine (NDMVFX), both of them detected after two days of experiment, and the *N,N*-didesmethylvenlafaxine (NNDMVFX) detected after 3 days of treatment time with *T. versicolor* and after 10 days with *G. lucidum*.

Table 6.2: concentrations of *N*-desemthylvenlafaxine identified during biodegradation of venlafaxine with *T. versicolor* and *G. lucidum*.

	T. versicolor		***G. lucidum***	
Days	**Conc. (µg/L)**	**%RSD**	**Conc. (µg/L)**	**%RSD**
0	0	0	0	0
3	21.5	4.46	11.4	6.33
7	18.4	4.31	15.3	11.4
10	17.4	4.61	19.6	27.6
15	14.3	8.48	30.8	15.8

%RSD: percentage of relative standard deviation

The generation of O- and NDMVFX could potentially be regioselective or regiospecific since more NDMVX is detected at the end of the experiments. Concentrations of ODMVFX were always below the method limit of detection for both *T. versicolor* and *G. lucidum,* while the concentration of NDMVFX was calculated and is compiled in Table 6.2. The concentration of NDMVFX reached a maximum of 21.5 µg/L after 3 days in the *T. versicolor* degradation experiment. Afterwards, the TP was degraded by the fungal activity leaving 14.3 µg/L at the end of the experiment. In the case of *G. lucidum,* NDMVFX was detected after 3 days of experiment and its concentration gradually increased until the end of the experiment (i.e., 30.8 µg/L). ODMVFX concentration could not be quantified due to its weak signal below limits of quantification, although a similar ODMVFX detection pattern was detected for both fungi species (Figure 6.5). In this context, we can conclude that *T. versicolor* is more efficient for the elimination of VFX and its TPs (NDMVFX, ODMVFX) than *G. lucidum*. In the case of the ODMVFX generation, the demethylation of the methoxy group of VFX could be due to the activity of a non-heme iron-dependent demethylase enzyme LigX from lignin metabolism (Bugg et al., 2011; Sonoki et al., 2000) present in *T. versicolor* (Paice et al., 1993). On the other hand, demethylation of the dimethylamino group of VFX, which lead to the formation of NDMVX, is likely to be attributed to the activity of enzymes related to *N*-demethylation metabolic pathway from fungi, similar to human liver pathways (Jollow et al., 1977). Gasser et al. (2012) also identified NDMVFX as

a degradation product of VFX as well as ODMVFX and *N,O*-didesmethylvenlafaxine (NODMVFX) during the aerobic degradation of VFX in wastewaters with activated sludge. Nonetheless, in this case the presence of ODMVFX could not be only related to the degradation of VFX since ODMVX, as human metabolite of VFX, was already present in the wastewater used for the experiments. In contrast, high regioselectivity was observed for the experiments carried out under anaerobic conditions as the authors detected the generation of ODMVFX at higher amounts than NDMVFX.

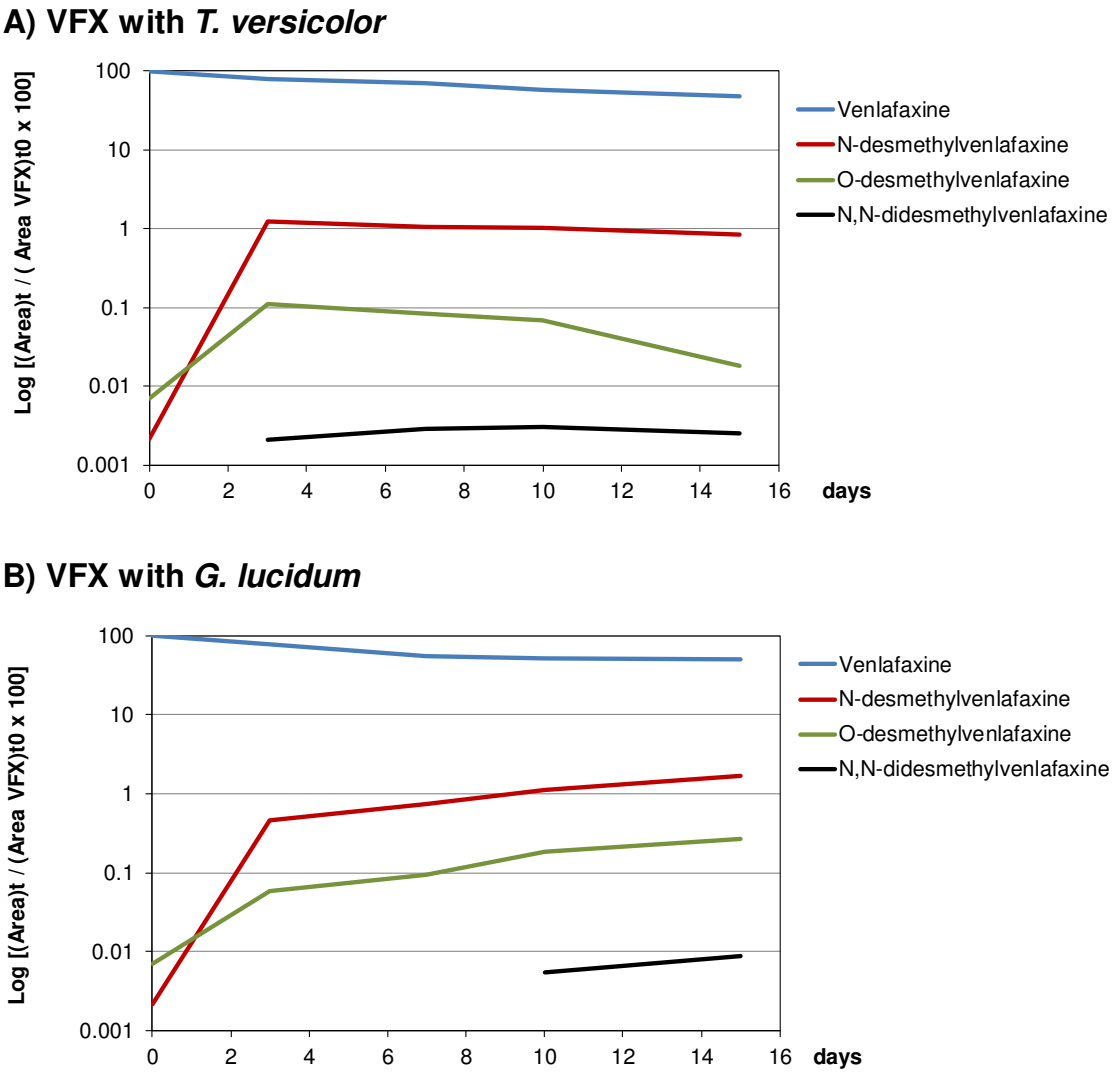

Figure 6.5: transformation products identified or postulated during removal treatments of venlafaxine with *T. versicolor* (A) and *G. lucidum* (B). The results are expressed as the log of the percentage of generated peak area in the chromatogram at time t *vs.* the peak area in the chromatogram of VFX at time 0.

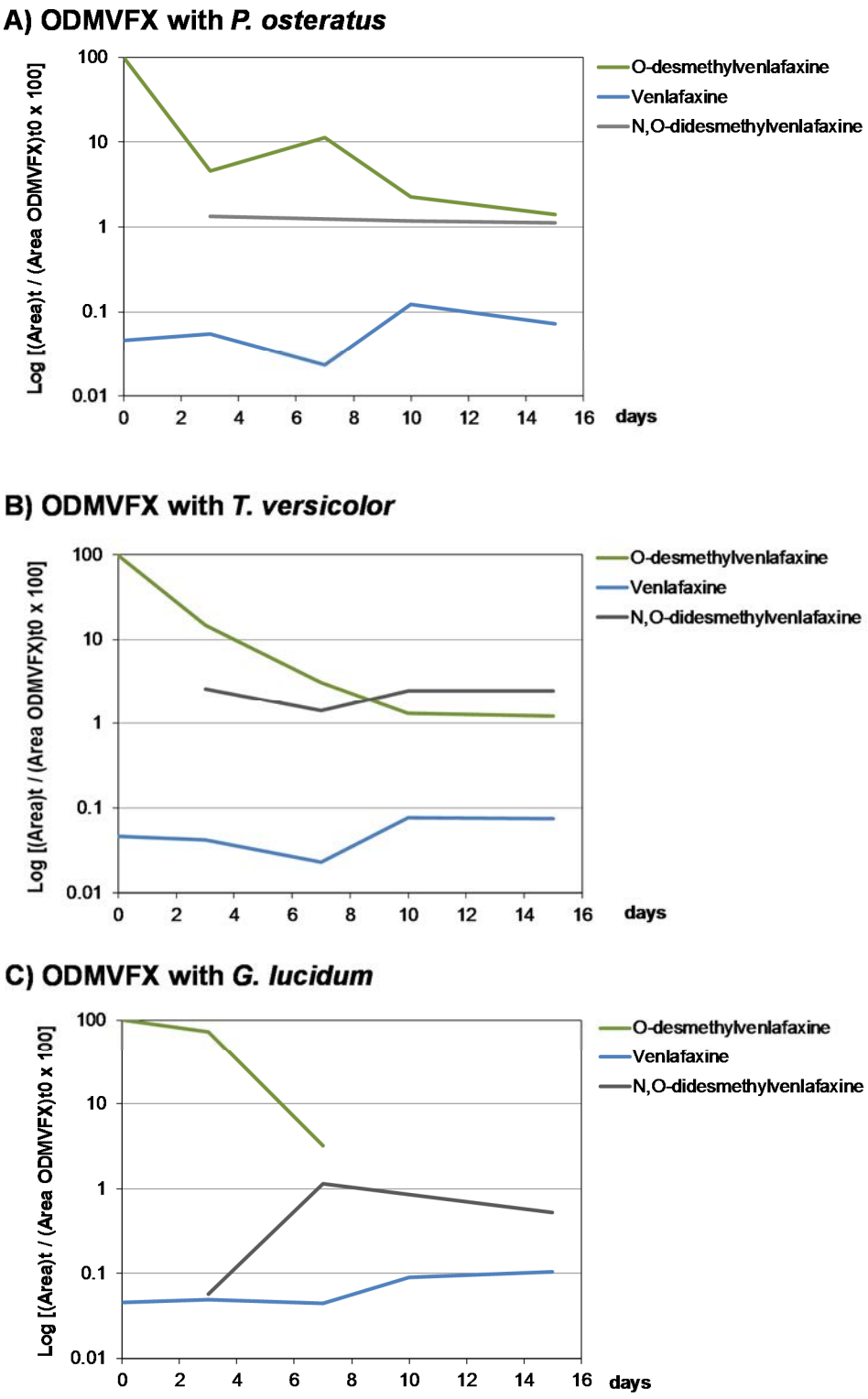

Figure 6.6: transformation products identified or postulated during removal treatments of *O*-desmethylvenlafaxine with *P. ostreatus* (A), *T. versicolor* (B) and *G. lucidum* (C). The results are expressed as the log of the percentage of generated peak area in the chromatogram at time t *vs.* the peak area in the chromatogram of ODMVFX at time 0.

Degradation experiments for ODMVFX in waters with *T. versicolor*, *G. lucidum* and *P. ostreatus* denoted the presence of two TPs including *N,O*-didesmethylvenlafaxine (NODMVFX) and venlafaxine (VFX) (Table 6.1 and Figure 6.6). It is suspected that the generation of NODMVFX was due to the activity of enzymes related to the *N*-demethylation, the same process implied in the amino demethylation of VFX. On the other hand, the main hypothesis for the generation of VFX from ODMVFX during fungal treatment was the activity of the regiospecific *O*-methylation of the *O*-methyltransferases of fungal systems (Wessjohann et al., 2013).

6.3.4. MTP and MTPA bioreactor

This promising performance of *G. lucidum* in the degradation experiments of MTP and MTPA, encouraged the scaling up of the process into bioreactor scale for a period of 7 days in non-sterile conditions with real hospital wastewater (HWW). Unlike the previous experiments, the fungus was exposed in this experiment to bacterial competition for nutrients and the presence of any other PhAC in real HWW that could affect its growth or metabolism. However, *G. lucidum* treatment of MTP and MTPA was successfully scaled up, and, in the case of MTPA, removals for the same period were even higher than in Erlenmeyer flask, with an elimination of 64% (compared to 48% obtained in Erlenmeyer flask for 7 days). On the contrary, MTP removals in this experiment were equivalent to the previous assays in Erlenmeyer flasks (slightly over than 32% compared to 37% in flasks for 7 days) (figure 6.7).

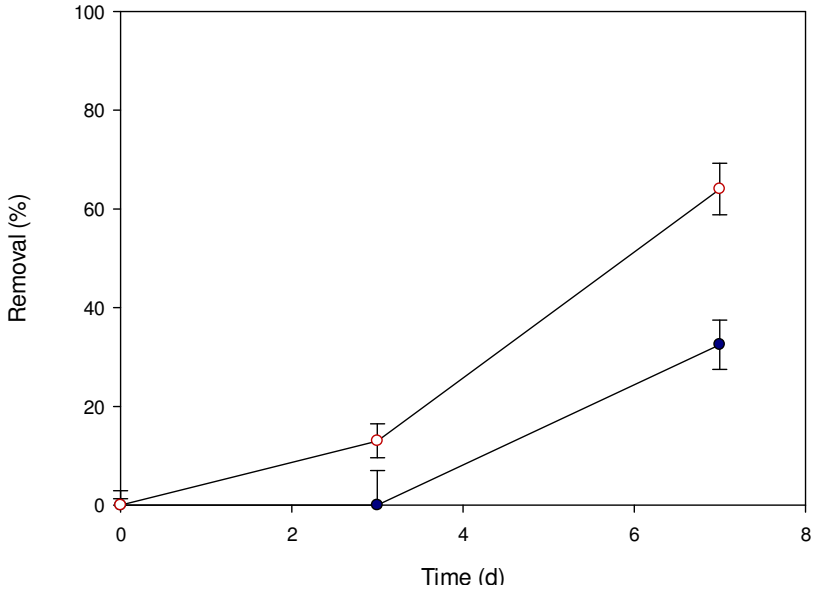

Figure 6.7 MTP (black filled circles) and MTPA (empty circles) removals during the batch reactor operation

6.4. Conclusions

Three WRF species were tested for the elimination of recalcitrant PhACs MTP, MTPA, VFX and ODMVFX from wastewaters.

- Highest removals for MTP and MTPA were achieved by *G. lucidum*, with 55% and 81% respectively, and although none of the fungi achieved the total removal, biodegradation mechanism was assumed to happen for all fungi with MTPA. Nevertheless, only in MTP *G. lucidum* showed biodegradation as the main removal mechanism.
- *T. versicolor* and *G. lucidum* were able to remove up to 70% of VFX in 15 days, whereas *P. ostreatus* was only able to remove 25% during the same time. In the case of *T.versicolor*, up to 30% of 70% removal was due to sorption processes, whereas *G. lucidum* biodegradation mechanisms could explain 97% of the removal. In contrast, all three fungi achieved a removal of 100% of ODMVFX with negligible sorption percentages, though *T. versicolor* and *P. ostreatus* did it within 3 days of experiment whereas *G. lucidum* needed 6 days.
- Laccase seem not to be strongly involved in the degradation of the studied PhACs because when its activity was high (*T. versicolor* with VFX and ODMVFX), similar removal rates were obtained for other non-laccase producer species. Besides, in MTP and MTPA experiments laccase activity remained low for all fungi, and highest removals (attributed to *G. lucidum*) coincided with the lowest laccase activities.
- Demethylation of the dimethylamino and methoxy moieties were the main degradation pathways identified for VFX, and two TPs (ODMVFX and NDMVFX) were detected and one tentatively identified (*N,N*-didesmethylvenlafaxine). In the case of ODMVFX, VFX was identified as degradation byproduct (back-transformation to the parent compound) and another one was also tentatively identified (*N,O*-didesmethylvenlafaxine).
- A scaling up from Erlenmeyer flask to fluidized bed bioreactor with G. lucidum for MTP and MTPA treatment was successfully achieved. The bioreactor was operated in fedbatch mode for 7 days and removals for MTP were similar whereas for MTPA results were even better. Further research on the scaling up of MTP and MTPA is

needed. The identification of MTP and MTPA TPs for this fungal treatment will also be studied in the future.

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7. Fluidized Bed Bioreactors for PhACs fungal biodegradation

7.1. Introduction

In submerged fungal bioreactors, three forms of fungal morphologies can be present, suspended mycelia (fungal filaments), clumps (small irregular agreggates) and pellets (biomass spherical granules). The type of fungal growth selected for a particular treatment depends on its application. In particular, pellet morphology has been the most common strategy in the removal of pollutants from wastewater. with fungal bioreactors due to factors such as low viscosity, easier manipulation requirements during continuous operation or better homogenization of oxygen and nutrients (Espinosa-Ortiz et al., 2016).

Fungal treatments have been investigated for the removal of pollutants and micropollutants in wastewaters for more than a decade (Grandclément et al., 2017), and during the last years several cases of membrane bioreactors have achieved succesful implementation in real scale hospitals (Beier et al., 2011; Kovalova et al., 2012), but only very few alternatives with pure cultures of fungal species have attempted PhACs degradation in real wastewaters under non sterile conditions (Cruz-Morató et al., 2013a), which would be the ultimate goal for the implementation of this technology.

Within the attempts to achieve this ultimate goal, several reactor configurations (e.g. stirred tank reactors, fixed bed bioreactors, enzymatic membrane bioreactors, etc.) have been used to work with fungal pelleted biomass in the removal of PhACs, however the most common system used for this application were fluidized bed bioreactors with fungal pellets (FBBs) (Espinosa-Ortiz et al., 2016). In particular, due to its high stability, Air pulsed FBBs have been used in the removal of several PhACs with promising results, even in real HWW and in different operational modes (Cruz-Morató et al., 2013a; Ferrando-Climent et al., 2015; Gros et al., 2014).

In this chapter the feasibility of treating several PhACs in FBB with *T. versicolor*, but also with other fungal species was assessed. In particular, three different studies were performed, each one working with a specific individual fungal species (*Trametes versicolor*, *Pleurotus ostreatus* and *Stropharia rugosoannulata*) and targetting the degradation of one or more PhACs (an antibiotic cocktail in the case of *T. versicolor*, a mix

of atenolol (ATL), diclofenac (DCF) and ketoprofen (KTP) for *P. ostreatus* and carbamazepine (CBZ), iopromide (IPD) and DCF for *S. rugosoannulata*).

In the first case, *T. versicolor* was used for the removal of an antibiotic cocktail that contained up to eleven different compounds, belonging to seven antibiotic families (sulfonamides, penicillins, fluoroquinolones, quinolones, tetracyclines, metroimidazoles and dihdrofolate reductase inhibitors).

In spite of being overlooked for many years, antibiotics are rising their presence and concentration as environmental micropollutants in surface and underground water bodies. The estimated annual consumption in the beginning of 2000s decade was among one hundred and two hundred thousand tons around the world (Wise, 2002). This high consumption coupled to its partial or low removal in WWTP that act as a source point or "hot spot" for the rest of natural water bodies, are the main causes of this accumulative effect (Gros et al., 2010; Kümmerer, 2009a; Ortiz de García et al., 2013). This effect may contribute to the worldwide's emerging appearance of antibotic resistances in microbiota, threatening future antibiotic effectiveness, which can be considered one of the biggest challenges that will face human and animal health in a nearby future (Kümmerer, 2009b). There are still big differences in removal efficiencies in WWTPs depending on the structure and the group of antibiotics (Verlicchi et al., 2012), and several removal technologies have been already applied to tackle this situation.(Abejón et al., 2015; Kovalova et al., 2013)

In this context, AOPs and membrane filtration have been evaluated and are some of the most implemented treatments nowadays (Méndez et al., 2017), but due to their high demands in energy, capital costs and maintenance complexity, biodegradation and enzymatic oxidation processes are gaining popularity. These two processes, when optimized, are able to target specific pollutants and transform them into less dangerous products or even mineralize them (García-Galán et al., 2011; Torres et al., 2003).

Enzyme oxidation processes can be more selective than other biological processes and can be easily scalable in efluents containing large amounts of micropollutants. On the contrary, complete transformation is difficult to achieve and maintain stability in different

pHs and temperatures involves enzymatic immobilization (De Cazes et al., 2014). Membrane immobilization has proven to be one of the most effective strategies, although enzymatic life-time remains as one of its main drawbacks (Abejón et al., 2015). Enzymes such laccases have been succesfully applied in these conditions for the removal of antibiotics at room temperature and active in a wide range of pH (Abejón et al., 2015; de Cazes et al., 2015).

Alternatively, the use of WRF (mainly *T. versicolor*) in the removal of antibiotics has also been reported (Gros et al., 2014; Prieto et al., 2011; Carlos E Rodríguez-Rodríguez et al., 2012). The use of fungal active biomass in the treatment of antibiotics allows the combination of several transformation mechanisms such as intracellular enzymatic complexes and secreted extracellular enzymes (Harms et al., 2011), besides the potential removal by sorption mechanisms of the micropollutants into the biomass (Blánquez et al., 2004).

Thus, this first part of this chapter was aimed at the removal evaluation of eleven antibiotics cocktail by the white-rot fungi *Trametes versicolor* in FBB (from now on referred as TVR). This experimental set was evaluated at lab scale in experiments carried out first in osmosed water spiked with antibiotics, and then with raw HWW, always under non-sterile conditions.

In the second part of this chapter, the capability of the fungal species *P. ostreatus* to remove PhACs was assessed. Two main family drugs were analyzed: on one side, ATL, belonging to β-blockers family, and on the other DCF and KTP, both belonging to non-steroidal anti-inflammatory drugs (NSAIDs). β-blockers are extensively used for the treatment of hypertension, angina and arrhythmias (Liu et al., 2013) and have been frequently detected in effluents of WWTPs and even in surface waters, with concentrations ranging from ng/L to µg/L (Maurer et al., 2007). Among the β-blockers, Atenolol (ATL) is one of the most toxic for humans and aquatic organisms (Murugananthan et al., 2011). NSAIDs represent a remarkable group of pharmaceuticals, which exhibit anti-inflammatory, analgesic and antipyretic activities. In particular, diclofenac (DCF) and ketoprofen (KTP) are two of the most used non-prescription drugs,

and they both have been detected in surface waters in concentrations ranging from ng/L up to µg/L (Tixier et al., 2003).

Although quite promising results have been obtained in continuous treatment of real wastewater with *T. versicolor*, several strategies have been implemented in order to maintain the operation up to 2 months. Organic load measured as chemical oxigen demand (COD) is not reduced during continuous treatment of urban wastewater by *T. versicolor* (Cruz-Morato et al., 2013). Consequently, glucose or any other carbon source must be added for fungus maintenance of the primary metabolism. Another requirement to maintain the operation is the reduction of bacterial load in the influent by pretreatment of the HWW, preventing nutrient competition with bacteria, and therefore extending the treatment period (Mir-Tutusaus et al., 2016). Finally to maintain fungal activity under growth limiting conditions is necessary to renew the biomass to avoid biomass ageing (Mir-Tutusaus et al, 2017). Considering these multiple operational requirements, it is interesting to explore the use of other fungi to treat HWW. The capacity of *Pleurotus ostreatus* to degrade organic pollutants has been reported through fluorene (Akdogan & Pazarlioglu, 2011), textile dye (Akdogan & Canpolat, 2014), polyphenols in olive mill wastewater (Olivieri et al., 2006) or naphthalene sulfonic acid polymers of petrochemical wastewater (Palli et al., 2014, 2016). Nevertheless only the degradation of the psychiatric drug carbamazepine by *P. ostreatus* in solid state fermentation has been evidenced (Golan-Rozen et al., 2011, 2015). In addition, *P. ostreatus* treatment has been usually carried out in airlift bioreactor (Olivieri et al, 2006) or packed bed bioreactor (Akdogan & Pazarlioglu, 2011, Palli et al, 2016), but this is the first time reported of being performed in FBB. Hence, the capability of *Pleurotus ostreatus* to biodegrade selected pharmaceuticals (ATL, DCF and KTP) in real wastewater was evaluated. In this study *P. ostreatus* was used in pellet morphology as other fungi used in FBB, due to its easier retention and settling velocity during continuous operation. This morphology, compared to suspended one, increases the settling velocity of the biomass and improves its retention inside the bioreactor (Espinosa-Ortiz et al., 2016). Biodegradation experiments were conducted in air-pulsed FBB, operating in batch and in continuous mode, treating hospital wastewater spiked with selected pharmaceuticals. Further experiments were carried out in flasks, with the purpose of identifying the enzymatic system involved in the

degradation process and the detection of the fungal degradation intermediates by nuclear magnetic resonance (NMR).

Finally, in the last part of this chapter, the application of a biodegradation treatment with *Stropharia rugosoannulata* in a FBB for the removal of CBZ, DCF and IPD in HWW, under sterile and non-sterile conditions in batch configuration, was also evaluated. *Stropharia rugosoannulata* was one of the promising species according to the results obtained in the screening of six PhACs against different fungi (chapter 5).

Several removal experiments with these PhACs have been carried out by usign different species of ligninolytic fungi. For instance, in the case of the anticonvulsant drug CBZ, a PhAC commonly used as a model for recalcitrant compounds, degradation experiments with *P. ostreatus* (Golan-Rozen et al., 2011) and *T. versicolor* (Jelić et al., 2012) obtained promising results. In particular, this last experiment was the closest attempt to a real application treatment for CBZ, being performed in a FBB and achieving a removal of 96% from the initial 9 mg/L after 4 days. Moreover a continuous reactor was operated for 25 days, managing to eliminate 54% of the inflow concentration. However, this treatment was performed under sterile conditions in a spiked defined medium, and the application of this kind of treatments, with real HWW and under non-sterile conditions, remained unexplored.

The removal of the contrast media agent IPD has also been previously explored in FBB with ligninolytic fungus *T. versicolor* (Badia-Fabregat et al., 2016; Gros et al., 2014), and despite the use of real HWW and their performance under non-sterile conditions in both cases, removals did not exceed 65%. Thus, considering the promising performance of the fungus *S. rugosoannulata* with more than 80% of IPD removal (chapter 5), the treatment of spiked real HWW with *S. rugosoannulata* in FBB under non-sterile conditions was assessed.

Preliminary assays of *S. rugosoannulata* treatment with the anti-inflammatory drug DCF also showed high removal rates for this PhAC (almost 80%), therefore, the removal of this PhAC was also implemented by FBB with this fungus in real wastewater and under non-sterile conditions. Previous studies with real non-sterile conditions with *T. versicolor*

adding carbon and nitrogen sources, showed complete removals of this PhAC (Cruz-Morató et al., 2013a), and also in a FBB in non-sterile, wastewater-native microorganisms, Mir-Tutusaus et al. (2017) obtained complete removal of this PhAC. In this chapter, two FBBs batches were operated with *S. rugosoannulata,* studying their glucose consumption, COD and pH variation, and PhACs removal under non-sterile conditions.

Hence, the general aim of this chapter is to provide different examples that demonstrate the feasibility of the fungal biodegradation in PhACs removal, and to highlight promising combinations among bioreactor operational modes, PhACs, and fungal species for a future real scale implementation.

7.2. Materials and methods

7.2.1. Microorganisms and chemical reagents

Microorganisms and their culturing methods used in this chapter can be consulted in general methodology.

Pure standards of the following target antibiotics were purchased in high purity grade (>90%) from Sigma-Aldrich (Barcelona, Catalonia): Sulfamethoxazole, Amoxicillin, Penicillin V, Cloxacilin, Ofloxacin, Ciprofloxacin (CPX), Norfloxacine, Pipemidic acid, Tetracycline, Metronidazole and Trimethoprim. Individual stock standard, isotopically labeled internal standard and surrogate standard solutions were prepared at a concentration of 1000 mg/L, by dissolving 10 mg of solid reference standard in 10 mL of an appropriate solvent. After preparation, standards were stored at -20 ºC.

ATL, CBZ, DCF, IPD and KTP were of high purity grade and obtained from Sigma-Aldrich (Barcelona, Catalonia). Purified laccase was obtained from Fluka (Barcelona, Catalonia). The chemicals acetonitrile supergradient HPLC grade, acetic acid (96%, v/v) reagent grade (Scharlau-Chemie) and CD_3OD (99.80% D) (CortecNet, France) were used in the analyses for NMR identification of metabolites. All other chemicals used were of the highest available purity and were purchased from Sigma-Aldrich.

Standard solutions, containing all antibiotics were prepared in methanol/water (50:50, v/v) and were renewed before each analytical run by mixing appropriate amounts of intermediate standard solutions.

All the solvents were of high purity. HPLC grade methanol, acetonitrile and water were supplied by Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) solution was from Panreac and formic acid 98% was from Merck (Darmstadt, Germany). For high-performance-liquid chromatography-high resolution mass spectrometry HPLC grade methanol and water (Lichrosolv) were supplied by Thermo Fisher Scientific. Solid phase extraction cartridges for off-line sample treatment Oasis HLB (60 mg 3 mL) were from Waters Corporation (Miltford, MA, U.S.A).

Removal of antibiotic cocktail by *T. versicolor* (and laccase)

Two glass FBBs of 1500 mL each were used to carry out the removal of a spiked antibiotic cocktail, operating in batch mode during 7 days. In the first set of experiments two reactors (A and B) were ran in parallel. Reactor A was inoculated with pellets of *T. versicolor* at approximately 3 g of DCW/L, whereas reactor B, was uninoculated and used as a control with the same conditions, containing only native microbiota.

Reactors were operated at a controlled constant temperature of 25°C and autoclaved before use, but the influents used (either osmosed water or HWW) were fresh and not sterilized.

In order to maintain the biomass, in both experiments glucose and ammonium tartrate were fed continuously from stock solutions (at concentrations of 100 g/L and 2.25 g/L, respectively) at an approximate rate of 440 mg/g cell dry weight (CDW) per day and 20 mg·/g CDW·day, respectively, equivalent to the measured consumption rate of the fungus. Up to 1 mL of Antifoam Tween 80 at 1.2 µM, provided by Sigma-Aldrich (Barcelona, Catalonia), was added during the experiment.

Removal of ATL, DCF and KTP with *P.ostreatus*

A glass FBB with a working volume of 1500 mL, was used to carry out the HWW treatment in batch and continuous mode. Bioreactor was autoclaved at 121ºC for 30 min before every biodegradation experiment. The first experiment was conducted in discontinuous (fed-batch) mode during 7 days, and inoculated with 158 g of *P. ostreatus* pellets (wet weight), equivalent to a biomass concentration of approximately 3.5 g/L (dry weight). A second experiment was conducted in continuous mode for 32 days, and it was inoculated with 205 g of *P. ostreatus* pellets (wet weight), approximately equivalent to 3.8 g/L (dry weight). In both experiments glucose and ammonium tartrate were fed continuously from stock solutions (at concentrations of 100 g/L and 1 g/L, respectively) with a peristaltic pump at an approximate rate of 100 mg/g CDW·day and 10 mg/g CDW·day, respectively, equivalent to the measured consumption rate of the fungus. In continuous treatment, the bioreactor was fed with sterilized wastewater spiked with 10 mg/L of each drug, and the influent flow rate was adjusted to provide a hydraulic retention time (HRT) of 1.63 d or 3.00 d, depending on the stage of the experiment. The biomass, in pellet form, was retained in the bioreactor throughout the experiment with no loss in the effluent.

Removal of CBZ, DCF and IPD with *S. rugosoannulata*

Two 500 mL glass FBBs were inoculated with *S. rugosoannulata.* The first FBB was operated under sterile conditions for 7 days, whereas the second one was kept under sterile conditions during 10 days. Fluidization was achieved by a cyclic timer connected to an electrovalve that was opened during 1 second and closed for the following 3, for a total air flow of approximately 12 NL/h. Around 20 µL of Tween 80 at 0.012 µM were added to the reactors prior to the beginning of the experiment to prevent foam generation.

In the case of reactor 2, NH_4Cl was fed into the reactor at an approximated consumption rate of 40 mg/g CDW·day.

7.2.2. Hospital wastewater (HWW)

Hospital wastewater for TVR and POR experiments was collected from the main sewer manifold of Dr. Josep Trueta University Hospital (Girona, Catalonia). For SRR experiment, HWW was collected from the main sewer manifold of Sant Joan de Déu Hospital (Barcelona, Catalonia).

7.2.3. Additional biodegradation experiments

In order to identify the enzymatic system involved in the degradation process of selected pharmaceuticals, some batch experiments were carried out in 250-mL flasks containing about 200 mg of pellets (dry weight) and 50 mL of the defined medium whose pH was adjusted to 4.5. ATL, DCF and KTP were added to the medium from a stock solution in ethanol (1000 mg/L) to give final concentrations of 10 mg/L each. The flasks were maintained in the dark, under orbital shaking (130 rpm) and at 25°C. Flasks inoculated with autoclaved pellets of *P. ostreatus* were used as heat-killed controls to measure the sorption of the drugs on the biomass. During experiments, 1 mL samples were withdrawn at different times, filtered through Millipore 0.45-µm nylon filters and subsequently analyzed by HPLC. In order to study the role of the CYP450 system in the degradation of the pharmaceutical compounds, further experiments were conducted under the same conditions as the previous, but with the addition of 1-aminobenzotriazole (1-ABT), a well-known CYP450 inhibitor, at a final concentration of 5 mM. Every experiment was performed in triplicate.

7.2.4. Analytical procedures

To analyze the antibiotic cocktail compounds, samples were analyzed following the protocol previously described by Gros et al (2013). Briefly, water samples were successively filtrated through 2.7 mm (Glass fiber), 1.0 mm (Glass fiber) and 0.45 mm (PVDF) pore-size membranes (Millipore; Billerica, MA, USA). After pH adjustment to 3 with the addition of HCl (0.1M) and EDTA (3 %), a volume of 50 mL of sample was pre-concentrated into Solid Phase Extraction (SPE)-HLB cartridges (60 mg, 3 mL) (Waters Corp.; Mildford,MA, USA). After the evaporation under nitrogen stream samples were

reconstituted with 1 mL of methanol–water (50:50 v/v). Then, 10 µL of internal standard mix at 1 ng/L were added in the extracts for internal standard calibration. Chromatographic separation was carried out with a Ultra-Performance liquid chromatography system (Waters Corp. Mildford, MA, USA), using an Acquity HSS T3 column (50 mm × 2.1 mm i.d. 1.7 m particle size) for the compounds analyzed under positive electrospray ionization (PI) from Waters Corporation. The UPLC instrument was coupled to a 5500 QTRAP hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, Foster City) with a turbo Ion Spray source. The solvents applied were Acetonitrile (A) and HPLC grade water (B) acidified at 0,1% with formic acid according to Gros et al (2013). The sample volume injected was 5 µL.

In the analysis of ATL, DCF and KTP, PhACs were analyzed by HPLC following the same conditions reported in general methodology. For the quantification of DCF and KTP, the mobile phase consisted of 6.9 mmol/L of acetic acid adjusted to pH 4 (by NaOH) with 35% v/v acetonitrile. It was delivered isocratically at 1 mL/min. On the contrary, for the quantification of ATL, the mobile phase A of 0.01 M ammonium acetate (pH 7) and the mobile phase B acetonitrile were delivered at a flow rate of 1.2 mL/min in a gradient elution (t =0 min A=95%, t=20 min A=80%). The detection limit of ATL, DCF and KTP was 0.1 mg/L.

Analysis of metabolites by NMR

To detect the major metabolites of the degradation of DCF and KTP by *P. ostreatus*, 80 mL of defined medium was added to 500-mL Erlenmeyer flasks and subsequently the flasks were inoculated with 20 g of wet pellets of *P. ostreatus* (equivalent to 1 g of dry weight). Each flask contained only one PhAC at a final concentration of 10 mg/L. Flasks were incubated in darkness under shaking conditions (130 rpm) at 25ºC. At each time point (0, 1, 3, 6, 24 h and 7 d), one flask was sacrificed, with its contents being filtered through a 0.45-µm glass fiber filter (Whatman) and cooled immediately. Target compounds in the liquid phase were extracted in one step by solid-phase extraction with Oasis HLB cartridges (60 mg adsorbent, Waters), as described in general methodology. After the

extraction, samples were reconstituted in 0.6 mL of CD_3OD and transferred to 5-mm-diameter NMR tubes. The analyzed samples consisted of aliquots of the degradation experiments of KTP and DCF at each time point. A Bruker Avance II 600 nuclear magnetic resonance spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 5 mm TBI probe with Z-gradients, operating at a 1H NMR frequency of 600.13 MHz and at 298.0ºK of temperature, was used for the NMR experiments. 1D 1H NMR spectra were acquired using a standard 90° pulse sequence, with an acquisition time of 1.71 s and a relaxation delay of 2 s. The data were collected into 32 K computer data points, with a spectral width of 9590 Hz and as the sum of 1024 transients. The resulting free inductions decays (FIDs) were Fourier transformed, manually phased, and baseline corrected. All the spectra were calibrated using the residual solvent signal (CHD_2OD, 3.31 ppm).

The analyses of CBZ, DCF and IPD concentration were done with the same conditions reported for *P. ostreatus* reactor except for the mobile phase, that consisted on formic acid 0.02% (v/v) as eluent A, and acetonitrile as eluent B. The gradient program was also specific and started with a flow of 0.6 mL/min of 100% A, following a linear gradient to 1.2 mL/min and 45% A at 20 min. After 5 minutes, conditions were set back to initial point of 0.6 mL/min and 100% A in 1 min and kept during 4 min for reequilibration. The range of measurable concentrations was 0-10 mg/L, with a limit of detection of 0.1 mg/L.

7.3. Results and discussion

7.3.1. Removal of antibiotics with *T. versicolor*

Results for *T. versicolor* reactor during the first batch with osmosed water achieved high removals of antibiotics. In particular, dihdrofolate reductase (DHFR) inhibitors (100%), tetracyclines (99.7%), sulfonamides (98%), quinolones (98%) and penicilins (96%), were almost completely removed after 168 h of treatment (Table 7.1), while metroimidazole antibiotics (91%) and fluoroquinolones (81%), showed lower efficiencies. The overall removal efficiency obtained by the TVR was of approx. 93% after 168h of treatment (Figure 7.1), where 56% were removed in the first 24h and 78% after 72h of treatment. Considering individual removals, the highest efficiencies were observed for trimethoprim (almost 100% removal) after 168 h, while CPX (71%) showed the lowest removals.

In the European Institute of Membranes at the research group of Matthias De Cazes, a parallel experiment with an enzymatic membrane reactor (EMR) was set to compare reactor's performances for the same infuents and equivalent conditions (EMR operation time was of 24 h instead of 168 h).

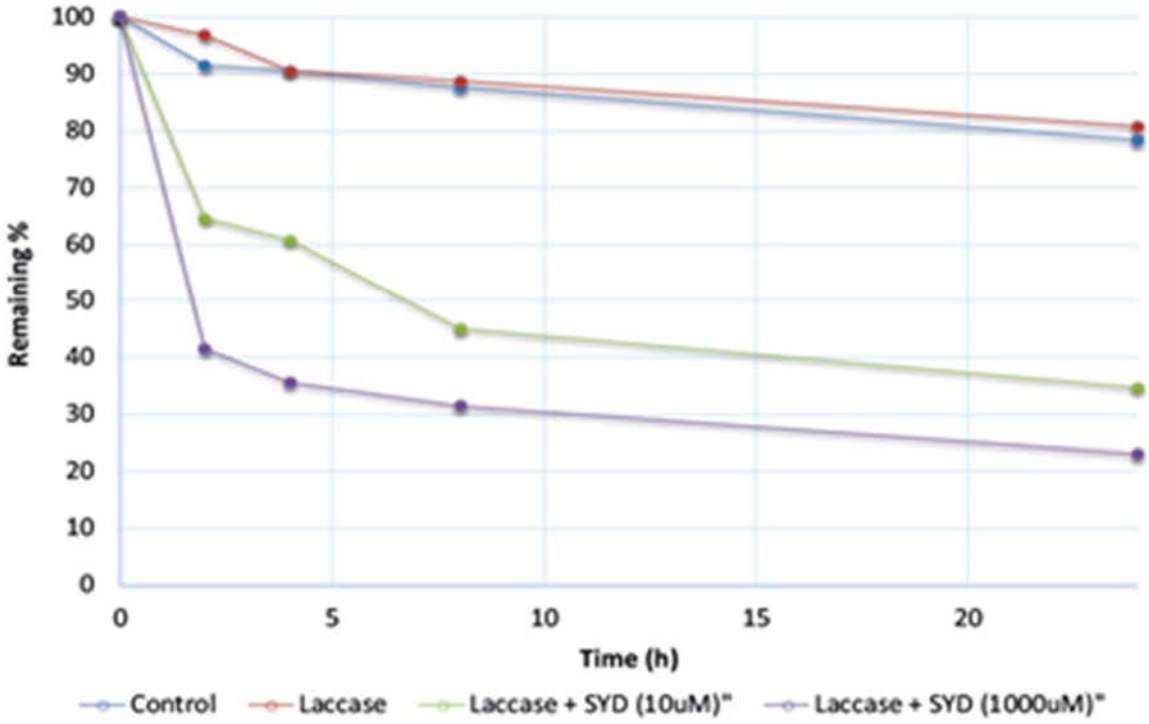
Results from De Cazes (Becker et al., 2016) (Table 7A.1 in the annex) also presented high removals for particular antibiotics such as sulfamethoxazole (97%), however removals for certain antibiotics were considerably much low (metronidazole was only removed by 9%). Results of the TVR (Table 7.1) showed a general higher removal efficiency for most of the antibiotics. EMR had a lower overall removal (73%) at the end of the experiment (24 h), and only higher removals than TVR were obtained for amoxicilin (95%) and CPX (93%) specifically. In spite of this, EMR removal was considerably faster than TVR, achieving more than 50% removal after 2 h, whereas in 24 h the overall removal difference was almost 20% more in EMR.

Table 7.1. Antibiotic removal percentages achieved by *T. versicolor* reactor in the osmosed water matrix.

Antibiotic		Time (h)			
		0	24	72	168
Sulfonamides	Sulfamethoxazole	0.00	72.93	91.99	97.57
	Amoxicillin	0.00	62.90	79.46	91.08
Penicillins	Penicillin V	0.00	71.07	94.57	99.12
	Cloxacilin	0.00	99.36	98.83	98.80
	Ofloxacin	0.00	75.50	95.17	91.49
Fluoroquinolones	Ciprofloxacin	0.00	33.68	85.87	70.95
	Norfloxacin	0.00	17.67	50.37	82.13
Quinolones	Pipemidic acid	0.00	46.26	59.74	98.28
Tetracyclines	Tetracycline	0.00	83.45	93.68	99.67
Metroimidazole	Metronidazole	0.00	-22.88	17.10	91.38
DHFR inhibitor	Trimethoprim	0.00	76.74	90.30	100.00

Figure 7.1 –Percentage of antibiotic concentration remaining accross time in osmosed water for (a) EMR and (b) TVR.

(a)



(b)

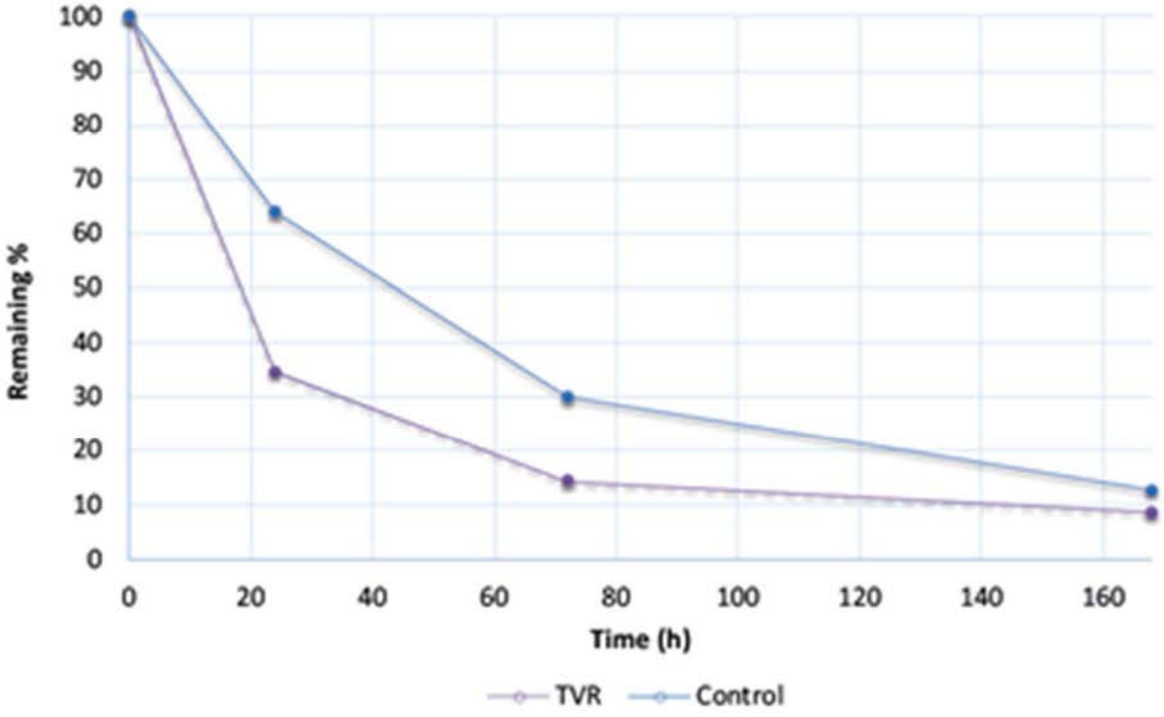
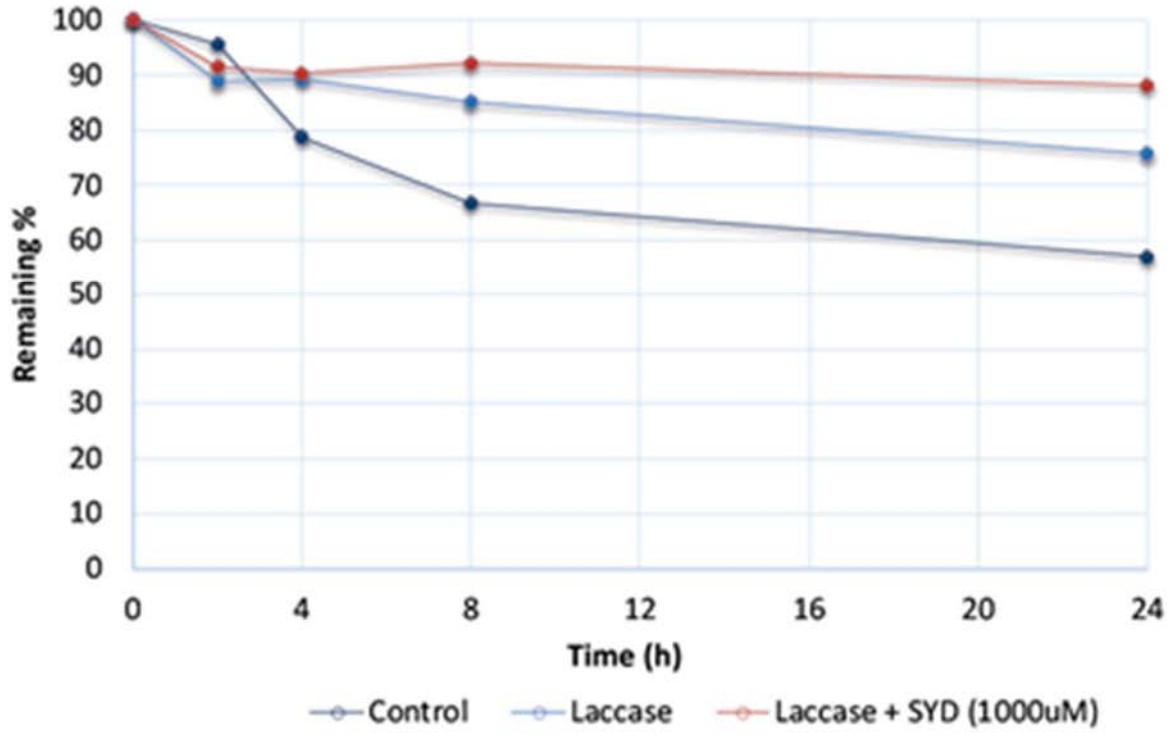
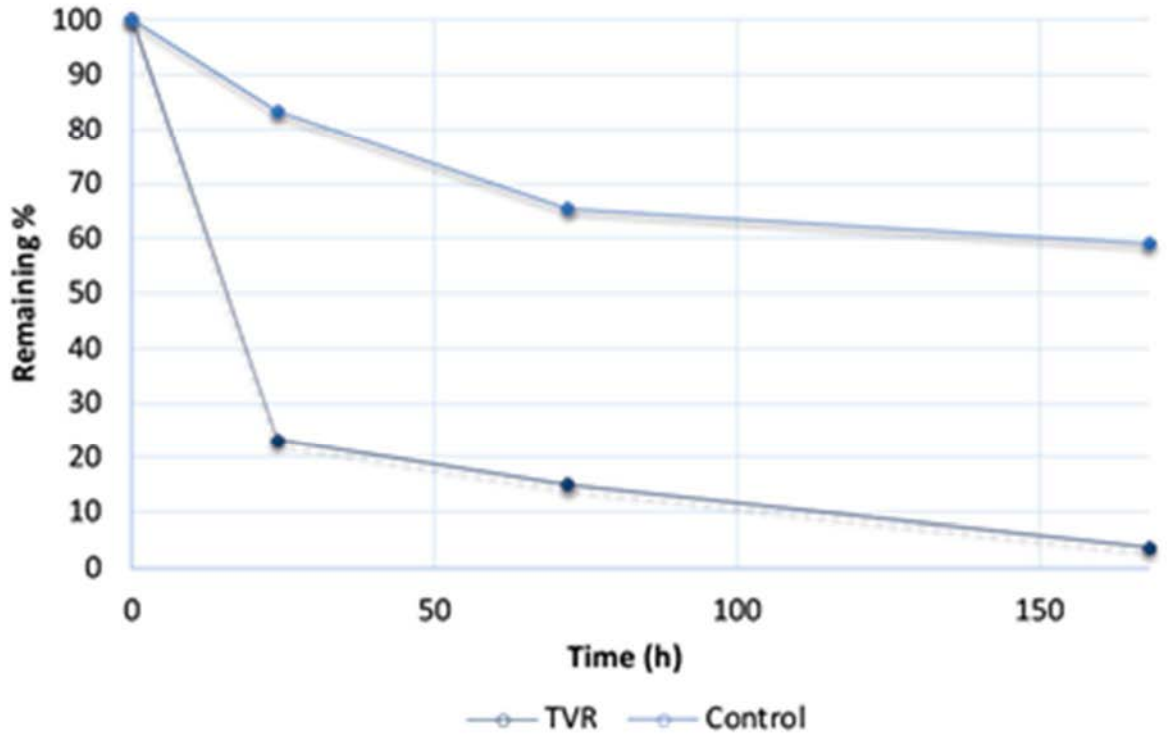


Figure 7.1 (continuation) –Percentage of concentration remaining accross time in HWW for EMR (c) and TVR (d).

(c)



(d)



These results show accordance with previous studies that obtained high antibiotic removals for *T. versicolor* in liquid media (Gros et al., 2014; Carlos E Rodríguez-Rodríguez et al., 2012). In particular, Prieto et al. (2011) achieved removals higher than 90% for CPX

and norfloxacin, confirming that under optimal conditions, *T. versicolor* can almost completely remove these antibiotics as well.

Despite of the high efficiency obtained by the TVR at 168h, the comparison of the removals obtained with the ones observed in the uninoculated reactor B (Table 7A.2 in the Annex) do not show big differences (final removal for TVR was 93% vs. 87% in reactor B at 168h). A closer analysis to these results shows that the degradation of the antibiotics by the TVR was faster specially in the first 24h of treatment (56%) compared to the ones observed for the reactor B in the same time steps (37%). After these first 24-72 hours, the removals in both reactors A and B became very similar, which could be explained by the proliferation of microorganisms in reactor B that were able to remove antibiotics (either by sorption or biodegradation). According to the monitored laccase activity and glucose consumption (Figures 7.2 and 7.3), no laccase activity was detected in reactor B during the experiment with osmosed water and low values were detected in TVR, aiming to antibiotic removal mechanisms not driven by laccase activities. Besides, despite of existing, glucose consumption showed minimal levels in reactor B. Thus, glucose was accumulated during the first days and kept rising (although at a minor rate) until the end of the experiment, paradoxically indicating a low concentration of microorganisms.

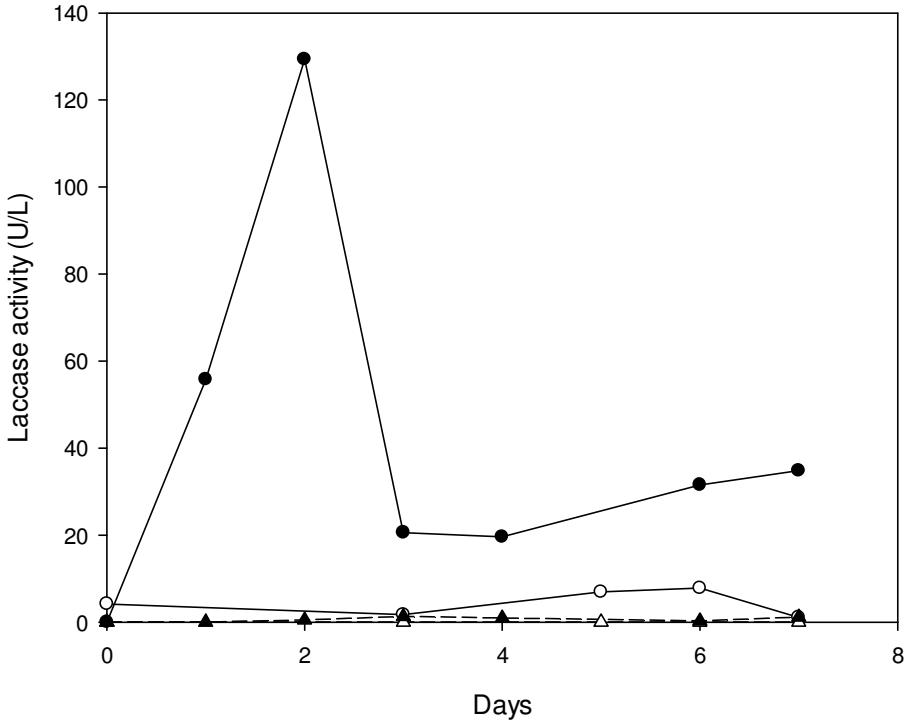


Figure 7.2 Laccase activity in reactor A (straight lines and circles) and reactor B (dashed

lines and triangles). Filled dots correspond to HWW whereas osmosed water is represented by empty dots.

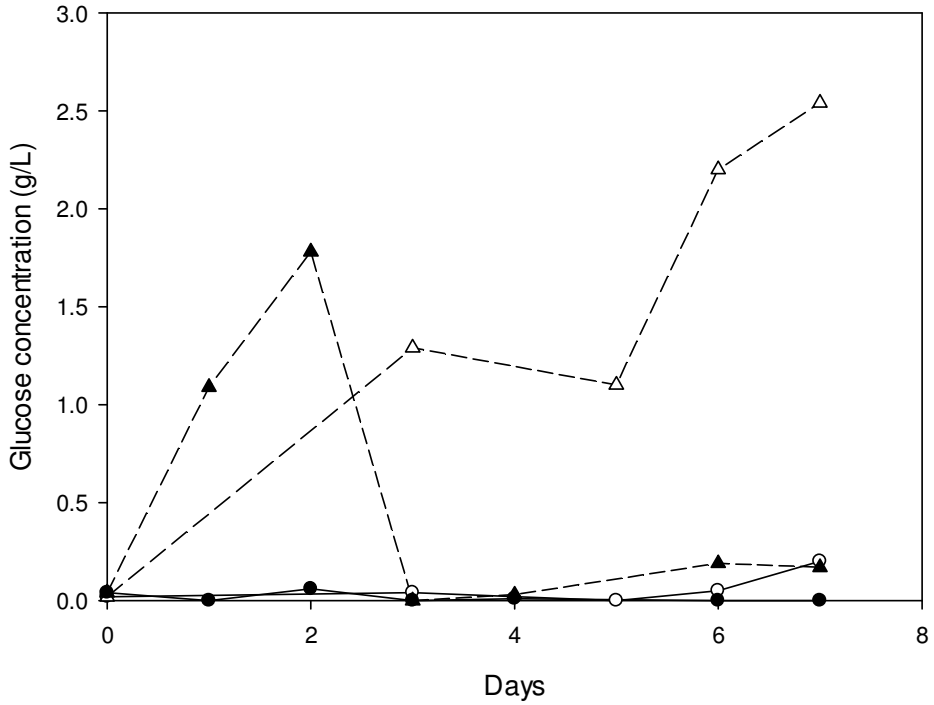


Figure 7.3 Glucose concentration for reactor A (straight lines and filled circles in HWW, empty circles in osmosed water) and reactor B (dashed lines and empty triangles in osmosed water whereas filled traingles in HWW).

TVR with HWW

When exposed to HWW, TVR removed 85% of the antibiotics after 168 h of treatment (Figure 7.1). However, in this case, removal in reactor B was considerably lower, with only 37% from the total inital antibiotic content. This difference could be attributable to the different composition of the HWW when compared to osmosed water, in terms of chemical composition, since apart from antibiotics, HWW can contain bacterial inhibitors, cytostatics and other PhACs (Gros et al., 2013; Santos et al., 2013; Verlicchi et al., 2010), but also concerning microbiota, because microorganisms present in osmosed water may not be detected in HWW. Sulfamethoxazole was the only sulfonamide detected in the HWW used before spiking (0.76 µg/L). In the case of reactor B, the removal efficiency for this particular antibiotic was around 34% at 24h and reached 36.0% at the end of the treatment, apparently reaching an equilibrium, which could suggest sorption mechanisms as the cause of this removal (Table 7A.2 in the Annex). On the contrary, this compound

was totally removed by the *Trametes versicolor* reactor as it was not detected after 24h (Table 7.2). Besides, laccase activity for TVR in HWW was considerably high reaching peaks of more than 120 U/L (Figure 7.2), probably induced by PhACs present in HWW (as previously reported in chapter 5), and thus, probably participating in antibiotic removal. On the other hand, reactor B did not show laccase activity, and unlike the osmosed water experiment, glucose was almost totally consumed from the third day until the end of the experiment (Figure 7.3)

Table 7.2. Antibiotic removal percentages achieved by *T. versicolor* reactor in HWW.

Antibiotic		Time (h)			
		0	24	72	168
Sulfonamides	Sulfamethoxazole	0.0	100.0	100.0	100.0
	Amoxicillin	0.0	31.8	58.1	70.4
Penicillins	Penicillin V	0.0	100.0	100.0	100.0
	Cloxacilin	0.0	91.0	74.9	31.9
	Ofloxacin	0.0	99.8	99.4	99.2
Fluoroquinolones	Ciprofloxacin	0.0	100.0	99.3	98.8
	Norfloxacin	0.0	100.0	100.0	100.0
Quinolones	Pipemidic acid	0.0	30.8	81.1	39.7
Tetracyclines	Tetracycline	0.0	100.0	100.0	100
Metroimidazole	Metronidazole	0.0	100.0	100.0	100.0
DHFR inhibitor	Trimethoprim	0.0	100.0	100.0	100.0

Regarding the particular elimination of antibiotics, removals of 100% at the end of the experiment were obtained by sulfamethoxazole, penicillin V, norfloxacine, tetracycline, metronidazole and trimethoprim. It is specially relevant that for all of these antibiotics the removal took place in less than 24 hours. Besides, ofloxacin and CPX also obtained almost complete eliminated, with removals taking place as well during the first 24 hours.

Few antibiotics were not completely removed such as amoxicilin, cloxacilin and pipemidic acid, although during the treatment their concentrations showed dramatic shifts. These relevant changes in the antibiotic concentration could be explained by conjugation and deconjugation phenomena occurring by fungal (and microbial) metabolism. In particular CPX, sulfamethoxazole and tetracycline have been reported to present retransformable excreted forms that could be present in HWW (Polesel et al., 2016).

Regarding the comparison with EMR (Table 7A.1 in the annex), eliminations of antibiotics were clearly lower than TVR. The overall removals of antibiotics was around 21%, being in particular amoxicillin and pipemidic acid the most eliminated antibiotics (90% and 70% respecitively). However, for the rest of antibiotics, removals below 36% were detected. These low elimination rates, some of them even negative, can be explained similarly to the case of TVR, by retransformation processes such as deconjugation from antibiotics already present in HWW initially, but could not be detected due to conjugation. Deconjugation is one of the possible retransformation mechanisms that can turn antibiotics into recalcitrant PhACs. Retransformation potentials have been described for several species of sulfonamides, CPX and sulfamethoxazole (Polesel et al., 2016) and according to some authors they could represent formation potentials of up to 22% from the initial parent compound (Testa et al., 2012). This low elimination capacity of EMR with HWW compared to the experiment with osmosed water, can also suggest the possible presence of laccase inhibitors that could prevent the antibiotic oxidation (Johannes and Majcherczyk, 2000).

7.3.2. Removal of ATL, DCF and KTP with *P. ostreatus*

To assess DCF, KTP and ATL degradation by pellets of *P. ostreatus*, the air-pulsed FBB was operated in batch mode for 7 days.

In this experiment DCF was completely removed in 18 hours while removal of KTP and ATL was only 36% and 8%, respectively, after almost 42 hours (Fig. 7.4). Right after, at day 2, a new pulse of PhACs was added to the bioreactor in order to increase the concentration of each of the PhACs up to approximately 10 mg/L, with similar results obtained. ATL removal was negligible during the 2 first days after the second spike, but it was increased up to 25% by the end of the experiment. In the same way, 39% of the KTP was removed after 2 days, but the final removal achieved 50% of 10 mg/L at the end. On the contrary, DCF was again completely removed after 1 day.

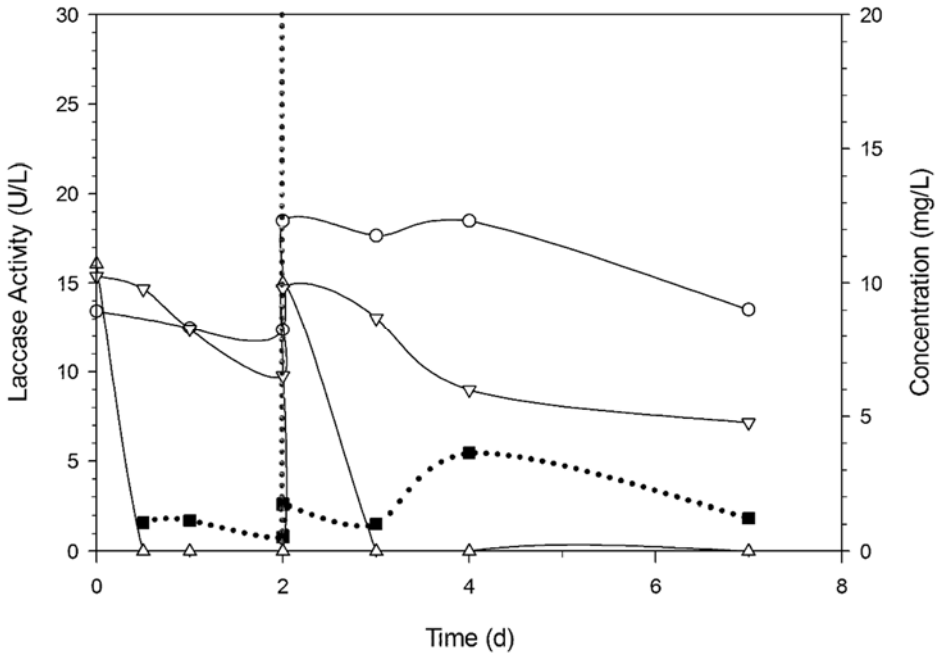


Figure 7.4. Time course of PhACs concentration and laccase activity during the batch degradation by pellets of *P. ostreatus* in a FBB. Vertical line indicates a new PhACs pulse added. Symbols: ATN (○), DCF (△), KTP (▽), and laccase activity (■).

Lac assays gave very low extracellular enzymatic activity (<2 U/L), although a slight improvement (5 U/L) after the second addition of pharmaceuticals was detected. Glucose concentration was always below 0.02 g/L.

Carbon and nitrogen were continuously fed at growth limiting conditions, but a pump malfunction probably caused a nutrient supply below the maintenance requirement and consequently after 7 days the biomass concentration had been reduced by 40%.

Another FBB was set to operate first in fed-batch process, but after 7 days, it was shift into continuous mode, feeding spiked hospital wastewater and operating with a HRT of 1.63 days.

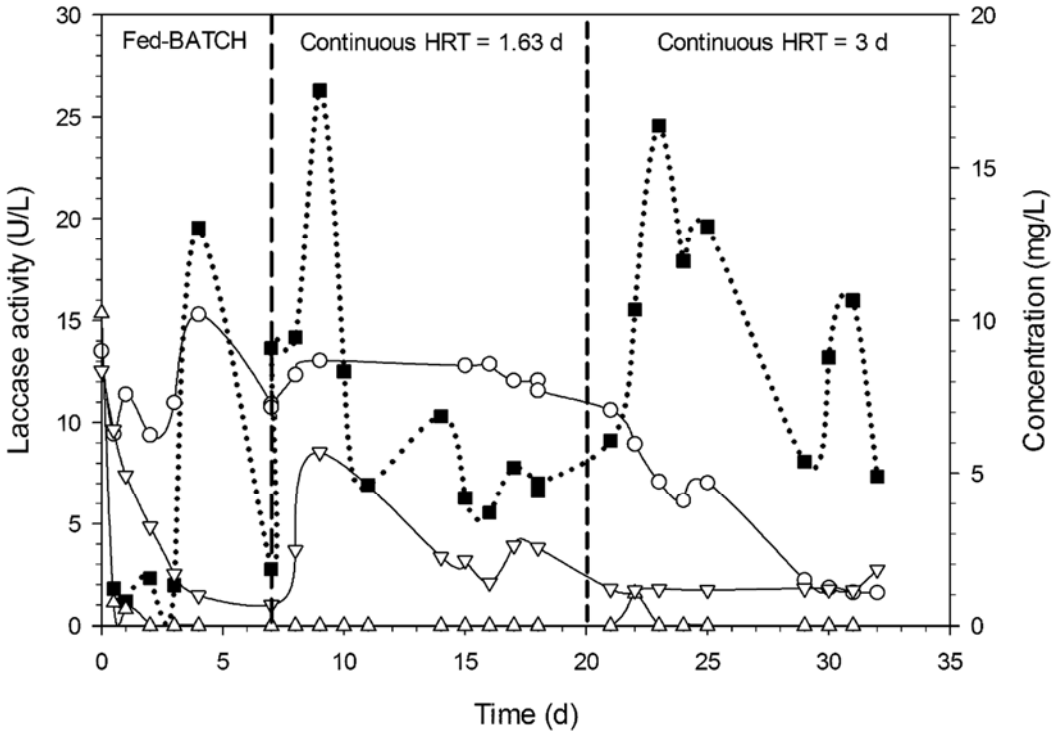


Figure 7.5 Time course of PhACs concentration in a continuous treatment of a hospital wastewater by pellets of *P. ostreatus* in a FBB. Laccase activity is represetend in a dashed line. Symbols: ATL (○), DCF (△), KTP (▽), and laccase activity (■).

Figure 7.5 depicts the concentration profiles of the PhACs in this bioreactor and the extracellular laccase activity. During batch stage, after 2 days, DCF and KTP were removed by 100% and 60%, respectively. At the end of the batch stage, KTP was removed by 90% while the ATL concentration was barely affected (<20% of removal). In addition, a peak of laccase was detected after 5 days. During the continuous treatment, DCF was fully removed while KTP removal was about 70% after day 15, when the hydraulic steady state

was reached. Again, no ATL degradation was detected. Although Lac activity oscillated significantly, a minimum threshold of 7 U/L was achieved at steady state. In order to improve the degradation of KTP and ATL, the HRT was increased to 3 days at day 20. KTP removal was increased up to 85% and also ATL removal reached 80% at the new hydraulic steady state. During this period, Lac activity was maintained at high level; this increase in laccase activity can be explained by biomass growth, since, at day 32, the biomass concentration was 8.75 g dry weight/L, which meant an increase of 130% compared to the initial inoculum. With such a high level of fungal biomass, however, severe operating problems occurred, such as a lack of dispersion of pellets, blockage of the outlet ports and accumulation of biomass on both the ceramic air distribution plate and in the upper part of the bioreactor. These problems forced the end of the experiment.

Nevertheless, it is important to underline that this experiment constitutes the first time that pellets of *P. ostreatus* have been used to degrade these PhACs in a bioreactor during continuous operation. These results also show that the rest of PhACs from real HWW do not interfere with their degradation capacity.

Total and soluble COD samples were measured across different states of the reactor (Table 7.3). The main fraction of COD was soluble, and although initial COD had considerably high values for a HWW, changes in operational mode (from batch to continuous) but specially a higher HRT were apparently crucial for the decreasing of COD values at the end of the experiment. This supports that *P. ostreatus* may be reducing COD through metabolism. COD analysis of samples from the continuous mode also showed noticeable reductions in soluble COD of up to 53% (for HRT=1.63 d) and 85% (for HRT=3 d). (Total COD reductions were 47% and 86% respectively)

Table 7.3 Total COD and soluble COD sample comparison (± standard deviation).

Sample	Mean Total COD (mg/L O_2)	Mean Soluble COD (mg/L O_2)
Inlet (32 d)	>5000	4235 ± 236.1
Continuous (HRT=1.63 d)	2665 ± 615.19	2002.5 ± 321.73
Continuous (HRT=3 d)	700.67 ± 115.76	628.67 ± 143.5

These results are different than those reported by *T. versicolor* (Mir-Tutusaus et al., 2017, 2016) where COD was not removed and biomass concentration was maintained constant when glucose was added at uptake rate for fungus maintenance. The evidence of COD reduction by *P. ostreatus* opens the possibility to use a completely different strategy than the described by Mir-Tutusaus et al. (2016) to develop a PhACs degradation process from HWW. This means glucose (and potentially nitrogen) addition may not be necessary to maintain fungal activity, but purging may be needed instead to avoid biomass accumulation. Periodic biomass purge would reduce operational costs ensuring biomass maintenance, preventing both the need of fresh biomass and the addition of carbon or nitrogen sources. Finally, without glucose nor nitrogen addition, the pretreatment of the HWW in order to reduce bacterial load in the fungal influent could be also avoided, preventing the proliferation of nutrient competitors under non sterile conditions. As a result, this study evidences a good potential of *Pleurotus ostreatus* as PhACs degrader from HWW but further research is necessary to approach real conditions.

Enzymatic system involved in the degradation

Further experiments were conducted with the purpose of studying the role of extracellular laccase of *P. ostreatus* in the degradation process. Although its expression was low during batch degradation (Figure 7.4), and the laccase peak appeared after most pharmaceuticals biotransformation was obtained (Figures 7.4 and 7.5), laccase still may have played a role in drugs removal. For this reason, the effect of this enzyme on the pharmaceuticals was assessed by using commercial pure laccase. As it can be observed in Figure 7.6A, ATL was completely removed in 1 h. On the contrary, laccase apparently does not degrade KTP (Fig. 7.6C), even in presence of a chemical mediator. Since in vivo experiments led to a removal of this compound, probably the degradation pathway involves other enzymatic systems. On the other hand, in the case of DCF (Fig. 7.6B) full degradation required 4 h, but this time could be reduced to 15 min by adding a mediator, such as HOBT, to the reaction medium.

The involvement of one of the main enzymatic systems of *P. ostreatus,* the cytochrome P450 monooxygenase system (CYP450) in the degradation of the three selected pharmaceuticals was also investigated.

Time-course of the pharmaceuticals concentration in the experiments is shown in Figure 7.7. Results show that about 30% of DCF is removed by sorption on the biomass while for ATL and KTP this phenomenon is negligible. Marco Urrea et al (Marco-Urrea et al., 2010a, 2010b) reported that DCF and KTP were highly stable through uninoculated experiments performed in similar conditions. These authors also reported that sorptions of DCF and KTP on *Trametes versicolor* biomass were 47% and 15%, respectively, obtained from the heat-killed control, but these percentages were reduced to 10% and 0%, respectively, when sodium azide was used to inactivate biomass, by blocking active transport across membrane or vesicular pathways. As a result, we can state that measured removals are caused mainly by *Pleurotus* degradation. Figure 7.7 shows identical results in the experimental flasks and the inhibited flasks, so it can be concluded that CYP450 is not involved in the fungal degradation of ATL. However, it could be involved in the degradation of KTP and DCF. Figure 7.7B (DCF) shows the highest difference among the three cases; in just 1 day an almost complete degradation (> 90%) was observed in inhibitor-free flasks while only less removal than 25% was obtained in inhibited flasks. In spite of this, the effect was less notorious at the end. On the other hand, in the case of KTP (Figure 7.7C), both inhibited and inhibitor-free experiments showed no differences within the first 3 days, but after 6 days the inhibited experiment reached lower degradation percentages than the inhibitor-free culture. These results are similar to those obtained by Marco-Urrea et al. (Marco-Urrea et al., 2010b) with *T. versicolor*: they observed a delay in the DCF degradation in the inhibited flasks comparing to inhibitor-free flasks. This behavior indicates that CYP450 may play a role in degradation of KTP and DCF by *P. ostreatus*.

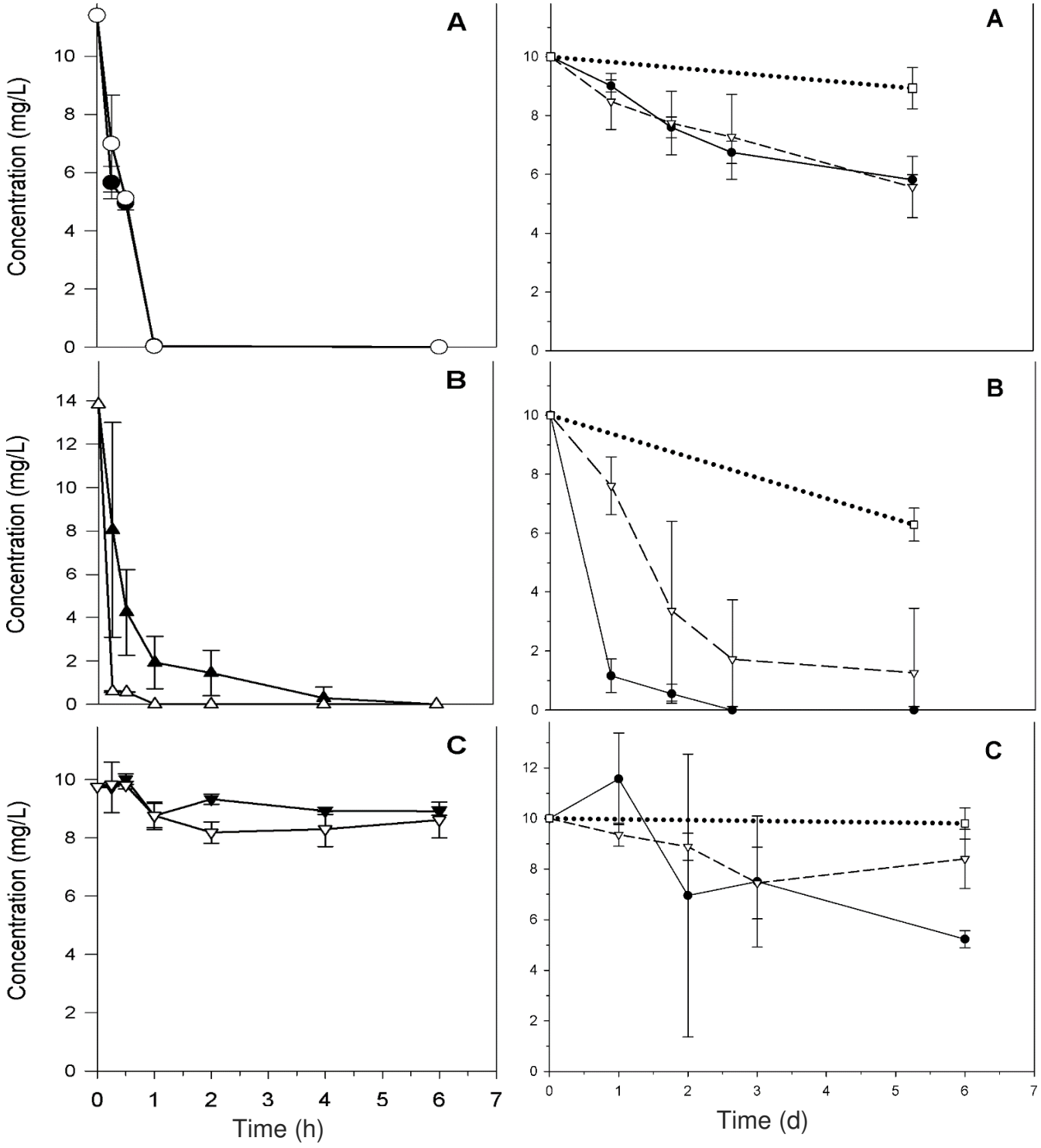


Figure 7.6 (left) PhACs concentration of in vitro treatment with purified laccase (500U/mL) without (ATL: ●; DCF: ▲**;** KTP: ▼) and with 1mM of HOBT (ATL: ○;DCF: △; KTP: ▽).

Figure 7.7 (right) Influence of the cytochrome P450 inhibitor 1-ABT (5 mM) on the degradation of the selected PhACs: ATL (A), DCF (B) and KTP (C). Symbols: heat-killed control (□), inhibitor-free controls (●), and cultures containing 1-ABT (△). Values plotted are means ± standard deviation for triplicate cultures.

Detection of metabolites

NMR spectroscopy allowed the detection and the identification of compounds originated from the degradation of KTP and DCF produced by *P. ostreatus* in the liquid medium. The kinetics of the degradation processes were monitored by 1H NMR experiments. Samples at time points 0, 1, 3, 6, 24 h and 7 days were prepared following the procedure described in section 2.7.2.4.2.1. Then, a 1H NMR spectrum of each sample were obtained and compared. Figures 7.8 and 7.9 show the aromatic region of the spectrum corresponding to the degradation products of KTP and DCF, respectively.

For both pharmaceuticals, from time point 0 to 6 h new resonances corresponding to degradation products appeared. After 24 h, the pharmaceuticals had been completely removed and their degradation compounds not detected at 7d.

Degradation compounds of KTP and DCF were identified by the comparison of the new resonances with the results described in previous works (Marco-Urrea et al., 2010a, 2010b), in which these drug metabolites were fully characterized by NMR spectroscopy. The compounds identified during the degradation of KTP by *P. ostreatus* were 2-[3-(4-hydroxybenzoyl)phenyl]propanoic acid (K1) and 2-[(3-hydroxy(phenyl)methyl)phenyl]-propanoic acid (K2), coming from the hydroxylation of an aromatic ring and from the reduction of the keto group, respectively (Figure 7.8). Similarly, the compounds identified during the degradation of DCF came from the hydroxylation of the drug in two different positions, 4'-hydroxydiclofenac (D1) and 5-hydroxydiclofenac (D2) (Figure 7.9). In both cases, the formation of other non-identified degradation molecules cannot be ruled out.

The detected metabolites for KTP and DCF are different from those identified after a photodegradation treatment where one stable product for each pharmaceutical compound was accumulated (Salgado et al., 2013). Hence, fungal degradation exhibits additional advantages, because further transformation of the pollutants is obtained.

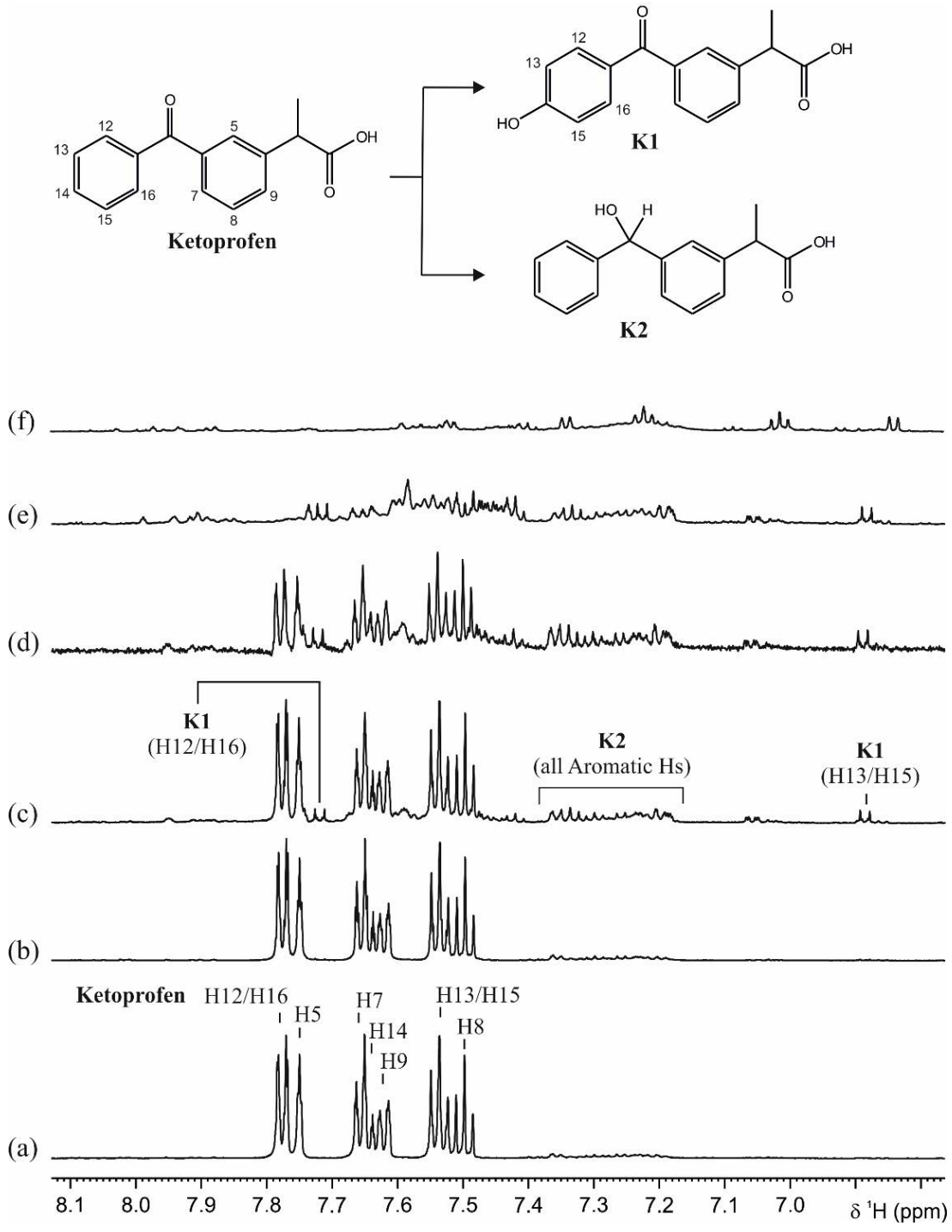


Figure 7.8 Structure of KTP and degradation compounds 2-[3-(4-hydroxybenzoyl)phenyl]propanoic acid (K1) and 2-[(3-hydroxy(phenyl)methyl)phenyl]-propanoic acid (K2) and the aromatic region of the 1H NMR spectra of samples at 0h (a), 1h (b), 3h (c), 6h (d), 24h (e) and 7 days (f) of experiment. Most characteristic peaks of each compound are indicated. Spectra were acquired at 298.0 K and at a magnetic field of 600 MHz

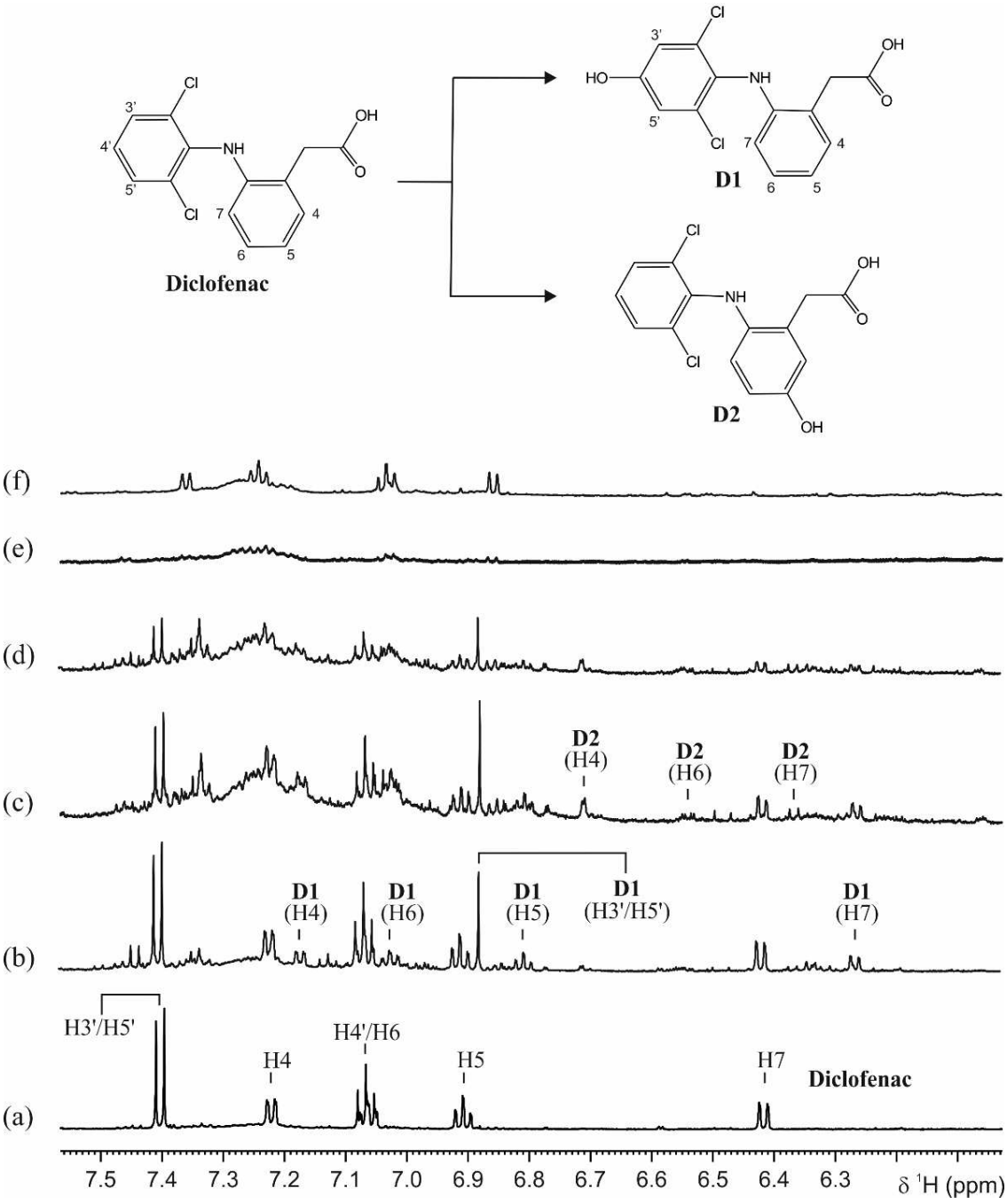


Figure 7.9 Structure of DCF and degradation compounds 4'-hydroxydiclofenac (D1) and 5-hydroxydiclofenac (D2) and the aromatic region of the 1H NMR spectra of samples at 0h (a), 1h (b), 3h (c), 6h (d), 24h (e) and 7 days (f) of experiment. Most characteristic peaks of each compound are indicated. Spectra were acquired at 298.0 K and at a magnetic field of 600 MHz.

7.3.3. Removal of DCF, CBZ and IPD with S. rugosoannulata

In preliminary experiments under sterile conditions, *S. rugosoannulata* was proven able to obtain a carbon source from HWW with high COD content (Annex Figure 7A.1), thus avoiding the need for glucose addition[33]. However, nitrogen content in HWW was not bioavailable for *S. rugosoannulata*, therefore, the feeding of NH_4Cl was required for an optimal fungal performance.

On the other hand, in order to approach real scale implementation, new attempts towards higher stability and lower operational costs were studied. Thus, in the first FBB where the removal of CBZ, DCF and IPD was studied, pH was not controlled, and instead of keeping stable (HWW pH>8), it progressively decreased to its optimal range of 4-5 driven by fungal metabolism (Figure 7.10). This effectively suggests the possibility of eliminating pH control at least during the first two/three days, until an excessive acidification could reduce or inactivate fungal performance. Despite of this promising step forward, in the beginning of the third day of the experiment, an excessive biomass growth caused a dramatic decrease in dissolved oxygen values in the HWW. To prevent fungal inactivation, the aeration flow was increased by around 5 NL/h. The consequent increased agitation and unstability in dissolved oxygen seemed to negatively affect fungal biomass, apparently unable to ricover its optimal pH. Enzymatical activity maximums were detected by day three (both for laccase and MnP), but this incremented activity apparently was not reflected in PhACs removal (Figure 7.11).

[33] Glucose consumption rate was calculated as approximately 0.94 g/g DCW·day

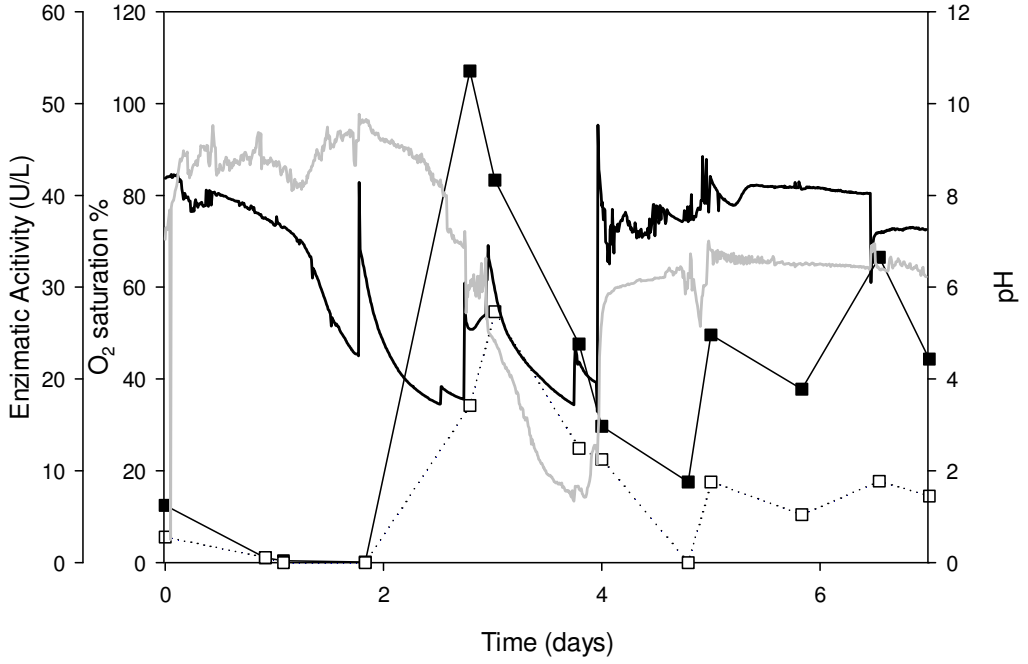


Figure 7.10 Time course of laccase (■) and manganese peroxidase (□) activity, pH (straight black line) and oxygen saturation (grey line) from *S. rugosoannulata* FBB reactor under sterile conditions.

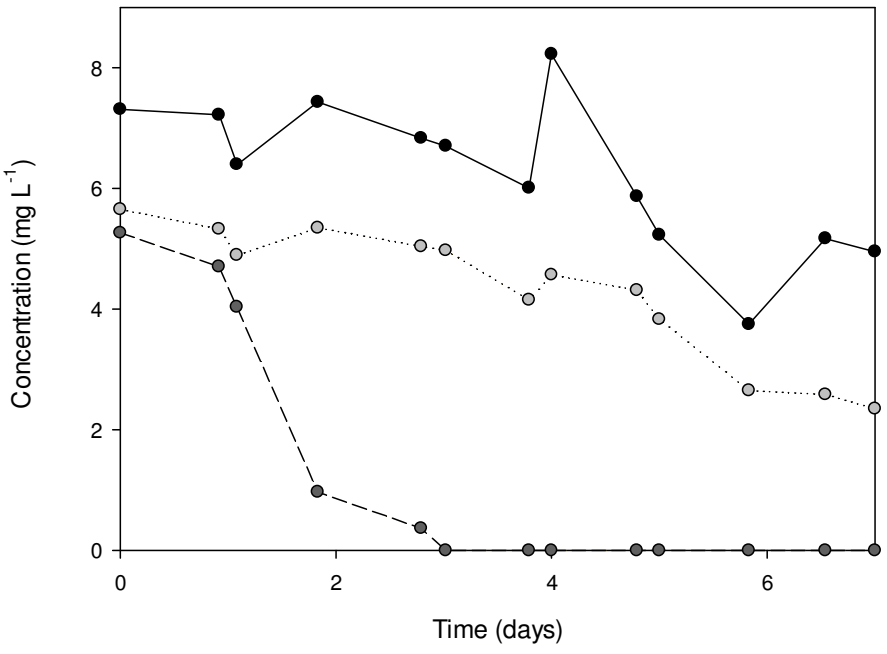


Figure 7.11 Time course of PhACs in the SRR sterile reactor, including CBZ (in black dots, straight line), DCF (in grey dots, dashed lines) and IPD(in pale grey dots and dotted lines)

Concerning PhACs removals, DCF was removed after 3 days of treatment, whereas CBZ and IPD were removed up to 60% and 33%, respectively. These results are considerablly lower than removals obtained before (chapter 5) but unstability in pH and aeration from the third day of the experiment could have influenced this lower elimination rates.

A new experiment was performed with the same batch for HWW as used in previous reactor, but in non-sterile conditions. Removal of CBZ, DCF and IPD was also assessed.

Sterility negatively affected PhAC elimination in all of the three PhACs (Figure 7.12): only 32% of CBZ was removed (almost half of the elimination in sterility); DCF, in spite of being completely removed, needed up to 10 days (whereas in sterile conditions total elimination took 3 days) and IPD concentration oscillated during the experiment with almost none removal probably due to retransformation phenomena or sorption/desorption equilibrium (Polesel et al., 2016). This erratic removal behaviour agrees with other studies performed under non-sterile conditions with real hospital wastewaters using iopromide (Kovalova et al., 2012).

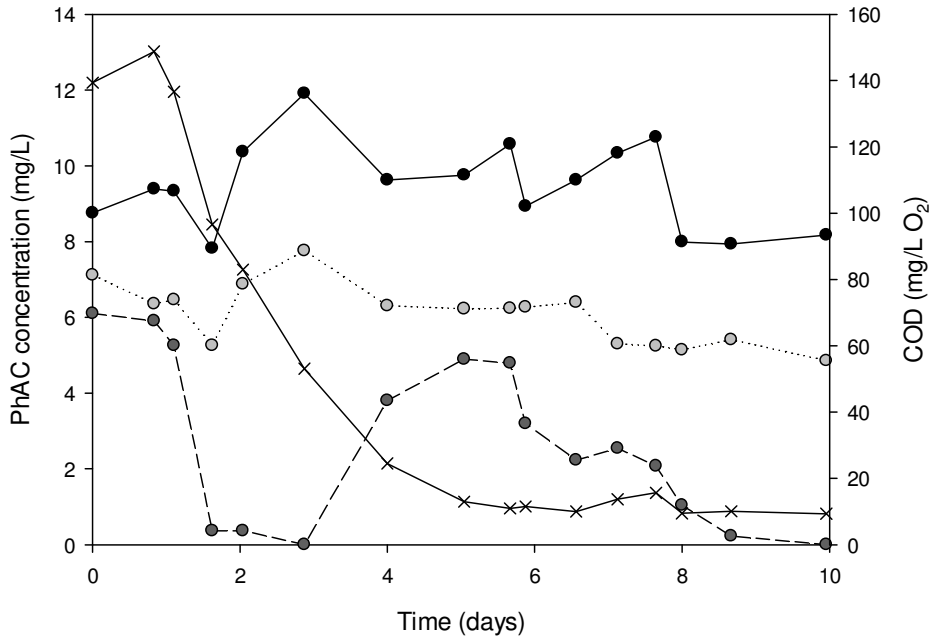


Figure 7.12 Time course of PhACs in the SRR non-sterile reactor, with CBZ (in black dots, straight line), DCF (in grey dots, dashed lines) and IPD(in pale grey dots and dotted lines). COD is also represented with crossed dots and straight lines.

Unlike previous SRR experiment, enzymatical activity was only reported for laccase with a maximum peak of 6 U/L in the fifth day. A similar oxygen limitation as reported in the first SRR experiment also took place after the first days of operation and aeration was increased accordingly, and this could have contributed to the lower removals obtained, which could be added to the lisis of some *S. rugosoannulata* pellets, possibly affected by microbial contamination, during the last days of the experiment

7.4. Conclusions

Regarding the first part of the chapter, air-pulsed FBB containing pellets of *T. versicolor* has proven to remove antibiotics to almost 100%, both in osmosed water and HWW at considerably higher rates than EMR, in spite of non-sterile conditions and not necessarily dependant on laccase activity. On the contrary, EMR was not succesful in treating HWW since removals of antibiotics were generally low. Retransformation of antibiotics and PhACs should be deeply studied to improve biological and enzymatical treatments and effectively degrade PhACs.

Moreover, experiments with *P. ostreatus* showed that diclofenac can be rapidly degraded through a biological process where CYP450 is apparently involved, although also laccase showed to be able of degrading it even without a redox mediator. Ketoprofen was not transformed by using neither pure laccase alone neither the addition of a redox mediator, whereas CYP450 exhibited a potential role on its degradation. Finally, atenolol is hardly degraded by this fungus as it requires a long contact time and high levels of biomass or laccase production to evidence degradation, while laccase can degrade it even without mediator. *P. ostreatus* is able to reduce COD in wastewater which allows exploring different strategies other than the reported to remove PhACs from HWW through a long term continuous treatment without carbon source addition and without biomass renovation. As a consequence *Pleurotus ostreatus* must be considered a powerful candidate for developing new wastewater treatment processes and optimal needs must be studied.

Concerning reactors with *S. rugosoannulata,* it has been able to consume carbon sources from HWW with high values of COD and, as mentioned for *P. ostreatus*, this opens a new exploring strategy for operating with air-pulsed FBB without the need of carbon source addition and biomass renovation. However, in the case of *S. rugosoannulata,* ammonia present in HWW was not reduced, suggesting the need to maintain nitrogen addition in the reactor. On the contrary, this fungus was able to acidify the pH of HWW to reach optimal conditions for its growth, despite that, during bioreactor operation pH control was required to avoid an excessive acidification.

DCF total removal was obtained either under sterile or non-sterile conditions, whereas removals for CBZ and IPD were dramatically affected by non-sterility.

In conclusion, these three different examples demonstrate the effectiveness of fungal treatments for PhACs removal and open the road for promising future real scale implementations.

7.5. References

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CONCLUSIONS AND
FUTURE PROSPECTS

3.1. Conclusions

In this thesis, the biodegradation of Pharmaceutical Active Compounds (PhACs) has been studied from several points of view, considering the four main topics presented in the objectives (chapter 2), the main conclusions are being described below:

I. Identification of new potential candidates for PhAC biodegradation.

- *Stropharia rugosoannulata* and *Gymnopilus luteofolius* are two ligninolytic fungi able to grow in pellet morphology and to remove PhACs in submerged cultures. Specially *S. rugosoannulata* has proven good performance in PhACs removal (in overall more than 50%), being very versatile in the removal of different PhACs.

- On the other hand, Biosand filter was revealed as a source for potential candidates to PhAC removal. Native microbiota was able to degrade spiked PhACs in HWW to 90% of ciprofloxacin and 50% of ibuprofen (from an initil concentration of 7 mg/L).

- The distribution and diversity of fungal species in the biofilm section of the BSF was affected by PhAC exposition. Fungal species such as *Apiotrichum scarabaeorum* or *Trichosporon spp.* were promoted during PhAC spiking and could be involved in degradation processes.

II. Assessment of biodegradation capabilities for several ligninolytic fungi in the treatment of recalcitrant compounds and the study of their metabolites.

- Part of the removal in PhACs by fungi is eliminated by sorption mecanisms into biomass. In 6 days or less, there is not enough time for degrading sorpted PhACs. Therefore, in this conditions PhACs degradation determined by liquid phase is accurate enough to avoid the analyses of PhACs sorption into biomass.

- Laccase is involved in certain PhAC degradation, such as atenolol, but it does not play a role in the case of diclofenac, where cytochrome P450 is clearly involved. On the other hand, with reference to metoprolol (MTP), metoprolol acid (MTPA), venlafaxine (VFX) and O-desmethylvenlafaxine (ODMVFX), the low laccase levels produced by *G. lucidum*, in spite of their high removals, suggests scarce involvement of this enzyme in removal mechanisms for these PhACs.

- In the degradation study of metoprolol, metoprolol acid, venlafaxine and O-desmethylvenlafaxine, comparing *G. lucidum, P.ostreatus* and *T. versicolor*, the best removals: 55% for MTP, 81% for MTPA, 70% for VFX and 100% for ODMVFX were achieved by *G. lucidum*.

- Degradation products in the case of VFX and ODMVFX were investigated and two TPs (N-desmethylvenlafaxine and O-desmethylvenlafaxine) were also identified during the treatment with fungal species assessed.

- In controlled conditions, pellets of *Trametes versicolor* was able to achieve 100% elimination of several antibiotic compounds, in synthetic wastewater and real HWW, both under non-sterile conditions, and with overall

eliminations over 90%, compared to the 70% achieved under sterile conditions for enzymatic membrane reactors.

III. Implementation of the treatment of real hospital wastewater (HWW) in bioreactor.

- *G. lucidum* treatment for MTP and MTPA in a fluidized bed bioreactor, showed even higher effectiveness than the removal in Erlenmeyer.

- *Pleurotus ostreatus* in bioreactor was able to degrade ATL and DCF spiked to sterile HWW operating in continuous mode with hydraulic retention time of 3d for 32 days.

- *S. rugosoannulata* has shown promising performance when treated spiked HWW in fluidized bed bioreactor either in sterile or non-sterile conditions. Despite of that, several operational requirements need to improve towards its future implementation in a continuous treatment.

IV. Assessment of new strategies for the operation in fungal fluidized bed bioreactors.

- The use of Biosand Filter (BSF) as a fungal bioreactor pretreatment was able to reduce baterial load in hospital wastewater (HWW) up to 10^2, but this reduction was not sufficient to prevent the competition of other microorganisms.

- *P. ostreatus and S. rugosoannulata* are able to reduce COD from HWW while consuming it as a carbon source. This new approach for these two species, allows the proposal of new strategies towards the implementation of real scale process such as avoiding glucose continuous feeding, or the pH controll during the acidification of the HWW in the first steps of the treatment.

This Thesis evidences the importance of finding new species of microorganisms and to improve the knowledge of their degradative mechanisms, which will allow to implement different biodegradation strategies to face the still unknown recalcitrant pollution.

3.2. Future prospects

Despite promising results, big part of this thesis work refers to Erlenmeyer scale in sterile conditions, with several experiments where contaminants were spiked in the matrix instead of being pre-existent.

The use of real contaminated samples, with generally lower PhAC concentrations but increased complexity, can provide a better estimation of the potential environmental impact of the real application approaches. Therefore, future research should be focused on the application of the studied fungi under non-sterile conditions, with real (non-spiked) PhACs concentrations, where fungi will be competing against several microorganisms, as well as in the scale-up of the process, which would allow to consider at the end the implementation of a real scale plant.

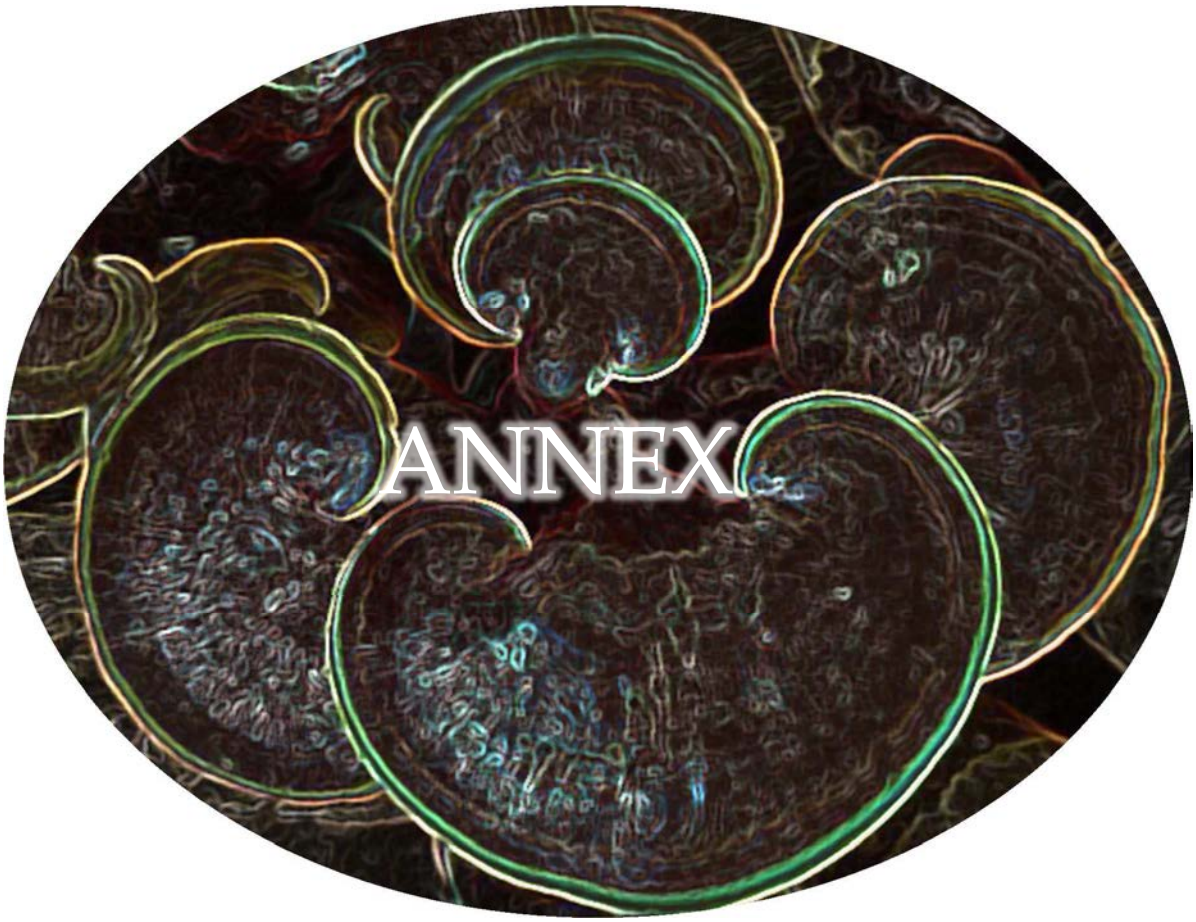
Fungal treatments have proven to be very effective for certain PhACs removal, however, the ultimate fate of PhACs after being treated with fungi has been rarely investigated. In this context, a short-term complete mineralization is apparently difficult to achieve for most of recalcitrant PhACs. For certain PhACs, fungal removal mechanisms can be based only on sorption processes, whereas when biodegradation effectively occurs, TPs and secondary metabolites are still an unresolved problem. In spite of several advances in their detection and ecotoxicological assessment, studies about TPs generation and transformation in fungal treatments are still scarce, and in those particular studies that have identified them, their toxicity can be higher than their parental compounds. Moreover, fungi have not always shown success in their elimination. Hence, before studying the application of fungal treatments on new PhACs, the fungal potentiality in TPs generation should be deeply assessed, and in case the subsequent TPs can cause any impact on human health and environment, investigate their ultimate fate through the fungal metabolism (techniques such as isotopic labelling have proven to be effective in studying this issue).

On the other hand, microorganisms able to grow in HWW can be suitable candidates for PhACs removal as it was proven in this thesis. Every year new PhACs go on the market without any previous environmental biodegradability studies, this fact and the lack of a

definitive solution to PhACs contamination in water, promises that the study of novel potential candidates for PhACs biodegradation has still a long way to go.

With the aim to obtain real approaches for the removal of Pharmaceutical Active Compounds (PhACs) from wastewater, further research should focus on:

- Identification of the enzymatic systems involved in the degradation of the PhACs by new fungal degraders.
- Determining the growth conditions to produce pellets of new fungal degraders in defined medium and in bioreactor.
- Improving the degradation yields through the knowledge of the better fungal requirements.
- Exploring the suitable conditions to implement a continuous treatment process with new fungal degraders.
- Study the relationship between fungi and native microbiota of the wastewater, and their combined effect on PhACs degradation.


ANNEX

4.1. Promising fungal species for PhACs removal in contaminated water

Table 5A.1. - Summary of the optimum values and MRM transitions used in the PhACs analysis.

	Rt (min)	Precursor ion	MRM		DP (volts)	EP (volts)	CE (volts)	CXP (volts)
Carbamazepine	2,66	273 [M+H]+	MRM 1	273>194	61	10	29	28
			MRM 2	273>193	61	10	49	14
Carbamazepine d10 (IS)	2,66	247 [M+H]+	MRM1	247>204	180	10	47	10
Cyclophosphamide	2,28	261 [M+H]+	MRM 1	261>140	101	10	31	20
			MRM 2	261>106	101	10	25	12
Cyclophosphamide 4H (IS)	2,28	266 [M+H]+	MRM1	266>141	61	10	29	20
Diclofenac	3,35	296 [M+H]+	MRM 1	296>214	61	10	49	10
			MRM 2	296>215	61	10	29	12
Fluoxetine d5 (IS)	2,84	315 [M+H]+	MRM 1	315>44	76	10	53	8
Ifosfamide	2,16	261 [M+H]+	MRM 1	261>154	76	10	31	14
			MRM 2	261>92	76	10	33	10
Iopromide	1,19	792 [M+H]+	MRM 1	792>573	156	10	35	20
			MRM 2	792>300	156	10	83	10
Ofloxacin d3 (IS)	4,97	365 [M+H]+	MRM1	365>321	180	10	47	10
Venlafaxine	2,29	278 [M+H]+	MRM 1	278>58	66	10	55	10
			MRM 2	278>260	66	10	17	12
Venlafaxine d6 (IS)	2,29	284 [M+H]+	MRM 1	284>64	180	10	47	10

Table 5A.2. Limits of detection (LOD) and quantification (LOQ) expressed in ppb, for the PhACs studied in this chapter.

PhAC	LOD	LOQ
Carbamazepine (CBZ)	0,01	0,04
Cyclophosphamide (CFD)	0,02	0,07
Diclofenac (DCF)	0,02	0,07
Ifosfamide (IFD)	0,02	0,06
Iopromide (IPD)	0,03	0,10
Venlafaxine (VFX)	0,02	0,06

Table 5A.3.-Concentrations measured in both water and biomass samples from experimental and killed control cultures..

	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 g^{-1})	Water (ng L^{-1})	Biomass (ng g^{-1})	Water (ng L^{-1})	Water (ng L^{-1})	Biomass (ng g^{-1})	Water (ng L^{-1})	Biomass (ng g^{-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 g^{-1})	Water (ng L^{-1})	Biomass (ng g^{-1})	Water (ng L^{-1})	Water (ng L^{-1})	Biomass (ng g^{-1})	Water (ng L^{-1})	Biomass (ng g^{-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 g^{-1})	Water (ng L^{-1})	Biomass (ng g^{-1})	Water (ng L^{-1})	Water (ng L^{-1})	Biomass (ng g^{-1})	Water (ng L^{-1})	Biomass (ng g^{-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

Table 5A.3 (Continuation).-Concentrations measured in both water and biomass samples from experimental and killed control cultures of the batch experiments performed with spiked ultrapure water. Data from water samples were taken from (Castellet-Rovira et al., 2017)

	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 ($ng\ g^{-1}$)	Water ($ng\ L^{-1}$)	Biomass ($ng\ g^{-1}$)	Water ($ng\ L^{-1}$)	Water ($ng\ L^{-1}$)	Biomass ($ng\ g^{-1}$)	Water ($ng\ L^{-1}$)	Biomass ($ng\ g^{-1}$)
Carbamazepine	123000	17220	2951	111930	2284	5000	3690	599	7380	1197
Diclofenac	113000	24182	11586	51980	508	3600	5650	619	6780	742
Iopromide	184700	11082	988	140372	428	9100	1847	370	20317	4065
Venlafaxine	82700	72776	810	74430	155	2700	7443	314	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 ($ng\ g^{-1}$)	Water ($ng\ L^{-1}$)	Biomass ($ng\ g^{-1}$)	Water ($ng\ L^{-1}$)	Water ($ng\ L^{-1}$)	Biomass ($ng\ g^{-1}$)	Water ($ng\ L^{-1}$)	Biomass ($ng\ g^{-1}$)
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	106000	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 ($ng\ g^{-1}$)	Water ($ng\ L^{-1}$)	Biomass ($ng\ g^{-1}$)	Water ($ng\ L^{-1}$)	Water ($ng\ L^{-1}$)	Biomass ($ng\ g^{-1}$)	Water ($ng\ L^{-1}$)	Biomass ($ng\ g^{-1}$)
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

Table 5A.4. Measured PhACs concentrations in the culture of the experimental and killed control flasks.

Trametes versicolor						
	t = 0 days		t = 6 days			
			Killed control		Experimental	
	Conc(µg/L)	Std Dev	Conc(µg/L)	Std Dev	Conc(µg/L)	Std Dev
Carbamazepina	47,7	3	43,9	1,9	20,3	3,7
Venlafaxine	53,6	2,7	50,4	3,2	24,2	7,4
Iopromide	174,4	2,1	161,0	8,7	93,1	1,8
Diclofenac	55,0	12,7	32,5	4,1	2,4	5,7
Cyclophosphamide	42,7	4,0	29,1	1,7	24,0	5,6
Ifosfamide	41,4	3,5	37,4	1,4	30,0	8,4
Total	**414,7**	**28,0**	**354,1**	**21,1**	**194,1**	**32,7**
Ganoderma lucidum						
	t = 0 days		t = 6 days			
			Killed control		Experimental	
	Conc(µg/L)	Std Dev	Conc(µg/L)	Std Dev	Conc(µg/L)	Std Dev
Carbamazepina	54,8	6,5	47,7	3,8	35,1	6,0
Venlafaxine	50,5	0,8	45,1	4,5	35,4	4,5
Iopromide	51,1	2,3	41,9	1,4	35,1	1,1
Diclofenac	61	1,6	40,0	1,3	1,1	0,3
Cyclophosphamide	43,5	2,6	29,6	3,5	26,5	1,7
Ifosfamide	39,1	2,2	28,2	3,9	25,0	4,3
Total	**300**	**16,0**	**232,4**	**18,4**	**158,2**	**17,9**
Irpex lacteus						
	t = 0 days		t = 6 days			
			Killed control		Experimental	
	Conc(µg/L)	Std Dev	Conc(µg/L)	Std Dev	Conc(µg/L)	Std Dev
Carbamazepina	83,4	8,4	79,2	2,5	31,7	1,7
Venlafaxine	104	11,0	97,6	0,0	88,4	0,0
Iopromide	105	8,2	99,8	4,2	76,7	8,4
Diclofenac	61	2,7	45,0	0,0	2,0	0,6
Cyclophosphamide	92	13,0	74,5	7,4	70,8	8,3
Ifosfamide	94,4	12,7	88,7	5,7	72,7	10,4
Total	**539,8**	**56,0**	**484,8**	**19,7**	**342,2**	**29,3**
Stropharia rugosoannulata						
	t = 0 days		t = 6 days			
			Killed control		Experimental	
	Conc(µg/L)	Std Dev	Conc(µg/L)	Std Dev	Conc(µg/L)	Std Dev
Carbamazepina	123	5	111,9	7,38	17,2	3,69
Venlafaxine	82,7	2,7	74,4	7,44	72,8	7,44
Iopromide	184,7	9,1	140,4	20,32	11,1	1,85
Diclofenac	113	3,6	52,0	6,78	24,2	5,65
Cyclophosphamide	80,7	4,1	55,7	4,84	55,7	5,65
Ifosfamide	84,3	6,5	74,2	5,06	71,7	8,43
Total	**668,4**	**31**	**508,6**	**51,82**	**252,6**	**32,71**

Table 5A.4 (Continuation) Mean PhACs concentrations in the liquid culture of the experimental and killed control flasks.

Gymnopilus luteofolius						
	t = 0 days		t = 6 days			
			Killed control		Experimental	
	Conc(µg/L)	Std Dev	Conc(µg/L)	Std Dev	Conc(µg/L)	Std Dev
Carbamazepina	71	11	63,9	7,1	32,0	8,5
Venlafaxine	106	16	90,1	11,7	90,1	13,8
Iopromide	82	20	79,5	2,5	3,3	5,7
Diclofenac	106	10	47,4	0,6	25,8	0,6
Cyclophosphamide	90	12	67,5	6,3	67,5	10,8
Ifosfamide	94	13	85,5	2,8	78,0	5,6
Total	**549**	**82**	**434,0**	**30,94**	**296,65**	**45,08**
Agrocybe erebia						
	t = 0 days		t = 6 days			
			Killed control		Experimental	
	Conc(µg/L)	Std Dev	Conc(µg/L)	Std Dev	Conc(µg/L)	Std Dev
Carbamazepina	114	2	106,0	6,8	62,7	11,4
Venlafaxine	106	9	103,9	2,1	95,4	10,6
Iopromide	159	5	139,9	9,5	71,6	17,5
Diclofenac	104	9,1	27,0	0,6	24,0	0,7
Cyclophosphamide	95	10	85,5	9,5	73,2	17,1
Ifosfamide	101	20	96,0	5,1	96,0	5,1
Total	**679**	**55,1**	**558,3**	**33,7**	**422,8**	**62,4**

Table 5A.5 Initial and final glucose concentration and biomass dry cell weight (DCW) plus minus standard deviation.

Fungus	Treatment	Initial glucose (g/L)	Final glucose (g/L)	Approximated initial biomass DCW (g/L)
-	Abiotic control	5.69 ± 2.83	5.38 ± 2.37	-
T.versicolor	Killed control	6.46 ± 1.91	6.16 ± 1.92	
T.versicolor	Experimental	6.04 ± 1.65	0.01 ± 0.01	4.69 ± 0.76
T.versicolor	Biotic control	8.72 ± 1.52	0.03 ± 0.01	
G.lucidum	Killed control	4.43 ± 2.20	3.24 ± 3.21	
G.lucidum	Experimental	7.22 ± 1.73	0.02 ± 0.02	4.78 ± 1.45
G.lucidum	Biotic control	9.76 ± 0.87	0.04 ± 0.02	
I.lacteus	Killed control	8.01 ± 0.28	9.37 ± 0.85	
I.lacteus	Experimental	6.89 ± 0.09	0.02 ± 0.00	4.64 ± 0.27
I.lacteus	Biotic control	9.24 ± 0.37	0.01 ± 0.02	
S.rugosoannulata	Killed control	6.88 ± 0.04	5.84 ± 1.36	
S.rugosoannulata	Experimental	6.09 ± 0.12	0.01 ± 0.03	4.28 ± 1.46
S.rugosoannulata	Biotic control	9.97 ± 0.68	0.01 ± 0.00	
G.luteofolius	Killed control	7.94 ± 0.11	8.83 ± 0.13	
G.luteofolius	Experimental	6.85 ± 0.06	0.02 ± 0.01	4.40 ± 0.91
G.luteofolius	Biotic control	7.14 ± 0.20	0.00 ± 0.01	
A. erebia	Killed control	8.49 ± 0.10	8.38 ± 0.02	
A. erebia	Experimental	5.94 ± 1.36	0.01 ± 0.01	4.33 ± 0.75
A. erebia	Biotic control	6.94 ± 0.13	0.01 ± 0.02	

4.2. Fluidized Bed Bioreactors for PhACs fungal biodegradation

7.1 TVR

Table 7A.1. Antibiotic removal percentages achieved by the EMR and TVR in osmosed water matrix and HWW.

EMR Osmosed water

Time	Sulfonamides		Penicillins		Fluoroquinolones			Quinolones	Tetracyclines	Metroimidazole	DHFR inhibitors
	Sulfamethoxazole	Amoxicillin	Penicillin V	Cloxacilin	Ofloxacin	Ciprofloxacin	Norfloxacine	Pipemidic acid	Tetracycline	Metronidazole	Trimethoprim
0 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2 h	98.71	68.64	33.20	23.76	27.82	79.89	41.14	73.64	74.28	5.35	35.76
4 h	99.30	97.66	31.90	28.82	45.30	92.05	69.55	83.13	63.99	19.69	50.22
8 h	99.22	93.42	39.52	35.44	67.58	92.09	77.17	85.63	50.27	15.20	55.03
24 h	97.22	94.66	70.56	54.32	77.66	93.00	82.37	85.48	69.66	9.42	66.77

EMR HWW

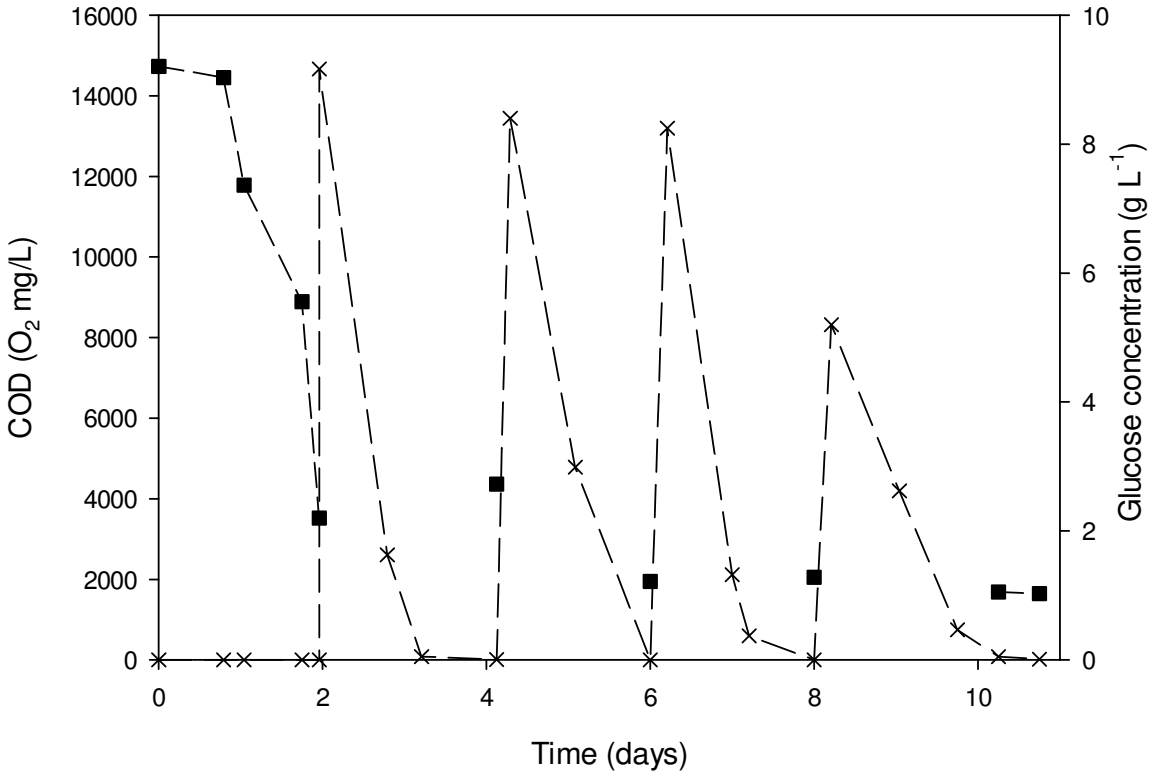
Time	Sulfonamides		Penicillins		Fluoroquinolones			Quinolones	Tetracyclines	Metroimidazole	DHFR inhibitors
	Sulfamethoxazole	Amoxicillin	Penicillin V	Cloxacilin	Ofloxacin	Ciprofloxacin	Norfloxacine	Pipemidic acid	Tetracycline	Metronidazole	Trimethoprim
0 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2 h	20.94	48.85	20.22	5.82	-3.88	4.78	8.32	13.04	19.47	6.44	6.44
4 h	16.11	58.26	19.57	1.59	-3.88	4.95	8.46	49.41	29.72	2.15	2.15
8 h	26.35	75.84	21.19	6.35	-4.85	-0.68	-2.82	39.92	36.60	-1.17	-1.17
24 h	27.96	90.39	20.86	-12.70	-3.88	3.58	5.50	70.16	35.87	-1.95	-1.95

Table 7A.2 Removals (in percentage) of reactor B (uninoculated TVR control) for the antibiotics detected in osmosed water and HWW respectively.

Reactor B Osmosed water											
Time	Sulfonamides		Penicillins			Fluoroquinolones		Quinolones	Tetracyclines	Metroimidazole	DHFR inhibitors
	Sulfamethoxazole	Amoxicillin	Penicillin V	Cloxacilin	Ofloxacin	Ciprofloxacin	Norfloxacine	Pipemidic acid	Tetracycline	Metronidazole	Trimethoprim
0 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
24 h	20.88	25.56	47.85	41.00	48.23	38.59	35.10	41.94	41.53	24.31	43.96
72 h	75.04	89.45	92.20	99.35	76.80	38.80	8.30	39.57	85.92	22.79	79.56
168 h	87.92	99.93	99.96	99.28	95.67	92.74	90.51	90.39	95.82	21.73	88.14
Reactor B HWW											
Time	Sulfonamides		Penicillins			Fluoroquinolones		Quinolones	Tetracyclines	Metroimidazole	DHFR inhibitors
	Sulfamethoxazole	Amoxicillin	Penicillin V	Cloxacilin	Ofloxacin	Ciprofloxacin	Norfloxacine	Pipemidic acid	Tetracycline	Metronidazole	Trimethoprim
0 h	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24 h	34.8	-50.6	8.5	24.6	6.6	10.2	12.1	37.9	26.6	18.2	17.0
72 h	27.4	69.9	1.8	32.3	18.1	27.2	25.2	37.9	46.2	42.2	42.6
168 h	36.0	87.5	33.5	50.0	22.1	33.2	32.7	-16.5	53.7	-28.5	100.0

7.2 SRR

Figure 7A.1 *S. rugosoannulata* reduction of COD from HWW with glucose addition under sterile conditions. COD is represented in a straight line, whereas glucose addition pulses are represented in dashed lines.



References

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